PROTOCOLS FOR FORENSIC RFLP ANALYSIS

VERSION 1.0

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GENERAL RFLP GUIDELINES

1. Notetaking is one of the most important aspects of casework; see the Forensic Biochemistry and Hematology Laboratory Manual for guidelines on documentation and notetaking. All RFLP worksheets, autorads, and membranes **must** be kept in the casefile, in a neat and organized manner.

   Documentation for validation or database samples **must** be kept in the appropriate files.

2. There are two basic methods for extraction of DNA:

   Non-organic extraction, Adapted from "A Simple and Efficient Method for the Isolation of High Molecular Weight Human Genomic DNA". Nucleic Acids Research 22 839 (1989).

   Organic extraction, Adapted from Procedures for the Detection of Restriction Fragment Length Polymorphisms in Human DNA. FBI Laboratory December 4, 1989.

   Most samples will be extracted according to the non-organic protocols. However, stains on denim, nylon and very dirty samples (i.e. visible grease or dirt mixed with the stains) may be extracted according to the organic protocols.

3. The DNA extraction of evidence samples must be done at a separate time from the extraction of exemplars.

4. Any sample which fails to either extract or restriction digest with the non-organic protocol may then be extracted according to the organic protocol. The judgement of which protocol to use will be made by the Forensic Analyst in consultation with the Forensic Scientist and/or Assistant Director.

5. For most casework, a single analytical gel is run. The number of samples allowed on the analytical gel is limited to 4 or 5, plus not more than 4 exemplars. Therefore, limit the quantity of samples extracted in a case to a manageable number (approximately 10).

6. If an exemplar yields highly degraded DNA and/or a yield of 1 ng/mL or less, it is not suitable for RFLP analysis. Other types of analysis (i.e., HLA-DQα) may be indicated for the case.

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7. At least one Cell Pellet Control (allelic control) and Negative Control will be analyzed with each case. The Cell Pellet Control will be carried along with the casework samples through the entire RFLP procedure. The Negative Control will be carried along with the casework samples up to the Test Gel. However, if the Cell Pellet Control appears normal on a yield gel and is lost during ethanol precipitation in the restriction digestion step then 100 ng Hae III digested K562 DNA may be substituted at the Analytical Gel.

8. Reviews **must** be obtained at the Yield Gel and Test Gel stages before continuing with casework.

9. Since RFLP analysis is a lengthy process, repeat analyses are not required. However, all autorads will be independently sized.

10. If less than one half of a sample was extracted and the sample yielded a conclusive RFLP result with all probes, then the extracted DNA is discarded after the case is completed and the sample is retained according to laboratory policy.

11. If one half or greater of a sample was extracted or a conclusive RFLP result was not obtained with all probes. then the extracted DNA is frozen at -80°C after the case is completed.

12. If any sample was extracted and had no DNA by yield gel **and** Quantiblot then that DNA extract may be discarded if less than one half of the sample was extracted.

13. All restriction digests are discarded after a case is completed.

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7. At least one Cell Pellet Control (allelic control) and Negative Control will be analyzed with each case. The Cell Pellet Control will be carried along with the casework samples through the entire RFLP procedure. The Negative Control will be carried along with the casework samples up to the Test Gel. However, if something unforeseen happens to the Cell Pellet Control during the RFLP procedure, 100 ng Hae III digested K562 DNA may be substituted at the Analytical Gel.

8. Reviews **must** be obtained at the Yield Gel and Test Gel stages before continuing with casework.

9. Since RFLP analysis is a lengthy process, repeat analyses are not required. However, all autorads will be independently sized.

10. If less than one half of a sample was extracted and the sample yielded a conclusive RFLP result with all probes, then the extracted DNA is discarded after the case is completed and the sample is retained according to laboratory policy.

11. If one half or greater of a sample was extracted or a conclusive RFLP result was not obtained with all probes. then the extracted DNA is frozen at -80°C after the case is completed.

12. If any sample was extracted and had no DNA by yield gel **and** Quantiblot then that DNA extract may be discarded even if one half or greater of the sample was extracted.

13. All restriction digests are discarded after a case is completed.

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7. At least one Cell Pellet Control (allelic control) and Negative Control will be analyzed with each case. The Cell Pellet Control will be carried along with the casework samples through the entire RFLP procedure. The Negative Control will be carried along with the casework samples up to the Test Gel. However, if something unforeseen happens to the Cell Pellet Control during the RFLP procedure, 100 ng Hae III digested K652 DNA may be substituted at the Analytical Gel.

8. Reviews **must** be obtained at the Yield Gel and Test Gel stages before continuing with casework.

9. Since RFLP analysis is a lengthy process, repeat analyses are not required. However, all autorads will be independently sized.

10. If less than one half of a sample was extracted and the sample yielded a conclusive RFLP result with all probes, then the extracted DNA is discarded after the case is completed and the sample is retained according to laboratory policy.

11. If one half or greater of a sample was extracted or a conclusive RFLP result was not obtained with all probes. then the extracted DNA is frozen at -80°C after the case is completed.

12. If any sample was extracted and had no DNA by yield gel **and** Quantiblot then that DNA extract may be discarded even if one half or greater of the sample was extracted.

13. All restriction digests are discarded after a case is completed.

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NON-ORGANIC EXTRACTION OF DNA FROM BLOOD AND BONE MARROW

1. Process a Cell Pellet Control (human cell line K562) and a Negative Control (0.5 mL) with every batch of extractions.

   When analyzing non-casework samples, extract at least one Cell Pellet Control and Negative Control for every 20 samples.

   When analyzing casework samples, extract one Cell Pellet Control and Negative Control per case.

2. Add 0.5 mL of well mixed blood or bone marrow if liquid, otherwise a 1.5 x 1.5 cm scoop of bone marrow, to a microcentrifuge tube.

3. Add 1.0 mL ice cold Cell Lysis Buffer (CLB). Vortex at high speed for one minute.

4. Centrifuge samples at 2700 x G at 4°C for 5 minutes.

5. Decant supernatant. (With a quick motion, hold tubes upside down to decant the supernatant.) Blot each tube on absorbent tissue. Keep tubes on ice.

6. Repeat steps 3-5 two more times with CLB.

7. Repeat steps 3-5 one more time substituting ice cold Protein Lysis Buffer (PLB) for the CLB. Keep tubes on ice.

8. Thaw a tube of Proteinase K (10 mg/mL) and mix thoroughly by gentle inversion of the tube.

9. Prepare a master mix of PLB and Proteinase K for N+2 samples:

	1 Sample	5 Samples	25 Samples	50 Samples
PLB	225 µL	1.13 mL	5.6 mL	11.3 mL
Proteinase K (10 mg/mL)	25 µL	0.125 mL	0.6 mL	1.3 mL

10. Add 250 µL of master mix. Pipet up and down to resuspend pellet. Mix well.

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11. Place tubes in 65°C heat block and incubate each tube for 2-2.5 hours. Vortex at high speed for ≈ 30 seconds every 15-20 minutes to ensure nuclear pellet is resuspended.

12. Vortex at high speed for ≈ 30 seconds following complete incubation.

13. Centrifuge samples 5 minutes in a microcentrifuge at room temperature to remove particles. Transfer supernatant to a new labeled tube. Measure and record the volume of the supernatant.

14. If a sample looks very discolored or still contains particles, further purify the sample by adding 125 μL 7.5 M LiCl and continuing the Organic Extraction (page 7) beginning at step 10. Do at least 2 organic extractions.

15. Store DNA at 4°C. It is stable for several months.

16. Continue with the protocol on page 14.

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NON-ORGANIC EXTRACTION OF DNA FROM BLOODSTAINS

1. Process a Cell Pellet Control (human cell line K562) and a Negative Control (0.5 mL) with every batch of extractions.

   When analyzing non-casework samples, extract at least one Cell Pellet Control and Negative Control for every 20 samples.

   When analyzing casework samples, extract one Cell Pellet Control and Negative Control per case.

2. Stains up to 1.5 cm x 1.5 cm can be extracted. Trim excess unstained fabric from stains.

   If a stain is very diffuse or is on a bulky substrate, the extraction can be scaled up appropriately.

   For swabs, remove cotton swab from applicator stick with a razor blade.

   For scrapings, use an amount to approximate 10 - 50 μL.

3. Cut stained fabric or swab into small pieces (3 x 3 mm).

4. Place fabric, swab, or scrapings into a microcentrifuge tube.

5. Add 1.5 mL ice cold Cell Lysis Buffer (CLB) to the tubes.

6. Incubate on ice 5-10 minutes. During this period vortex at least 3 times for $\approx$20 seconds each at high speed on the vortexer.

7. Centrifuge samples at 10,000 x G at 4°C for 5 minutes.

8. Decant supernatant. (With a quick motion, hold tubes upside down to decant the supernatant.) Blot each tube on absorbent tissue. Keep tubes on ice.

9. Repeat steps 5-8 two more times with CLB.

10. Repeat steps 5-8 one more time substituting ice cold Protein Lysis Buffer (PLB) for the CLB.

11. Remove residual PLB from the tube with a micropipette. Avoid the fabric or the bottom of tube. Keep tubes on ice.

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NON-ORGANIC EXTRACTION OF DNA FROM BLOODSTAINS

1. Process a Cell Pellet Control (human cell line K562) and a Negative Control (0.5 mL) with every batch of extractions.

   When analyzing non-casework samples, extract at least one Cell Pellet Control and Negative Control for every 20 samples.

   When analyzing casework samples, extract one Cell Pellet Control and Negative Control per case.

2. Stains up to 1.5 cm x 1.5 cm can be extracted. Trim excess unstained fabric from stains.

   If a stain in very diffuse or is on a bulky substrate, the extraction can be scaled up appropriately.

   For swabs, remove cotton swab from applicator stick with a razor blade.

   For scrapings, use an amount to approximate 10 - 50 μL.

3. Cut stained fabric or swab into small pieces (3 x 3 mm).

4. Place fabric, swab, or scrapings into a microcentrifuge tube.

5. Add 1.5 mL ice cold Cell Lysis Buffer (CLB) to the tubes.

6. Incubate on ice 5-10 minutes. During this period vortex at least 3 times for $\approx$ 20 seconds each at high speed on the vortexer.

7. Centrifuge samples at 10,000 x G at 4°C for 5 minutes.

8. Decant supernatant. (With a quick motion, hold tubes upside down to decant the supernatant.) Blot each tube on absorbent tissue. Keep tubes on ice.

9. Repeat steps 5-8 two more times with CLB.

10. Repeat steps 5-8 one more time substituting ice cold Protein Lysis Buffer (PLB) for the CLB.

11. Remove residual PLB from the tube with a micropipette. Avoid the fabric or the bottom of tube. Keep tubes on ice.

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12. Thaw a tube of Proteinase K (10 mg/mL) and mix thoroughly by gentle inversion of the tube.

13. Prepare a master mix of PLB and Proteinase K for N+2 samples.

	1 Sample	5 Samples	25 Samples	50 Samples
PLB	225 µL	1.13 mL	5.6 mL	11.3 mL
Proteinase K (10 mg/mL)	25 µL	0.125 mL	0.6 mL	1.3 mL

14. Add 250 µL of master mix to fabric or cotton swab. Make certain the fabric or swab is totally submerged. Pipet up and down to resuspend pellet. Mix well.

15. Place tubes in a 56°C heatblock and incubate overnight. Vortex at high speed for ≈ 30 seconds every 20-30 minutes for the first two hours to ensure nuclear pellet is resuspended.

16. Add 25 µL Proteinase K (10mg/mL) to each tube and incubate at 65°C for an additional 2-2.5 hours. Vortex at high speed for ≈ 30 seconds every 15-20 minutes to ensure nuclear pellet is resuspended.

17. Make sure the caps are on tight. Using a clean, sterile needle, punch a hole in the bottom of each tube (heating the needle in the flame of a Bunsen burner makes punching the hole much easier).

18. Piggyback the tube containing the sample inside a new labeled tube. Collect the lysate by centrifuging at 2700 x G in a swinging bucket rotor for 2 minutes. Save the tube containing the fabric or swab until after looking at the DNA on a yield gel.

19. Centrifuge the lysate 5 minutes in a microcentrifuge at room temperature to pellet any particles. Transfer supernatant to a new labeled tube. Measure and record the volume of the supernatant.

20. If a sample looks very discolored or still contains particles, further purify the sample by adding 125 µL 7.5 M LiCl and continuing the Organic Extraction protocol (page 7) beginning at step 10. Do at least 2 organic extractions.

21. Store DNA at 4°C. It is stable for several months.

22. Continue with the protocol on page 14.

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NON-ORGANIC DNA EXTRACTION FROM SEMEN STAINS OR SWABS

1. Fill out the extraction worksheet.

2. Pipette 1 mL of PBS into a 1.5 mL microcentrifuge tube for each sample. Cap all of the tubes.

3. A. Open one tube at a time with a Kimwipe and add one of the following:
   a) portion of semen stain about 3 mm square
   b) one half of a swab
   c) scrapings of a stain

   B. Cap the tube before adding sample to the next tube.

4. Mix by inversion or vortexing

5. Incubate at room temperature for 30 minutes.

6. Vortex or sonicate the substrate or swab for at least 2 minutes to agitate the cells off of the substrate or swab.

7. Remove the swab or other substrate from the sample tube, one tube at a time, using sterile tweezers and close tubes. Sterilize tweezers with ethanol before the removal of each sample.

   It is advisable not to discard the substrate until microscopic analysis (Step 11) shows that the sample contains sperm. Store swab or substrate in a sterile tube.

8. Spin in a microcentrifuge for 5 minutes at 10,000 to 15,000 x g.

9. Without disturbing the pellet, remove all but 50 μL of the supernatant. (The supernatant may be frozen or lyophilized and retained for analysis according to the Biochemistry and Hematology Laboratory Manual or discarded if not needed).

10. Resuspend the pellet in the remaining 50 μL by stirring with a sterile pipette tip.

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11. Remove about 3 μL of the resuspended sample for a Christmas Tree Stain (see Biochemistry and Hematology Laboratory Manual). After staining, the slide should be labeled and saved as evidence. If sperm are not visible microscopically, the substrate may be put back into the PBS and vortexed more vigorously (step 6) to try to dislodge additional sperm.

    Note: If epithelial cells are detected, proceed with the differential extraction procedure beginning with step 12. If no epithelial cells are observed, the differential extraction procedure may be omitted and the sample may be processed beginning with step 21.

12. To the approximately 50 μL of resuspended cell debris pellet, add 150 μL sterile deionized water (final volume of 200 μL).
13. Add 2 μL of 10 mg/mL Proteinase K. Vortex briefly to resuspend the pellet.

14. Incubate at 56°C for about 60 minutes to lyse epithelial cells, but for no more than 75 minutes, to minimize sperm lysis.

15. During the incubation step do the following:

    a. Label a new tube for each sample, including the extraction reagent control. Mark each tube as an epithelial cell fraction

16. Spin the extract in a microcentrifuge at 10,000 to 15,000 x g for 5 minutes.

17. Add 150 μL of the supernatant from each sample to its respective epithelial cell fraction sample tube. Store at 4°C or on ice until step 22.

    Note: The cell pellet control is stored with the epithelial fraction at 4°C on ice until step 22.

18. Wash the sperm pellet with Digest Buffer as follows:
    a. Resuspend the pellet in 0.5 mL Digest Buffer.
    b. Vortex briefly to resuspend pellet.
    c. Spin in a microcentrifuge at 10,000 to 15,000 x g for 5 minutes.
    d. Remove all but 50 μL of the supernatant and discard the supernatant.
    e. Repeat steps a-d for a total of 3-5 times.

19. Wash the sperm pellet once with sterile dH_2O as follows:
    a. Resuspend the pellet in 1 mL sterile dH_2O.
    b. Vortex briefly to resuspend pellet.
    c. Spin in a microcentrifuge at 10,000 to 15,000 x g for 5 minutes.
    d. Remove all but 50 μL of the supernatant and discard the supernatant.

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20. Resuspend the pellet by stirring with a sterile pipette tip. Remove about 3 μL of the resuspended sample and spot on a glass microscope slide for examination. Perform Christmas Tree stain (see Biochemistry and Hematology Laboratory Protocol). After staining the slide should be labeled and saved as evidence.

21. To the approximately 50 μL resuspended sperm fraction, add 150 μL of dH_2O, 2 μL of 10 mg/mL Proteinase K, and 7 μL of 1M DTT. Mix gently.

22. Vortex both the epithelial cell and sperm fractions. The following steps apply to both fractions.

23. Incubate at 65°C for approximately 120 minutes.

24. Vortex at high speed for 5 to 10 seconds.

25. Centrifuge the lysate 5 minutes in a microcentrifuge at room temperature to pellet any particles. Transfer supernatant to a new labeled tube. Measure and record the volume of the supernatant.

26. If a sample looks very discolored or still contains particles, further purify the sample by adding 125 μL 7.5 M LiCl and continuing the Organic Extraction protocol (page 7) beginning at step 10. Do at least 2 organic extractions.

27. Store DNA at 4°C. It is stable for several months.

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20. Resuspend the pellet by stirring with a sterile pipette tip. Remove about 3 μL of the resuspended sample and spot on a glass microscope slide for examination. Perform Christmas Tree stain (see Biochemistry and Hematology Laboratory Protocol). After staining the slide should be labeled and saved as evidence.

21. To the approximately 50 μL resuspended sperm fraction, add 150 μL of dH_2O, 2 μL of 10 mg/mL Proteinase K, and 7 μL of 1M DTT. Mix gently.

22. Vortex both the epithelial cell and sperm fractions. The following steps apply to both fractions.

23. Incubate at 65°C for approximately 120 minutes.

24. Vortex at high speed for 5 to 10 seconds.

25. Continue with the non-organic extraction after the proteinase K step.

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ORGANIC EXTRACTION OF DNA FROM BLOOD AND BONE MARROW

1. Process a Cell Pellet Control (human cell line K562) and a Negative Control (0.5 mL) with every batch of extractions.

   When analyzing non-casework samples, extract at least one Cell Pellet Control and Negative Control for every 20 samples.

   When analyzing casework samples, extract one Cell Pellet Control and Negative control for each case.

2. Add 0.5 mL of well mixed blood or bone marrow if liquid, otherwise a 1.5 x 1.5 cm scoop of bone marrow, to a microcentrifuge tube.

3. Add 1.0 mL ice cold Cell Lysis Buffer (CLB). Vortex at high speed on the vortexer for one minute.

4. Centrifuge samples at 2700 x G at 4°C for 5 minutes.

5. Decant supernatant. (With a quick motion, hold tubes upside down to decant the supernatant.) Blot each tube on absorbent tissue. Store tubes on ice.

6. Repeat steps 3-5 two more times with CLB.

7. Repeat steps 3-5 one more time substituting ice cold 0.2 M NaAcetate.

8. To the pellet add: 375 μL 0.2M NaAcetate
   25 μL 10% SDS
   5 μL Proteinase K (20 mg/mL)

   Vortex briefly.

9. Place tubes in a 56°C heatblock and incubate for 1 hour.

10. Add 120 μL Phenol, vortex $\approx$ 30 sec. This step must be carried out in the fume hood!

11. Centrifuge samples 2 minutes in a microcentrifuge at room temperature.

12. Carefully transfer the aqueous phase (top layer) to a new labeled tube. Do not disturb the layer of denatured protein that collects at the interface. Discard the old tube containing the phenol into a waste box in the hood.

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13. Repeat steps 10-12 with Phenol/Chloroform/Isoamyl Alcohol and then Chloroform/Isoamyl Alcohol.

14. If the sample is very discolored or dirty, repeat steps 10-13.

15. Add 1.0 mL cold, absolute EtOH. Mix by inversion of the tube. Place the tube at -20°C for at least 30 minutes. **The tubes can be stored indefinitely at this stage.**

16. Centrifuge for 15 minutes in a microcentrifuge at room temperature.

17. Decant supernatant. (With a quick motion, hold tubes upside down to decant the supernatant.) Blot each tube on absorbent tissue.

18. Add 180 μL TE to the pellets and vortex briefly.

19. Place the tubes in a 56°C heatblock and incubate for 10 minutes.

20. Add 20 μL 2.O M NaAcetate and mix for 5 seconds by hand.

21. Add 500 μL cold, absolute EtOH. Mix gently by hand to achieve a homogeneous solution.

22. Centrifuge 15 minutes in a microcentrifuge at room temperature.

23. Decant supernatant. (With a quick motion, hold tubes upside down to decant the supernatant.) Blot each tube on absorbent tissue.

24. To the pellet, add approximately 1.0 mL room temperature 70% EtOH. Centrifuge samples 5 minutes in a microcentrifuge at room temperature.

25. Decant supernatant. (With a quick motion, hold tubes upside down to decant the supernatant.) Blot each tube on absorbent tissue.

26. Dry the pellet in the Speed-Vac centrifuge for 10 minutes to remove remaining EtOH.

27. Add 250 μL TE, mix, and resolubilize by placing tubes in a 56°C heatblock and incubating for 14-18 hours.

28. Vortex at high speed for $\approx$ 30 seconds following complete incubation.

29. Store DNA at 4°C. It is stable for several months.

30. Continue with the protocol on page 14.

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ORGANIC EXTRACTION OF DNA FROM BLOODSTAINS AND TISSUES

1. Process a Cell Pellet Control (human cell line K562) and a Negative Control (0.5 mL) with every batch of extractions.

   When analyzing non-casework samples, extract at least one Cell Pellet Control and Negative Control for every 20 samples.

   When analyzing casework samples, extract one Cell Pellet Control and Negative control for each case.

2. Stains and tissues up to 1.5 x 1.5 cm can be extracted. Trim excess unstained fabric from stains.

   If a stain is very diffuse or is on a bulky substrate, the extraction can be scaled up appropriately.

   For swabs remove cotton swab from applicator stick with a razor blade.

   For scrapings, use an amount to approximate 10 - 50 μL.

3. Cut stained fabric into small pieces (3 x 3 mm). Mince tissues (< 1 mm^2) with a new razor blade into small pieces. The mincing should be done in a weigh boat in an ice bath.

4. Place fabric, swab, tissue, or scrapings into a microcentrifuge tube.

5. Thaw a tube of Proteinase K and mix thoroughly by gentle inversion of the tube.

6. Prepare a master mix of Stain Extraction Buffer and Proteinase K for N+2 samples.

	1 Sample	5 Samples	25 Samples	50 Samples
Stain Extraction Buffer	400 μL	2.0 mL	10.0 mL	20.0 mL
Proteinase K (10 mg/mL)	20 μL	0.1 mL	0.5 mL	1.0 mL

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7. Add 420 μL of master mix to fabric, tissue or cotton swab. Make certain the fabric, tissue, or swab is totally submerged.

8. Place tubes in a 56°C heatblock and incubate for 14-18 hours. Occasionally, vortex at high speed for $\approx$ 30 seconds to insure the fabric or tissue is resuspended.

9. If the tissue is not dissolved or the stain not removed from the fabric, add 20 μL Proteinase K (10 mg/mL). Place the tubes in a 65°C heatblock and incubate for 2-2.5 hours. During this period, vortex at high speed for $\approx$ 30 seconds every 15-20 minutes to insure tissue is resuspended.

10. Add 500 μL Phenol. This step must be done in the fume hood. Shake the tube vigorously by hand to achieve a milky emulsion in the tube.

11. Centrifuge the tube for 2 minutes in a microcentrifuge at room temperature.

12. Carefully transfer the aqueous phase (top layer) to a new labeled tube. Do not disturb the layer of denatured protein that collects at the interface. Discard the old tube containing the Phenol into a waste box in the hood.

13. Repeat steps 10-12 with Phenol/Chloroform/Isoamyl Alcohol and then Chloroform/Isoamyl Alcohol.

14. If the sample is very discolored or dirty, repeat steps 10-13.

15. Add 1.0 mL cold absolute EtOH. Mix by hand and place the tube at -20°C for at least 30 min. **The tubes can be stored indefinitely at this stage.**

16. Centrifuge for 15 minutes in a microcentrifuge at room temperature.

17. Decant supernatant. (With a quick motion, hold tubes upside down to decant the supernatant.) Blot each tube on absorbent tissue.

18. Place the tube in the Speed-Vac for 10 minutes to remove remaining EtOH.

19. Add 250 μL TE, mix, and resolubilize the DNA by placing the tubes in a 56°C heatblock and incubating for a minimum of two hours.

20. Store DNA at 4°C. It is stable for several months.

21. Continue with the protocol on page 14.

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ORGANIC EXTRACTION OF DNA FROM SEMEN STAINS OR SWABS

1. Process a Cell Pellet Control (human cell line K562) and a Negative Control (0.5 mL) for each batch of extractions.

   When analyzing non-casework samples, extract at least one Cell Pellet Control and Negative Control for every 20 samples.

   When analyzing casework samples, extract at least one Cell Pellet Control and Negative Control per case.

2. Stains up to 1.5 x 1.5 cm can be extracted. Trim excess fabric from stain.

   If a stain is very diffuse or is on a bulky substrate, the extraction can be scaled up appropriately.

   For swabs remove cotton swab from applicator stick with a razor blade.

3. Cut stained fabric or swab into small pieces (3x3 mm).

4. Place fabric or swab into a microcentrifuge tube.

5. Add 450 μL Phosphate Buffered Saline to the fabric or swab. Add 50 μL 20% Sarkosyl to the tube and mix.

6. Rock tube 14-18 hours at 4°C.

7. Centrifuge at 2700 x G in a swinging bucket rotor for 2 minutes. Pipet the supernatant into a new, labeled tube and save on ice.

   This supernatant is called the epithelial cell fraction and is enriched for non-sperm DNA.

8. To the tube containing the swab or fabric, add:

   400 μL Tris-EDTA-NaCl (TNE)
   50 μL 10% SDS
   50 μL H_20
   5 μL Proteinase K (20 mg/mL)

9. Mix, place the tubes in a 37°C heatblock, and incubate for 2 hours.

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10. Make sure the caps are on tight. Using a clean, sterile needle, punch a hole in the bottom of the tube (heating the needle in a Bunsen burner makes punching the hole much easier).

11. Piggyback the tube containing the sample inside a new labeled tube. Collect the lysate by centrifuging at 2700 x G in a swinging bucket rotor for 2 minutes. Save the tube containing the fabric or swab until after the DNA is examined on a yield gel.

12. Centrifuge the lysate for 5 minutes in a microcentrifuge at room temperature.

    The supernatant is part of the epithelial cell fraction and is enriched for non-sperm DNA. Combine it with the epithelial cell fraction collected at step 7. The epithelial cell fraction processing will continue at step 15.

13. To the pellet add:

    150 μL TNE
    100 μL 10% Sarkosyl
    40 μL 0.39M DTT
    100 μL H_20
    10 μL Proteinase K (20 mg/mL)

14. Place tubes in a 37°C heatblock and incubate for 2 hours.

15. To the sperm (pellet) and epithelial cell fraction, add 400 μL Phenol. This step must be done in the fume hood. Shake the tube vigorously by hand to achieve a milky emulsion in the tube.

16. Centrifuge samples for 2 minutes in a microcentrifuge at room temperature.

17. Carefully transfer the aqueous phase (top layer) to a new labeled tube. Do not disturb the layer of denatured protein that collects at the interface. Discard the old tube containing the Phenol into a waste box in the hood.

18. Repeat steps 15-17 with Phenol/Chloroform/Isoamyl Alcohol and then Chloroform/Isoamyl Alcohol.

19. If the sample is very discolored or dirty, repeat steps 15-18.

20. Add 1.0 mL cold absolute EtOH. Mix by hand and place the tube at -20°C for at least 30 min. **The tubes can be stored indefinitely at this stage.**

21. Centrifuge the samples for 15 minutes in a microcentrifuge at room temperature.

Initials: RCJ Date: 2/6/95

22. Decant supernatant. (With a quick motion, hold tubes upside down to decant the supernatant.) Blot each tube on absorbent tissue.

23. Dry the pellet in the Speed-Vac for 10 minutes to remove remaining EtOH.

24. Add 250 μL TE, mix, and resolubilize by placing tube in a 56°C heatblock for a minimum of two hours.

25. Store DNA at 4°C. It is stable for several months.

26. Continue with the protocol on page 14.

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ESTIMATION OF DNA QUANTITY AND QUALITY FROM YIELD GEL

1. Preparation of 20 x 25 cm yield gel (BRL Apparatus).

   The gels are poured with a 30 lane, 2 mm comb. The gel may have 2 or more origins if many samples are analyzed. The gels are cast in the electrophoresis tank using the combs and dams in the tank.

   a. For each gel, add 20 mL 10X TAE, 180 mL dH_2O and 1.8 g electrophoresis grade or DNA typing grade agarose to a flask of at least twice the liquid volume.

   b. Bring the flask to a boil in the microwave (2.5-3 minutes on high) to dissolve the agarose. Make sure the agarose is completely dissolved.

   c. Add 20 μL of ethidium bromide.

   d. Allow to cool to approximately 56°C.

   e. Pour agarose into the tank (be sure comb(s) are in place).

   f. Let the gel cool for approximately 30-60 minutes.

2. Pour approximately 1800 mL 1x TAE into the tank; enough buffer should be present to cover the gel. Remove comb(s).

3. Vortex samples including Cell Pellet Control, Negative Control, Yield Calibrators, Calibration Control and Lambda Marker tubes for 15 seconds.

4. Microcentrifuge briefly to bring contents to the bottom of the tube.

5. Place the tubes in a 65°C heatblock and incubate for 5 minutes.

6. Microcentrifuge briefly to bring contents to the bottom of the tube.

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7. Samples can be prepared in microcentrifuge tubes or in wells of a microtiter plate. Store the remainder of the DNA extracts at 4°C.

   a. Stains, Tissue, Post-Mortem Blood, and Swabs - Mix 10 μL of DNA sample and 2 μL of Yield Gel Loading Buffer.

   b. Whole Blood, Bone Marrow, and Fresh Sperm - Mix 2 μL of DNA sample, 8 μL of dH_2O and 2 μL of Yield Gel Loading Buffer.

   c. Cell Pellet Control - Mix 5 μL of DNA sample, 5 μL of dH_2O, and 2 μL of Yield Gel Loading Buffer.

8. If using a microcentrifuge tube, microcentrifuge briefly to bring contents to the bottom of the tube.

9. Load each row of each gel as follows:

Lane	Volume	Material	Description
1	10 μL	Lambda Marker	Hind III digested λ DNA
2	10 μL	Yield Calibrator A	30 ng/μL λ DNA (300 ng total)
3	10 μL	Yield Calibrator B	20 ng/μL λ DNA (200 ng total)
4	10 μL	Yield Calibrator C	10 ng/μL λ DNA (100 ng total)
5	10 μL	Yield Calibrator D	5 ng/μL λ DNA (50 ng total)
6	10 μL	Yield Calibrator E	2.5 ng/μL λ DNA (25 ng total)
7	10 μL	Yield Calibrator F	1 ng/μL λ DNA (10 ng total)
8	10 μL	Calibration Control	75 ng high M.W. human DNA
9-30	12 μL	Samples	Unknown samples

Include the Cell Pellet Control and Negative Control as samples.

9. Set the voltage at 100 volts on the dial. When the Bromophenol blue tracking dye has moved 4-5 cm (approx. 1.5-2 hours) from the origin, the run can be stopped.

Initials: LC Date: 6/21/95

10. Switch off the power supply and remove the gel from the tank. Examine the gel on the ultraviolet light transilluminator.

    Take a photograph of the gel using Polaroid film. For type 667 film the settings are *f*5.6 for 1/2-1 second. DO NOT EXPOSE YOURSELF TO THE UV LIGHT FOR AN EXCESSIVE AMOUNT OF TIME. ALWAYS WEAR U.V. GOGGLES WHEN WORKING WITH THE TRANSILLUMINATOR.

11. Interpretation:

    A. Quality - Intact DNA will move as a band not far from the origin. A smear from the origin to, or past, the dye front indicates that the DNA has been fragmented.

    B. Compare the intensity of the Calibration Control (lane 8) to that of the Yield Calibrators. If the intensity is between Yield Calibrators C and D (lanes 4-5), the result is valid. Otherwise the gel must be repeated.

    C. Examine the cell pellet control, it should have high molecular weight DNA with an intensity of the high molecular weight band of DNA greater than the intensity of Yield Calibrator F. Otherwise the extraction is inconclusive. "Trailing" of the cell pellet control band is allowed because the cell pellet controls were partially degraded when prepared.

    D. Quantity - Estimate the quantity (ng) and concentration (ng/μL) of high molecular weight DNA loaded for each sample by comparing the band intensity of the high molecular weight band of the unknowns with those of the Yield Calibrators. High molecular weight DNA should appear as a tight band slightly above the uppermost band of the Lambda Marker.

    If the sample shows "trailing", use only the region above the upper band of the Lambda Marker for quantifying high molecular weight DNA. If the band size is between two yield calibrators match the sample to the yield calibrator with the lowest amount of DNA. See table below for interpretation.

    E. Show the yield gel to another analyst to independently determine DNA quality, concentrations, and yields. Disagreements should be settled by a supervisor.

Initials: RCJ Date: 2/1/95

10. Switch off the power supply and remove the gel from the tank. Examine the gel on the ultraviolet light transilluminator.

    Take a photograph of the gel using Polaroid film. For type 667 film the settings are *f*5.6 for 1/2-1 second. DO NOT EXPOSE YOURSELF TO THE UV LIGHT FOR AN EXCESSIVE AMOUNT OF TIME. ALWAYS WEAR U.V. GOGGLES WHEN WORKING WITH THE TRANSILLUMINATOR.

11. Interpretation:

    A. Quality - Intact DNA will move as a band not far from the origin. A smear from the origin to, or past, the dye front indicates that the DNA has been fragmented.

    B. Compare the intensity of the Calibration Control (lane 8) to that of the Yield Calibrators. If the intensity is between Yield Calibrators C and D (lanes 4-5), the result is valid. Otherwise the gel must be repeated.

    C. Quantity - Estimate the quantity (ng) and concentration (ng/μL) of high molecular weight DNA loaded for each sample by comparing the band intensity of the high molecular weight band of the unknowns with those of the Yield Calibrators. High molecular weight DNA should appear as a tight band slightly above the uppermost band of the Lambda Marker.

    If the sample shows "trailing", use only the region above the upper band of the Lambda Marker for quantifying high molecular weight DNA. If the band size is between two yield calibrators match the sample to the yield calibrator with the lowest amount of DNA. See table below for interpretation.

    D. Show the yield gel to another analyst to independently determine DNA quality, concentrations, and yields. Disagreements should be settled by a supervisor.

Initials: RCJ Date: 6/6/95

	DNA CONCENTRATION[b]		
Sample Intensity[a]	2 μL Sample Loaded On Yield Gel	5 μL Sample Loaded On Yield Gel	10 μL Sample Loaded On Yield Gel
$\geq$A	see note c below	see note c below	Repeat yield gel loading 2 μL of sample
B	100 ng/μL	40 ng/μL	20 ng/μL
C	50 ng/μL	20 ng/μL	10 ng/μL
D	25 ng/μL	10 ng/μL	5 ng/μL
E	12.5 ng/μL	5 ng/μL	2.5 ng/μL
$\leq$F	Repeat yield gel loading 10 μL of sample	Repeat yield gel loading 10 μL of sample	$\leq$1 ng/μL

a. Yield Calibrator of closest intensity
b. For diluted samples, multiply DNA concentration by 10.
c. Dilute 5 μL of sample with 45 μL of TE and mix. Run 2 μL on a new yield gel. Save the dilution at 4°C.

Initials: RCJ Date: 2/6/95

Sample Intensity[a]	DNA CONCENTRATION[b]		
	2 μL Sample Loaded On Yield Gel	5 μL Sample Loaded On Yield Gel	10 μL Sample Loaded On Yield Gel
$\geq$ A	see note c below	see note c below	Repeat yield gel loading 2 μL of sample
B	100 ng/μL	40 ng/mL	20 ng/μL
C	50 ng/μL	20 ng/mL	10 ng/μL
D	25 ng/μL	10 ng/mL	5 ng/μL
E	12.5 ng/μL	5 ng/mL	2.5 ng/μL
$\leq$ F	Repeat yield gel loading 10 μL of sample	Repeat yield gel loading 10 μL of sample	$\leq$ 1 ng/μL

a. Yield Calibrator of closest intensity
b. For diluted samples, multiply DNA concentration by 10.
c. Dilute 5 μL of sample with 45 μL of TE and mix. Run 2 μL on a new yield gel. Save the dilution at 4°C.

Initials: RCJ Date: 2/1/95

RESTRICTION DIGESTION OF DNA

1. Before restricting samples, ensure that the Yield Gel results have been reviewed by a second analyst.

2. Prepare Hae III master mix for N+3 samples as follows and store on ice:

	1 Sample	5 Samples	25 Samples	50 Samples
10x Hae III Buffer	40 μL	200 μL	1.0 mL	2.0 mL
100 mM $MgCl_2$	40 μL	200 μL	1.0 mL	2.0 mL
Sterile dH_2O	66.5 μL	332.5 μL	1.7 mL	3.4 mL
Hae III (10 units/μL)	2.5 μL	12.5 μL	62.5 μL	125 μL

3. Pipet into microcentrifuge tubes the isolated DNA and sterile dH_2O water as described in Table II.

4. Pipet into a microcentrifuge tube 250 μL of Digestion Control. This contains 250 ng of human DNA from cell line K562.

5. Add 150 μL Hae III Master Mix to each tube.

6. Place the samples in a 37°C heat block and incubate for 2-2.5 hours.

7. Microcentrifuge briefly to bring contents to the bottom of the tube.

8. Add 200 μL 7.5 M LiCl to each tube and vortex briefly.

8. Place on ice for 10 minutes.

9. Microcentrifuge for 10 minutes, and pour the supernatant into a new, labeled microcentrifuge tube (Avoid transferring any of the precipitated debris. The pellet is often difficult to see). Discard the tube with the pellet.

10. To each tube containing the supernatant, fill to the top with room temperature absolute ethanol (approx. 1.2 mL) and mix by inversion. **The procedure may be stopped at this point and the samples stored at -20°C.**

Initials: RCJ Date: 2/6/97

11. Incubate at room temperature for 20-30 minutes.

12. Microcentrifuge for 10-20 minutes to pellet the DNA. Carefully decant supernatant. (With a quick motion, hold tubes upside down to decant the supernatant.) Blot each tube on absorbent tissue. Discard the supernatant.

13. To the pellet, add approximately 500 μL of room temperature 70% EtOH. Centrifuge for 5 minutes. Carefully decant the supernatant. (With a quick motion, hold tubes upside down to decant the supernatant.) Blot each tube on absorbent tissue.

14. Dry the pellet on a Speed-Vac for 5-10 minutes or if a vacuum pump is not available 5-6 hours without vacuum.

15. Resuspend the pellet in 20.5 μL sterile dH_2O.

16. Place the tubes in a 65°C heatblock and incubate for 10-15 minutes.

17. Vortex and microcentrifuge briefly to bring contents to the bottom of the tube.

18. Add 2.5 μL 10x Hae III Buffer to each tube.

19. Add 2 μL Hae III (20 units) to each tube.

20. Place the tubes in a 37°C heatblock and incubate for one hour.

21. Microcentrifuge briefly to bring contents to the bottom of the tube.

22. If a Test Gel is being done immediately, place samples on ice. For storage longer than 30 minutes, freeze at -20°C.

Initials: RCJ Date: 2/6/95

TABLE II- RESTRICTION DIGESTION OF SAMPLES

DNA Concentration of Original Sample From Yield Gel	Vol. of DNA Sample to Restrict	Vol. of dH_2O
≤ 1 ng /μL	215.0 μL	35.0 μL
2.5 ng/μL	200.0 μL	50.0 μL
5 ng/μL	100.0 μL	150.0 μL
10 ng/μL	50.0 μL	200.0 μL
12.5 ng/μL	40.0 μL	210.0 μL
20 ng/μL	25.0 μL	225.0 μL
25 ng/μL	20.0 μL	230.0 μL
40 ng/μL	12.5 μL	237.5 μL
50 ng/μL	10.0 μL	240.0 μL
100 ng/μL	5.0 μL	245.0 μL
125 ng/μL	(40.0 μL)[a]	210.0 μL
250 ng/μL	(20.0 μL)[a]	230.0 μL
500 ng/μL	(10.0 μL)[a]	250.0 μL
1000 ng/μL	(5.0 μL)[a]	245.0 μL

a. Use the 1/10 sample dilution which was prepared for the yield gel.

Initials: RCS Date: 2/1/95

TEST GEL: VERIFICATION OF DIGESTED DNA

1. Preparation of Test Gel

   BRL gels are poured with a 30 lane, 2 mm comb and Lifecodes (12 x 28 cm) gels are poured with a 25 lane comb. The gel may have 2 or more origins if many samples are analyzed. The BRL gels are cast in the electrophoresis tank using the combs and dams in the tank.

   a. For each gel, add 20 mL 10X TAE, 180 mL dH_2O and 1.6 g electrophoresis grade or DNA typing grade agarose to a flask of at least twice the liquid volume.

   b. Bring the flask to a boil in the microwave (2.5-3 min on high) to dissolve the agarose. Make sure the agarose is completely dissolved.

   c. Add 20 μL of ethidium bromide.

   d. Allow to cool to approximately 56°C.

   e. Pour agarose into gel form (be sure comb(s) are in place).

   f. Let stand 30-90 minutes to gel and then remove the tape.

2. Place gel into the electrophoresis tank. Pour 1x TAE into the tank (approx. 1800 mL for BRL or Lifecodes tanks). Enough buffer should be present to cover the gel. Remove comb.

3. Vortex samples including Cell Pellet Control, Calibration Control, Negative Control, Digestion Control, Test Gel Standards and Lambda Marker tubes for 15 seconds.

   Microcentrifuge briefly to bring contents to the bottom of the tube.

4. Place the tubes in a 65°C heatblock and incubate for 5 minutes.

5. Microcentrifuge briefly to bring contents to the bottom of the tube.

6. Samples can be prepared in microcentrifuge tubes or in wells of a microtiter plate.

   a. Add 5 μL of Test Gel Loading Buffer to each tube or well.

   b. Pipet 5 μL of each sample, Cell Pellet Control, Calibration Control, Negative Control, and the Digestion Control into separate tubes or wells of a microtiter plate.

Initials: RCJ Date: 2/1/95

7. If using a microcentrifuge tube, microcentrifuge briefly to bring contents to the bottom of the tube.

8. Load each row of each gel as follows:

Lane	Volume	Material	Description
1	10 μL	Lambda Marker	Hind III digested λ DNA
2	10 μL	Cal. Control	High M.W. human DNA (75 ng)
3	10 μL	Test Gel Standard	Hae III digested DNA (50 ng)
4	10 μL	Digestion Control	Digested along with samples
5	10 μL	Cell Pellet Control	Digested along with samples
6	10 μL	Negative Control	Digested along with samples
7-24	10 μL	Samples	Unknown samples

9. Set the voltage at 100 volts on the dial. When the bromphenol blue tracking dye has moved at least 4 cm (approximately 1.5-2 hours) from the origin, the run can be stopped.

10. Switch off the power supply and remove the gel from the tank. Examine the gel on the ultraviolet light transilluminator. If DNA cannot be visualized under UV light, return the gel bed to the gel box, add 100 μL Ethidium Bromide into the buffer and electrophorese for 15-30 minutes.

    Take a photograph of the gel using Polaroid film. For type 667 film the settings are *f*5.6 for 1/2-1 second. DO NOT EXPOSE YOURSELF TO THE UV LIGHT FOR AN EXCESSIVE AMOUNT OF TIME. ALWAYS WEAR U.V. GOGGLES WHEN WORKING WITH THE TRANSILLUMINATOR.

Initials: RCJ Date: 2/1/95

11. Interpretation of Test Gel

A. The test gel is used as an indicator of Hae III digestion. If enough high molecular weight DNA (≥ 250 ng) is present in a sample, the test gel should identify undigested and slightly digested samples. However, some partially digested samples may appear to be completely digested by test gel criteria. These samples will be identified later on the final autoradiograph.

B. Determine for each sample if the Hae III digestion appears complete by comparing its pattern with the Test Gel Standard (lane 3) and to the undigested Calibration Control (lane 2).

C. The Digestion Control should compare to lane 3 containing 50 ng of Hae III digested DNA. This control should reproducibly cut to completion under the conditions of the protocol with Hae III Enzyme and is a control of enzyme reliability. **If the digestion control is not completely digested by Hae III, all of the samples must be judged to be incompletely digested.**

D. Show the test gel to another analyst to independently verify complete restriction digestion. Disagreements should be settled by a supervisor.

13. A. For the samples completely digested by Hae III, proceed to the Analytical Gel section on page 24.

B. If the restriction digestion is not complete because the sample appears to contain too much DNA, dilute the sample appropriately and re-digest.

C. If the restriction digestion is not complete for any other reason, proceed as follows:

1. Combine the restriction digested sample with any leftover undigested sample.
2. Add 375 μL of Stain Extraction Buffer to each sample.
3. Continue with the protocol on page 7, step 10.

D. If the restriction digest is still not complete after 2 organic extractions, the sample may be further purified using other published or pre-validated techniques (i.e. microcentricon). The protocol for this further purification is at the discretion of the Forensic Analyst in consultation with a Forensic Scientist and/or Assistant Director. The entire protocol should be recorded on the appropriate data sheets or notebook along with a reference to the origin of the protocol.

Initials: RCJ Date: 2/1/95

ANALYTICAL GEL ELECTROPHORESIS

1. Preparation of 12 x 28 cm analytical gels:

   Pour a separate gel for each case. All gels are poured with a 17 lane comb. Seal the gel forms with tape before pouring the gels.

   a. For each gel, add 20 mL 10X TAE, 180 mL dH_2O and 2.0 g electrophoresis grade or DNA typing grade agarose to a flask of at least twice the liquid volume.
   b. Bring the flask to a boil in the microwave (4 minutes on high) to dissolve the agarose. Make sure the agarose is completely dissolved.
   c. Allow to cool to approximately 56°C.
   d. Measure volume of dissolved agarose and add dH_2O to bring to a volume of 200 mL per gel.
   e. Pour agarose into gel form (be sure comb is in place).
   f. Let stand 30-90 minutes to gel and then remove the tape.

2. Connect the recirculating hoses to the electrophoresis tank.

3. Up to three gels can be run in each electrophoresis tank. Add 1x TAE to the tank as follows:

# of gels/ tank	Vol. 1x TAE (mL)
1	1400
2	1600
3	1800

4. Place a gel bed in the tank and remove the comb. If running more than one gel, stagger the gel beds in the electrophoresis apparatus so all wells are accessible and remove the combs.

5. Add 13.2 μL of BRL Sizing Standard Solution A to 30.8 μL of BRL Sizing Standard Solution B for each gel to be run.

   Vortex and microcentrifuge briefly to bring contents to the bottom of the tube.

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6. Add 4 μL of Analytical Gel Loading Buffer to each digested sample and to each digested control except BRL Sizing Standard and Phi X marker.

   Vortex and microcentrifuge briefly to bring contents to the bottom of the tube.

7. Place samples, Phi X Marker, and BRL Sizing Standards in a 65°C heatblock and incubate for 5 minutes.

   Microcentrifuge briefly to bring contents to the bottom of the tube.

8. Load samples, Phi X Marker, and BRL Sizing Standards on gel according to Table III.

   Normally, 12 μL (half the restricted sample) of the sample will be loaded on the analytical gel. However, if the sample had a concentration of 1 ng or less, load the entire sample (24 μL).

   If running a database or validation gel, samples can be placed in all sample lanes.

   If running a case, exemplars can be placed side-by-side. Evidence stains must be separated from other samples by an empty lane or a BRL Sizing Standard lane.

9. Electrophorese at 75 volts as measured on the power supply until the bromophenol blue tracking dye has completely entered the gel (approximately 20 minutes).

10. Switch off the power supply and restack gel trays so that they are positioned evenly and in the middle of the gel box. Turn on the recirculation pumps. Make sure the buffer is recirculating! Turn the power supply back on.

11. Measure the voltage across the gels by placing the electrodes of the voltmeter into the buffer and holding them securely against the ends of the gel beds.

    Adjust the voltage to read 37 volts on the voltmeter and electrophorese for 17 ± 0.5 hours.

12. Approximately one hour before electrophoresis is stopped, add 115 μL of 10 mg/mL Ethidium Bromide into the buffer (57.5 μL at each end of the box). Mix gently with a pipette.

Initials: RO Date: 6/1/95

13. Switch off the power supply and remove the gel from the tank. Examine the gel on the ultraviolet light transilluminator.

DO NOT EXPOSE YOURSELF TO THE UV LIGHT FOR AN EXCESSIVE AMOUNT OF TIME. ALWAYS WEAR U.V. GOGGLES WHEN WORKING WITH THE TRANSILLUMINATOR.

a. To assess whether electrophoresis is complete, measure from the bottom of the wells to the second band (1078 kb) in the Phi X Marker lane. If this band has migrated at least 17.5 cm, a photograph of the gel may be taken using Polaroid film. For type 667 film the settings are *f*5.6 for 1/2-1 second.

b. If the band has not migrated 17.5 cm from the origin return the gel to the electrophoresis tank and continue to electrophorese until the second (1078 kb band) in the Phi X Marker lane has migrated at least 17.5 cm but not more than 20 cm.

Initials: RCJ Date: 2/6/95

TABLE III- ANALYTICAL GEL LOADING PATTERN

Lane	Volume	Material	Description
0	10 μL	Phi X Marker	Hae III digested φX174 DNA
1	10 μL	BRL Sizing Standard	DNA Sizing Ladder
2	12 μL	Cell Pellet Control	100 ng Hae III digested K562 DNA
3	12 μL	*	
4	12 μL	Sample	Unknown samples
5	12 μL	*	
6	12 μL	Sample	Unknown samples
7	10 μL	BRL Sizing Standard	
8	12 μL	Sample	Unknown samples
9	12 μL	*	
10	12 μL	Sample	Unknown samples
11	10 μL	BRL Sizing Standard	DNA Sizing Ladder
12	12 μL	Sample	Unknown samples
13	12 μL	*	
14	12 μL	Sample	Unknown samples
15	10 μL	BRL Sizing Standard	DNA Sizing Ladder
16	10 μL	Phi X Marker	Hae III digested φX174 DNA

* The following may be loaded in these lanes:

a. Casework exemplars (lanes 3-6)

b. Validation samples if the gel is not used for casework.

c. Database samples if the gel is not used for casework.

Initials: RCJ Date: 2/1/95

TRANSFER OF DNA FROM GEL TO MEMBRANE

1. Cut off nicks from the sides of the gel beds. Carefully place gels (still on beds) into a plastic container with a lid.

2. Add enough Denaturation Solution to cover the gels. Place an empty gel tray on top of the upper gel to prevent it from floating. Cover tightly and shake gently (speed approx. 35 rpm) for 30 minutes.

3. While gels are denaturing prepare membranes and transfer apparatus:

   Membranes should be handled only by gloved hands.

   A. Place 14 x 28 cm Pall Biodyne B membranes on a flat surface so that the nicks are in the top left corner. Label the membranes in pencil with the membrane number, staff member's initials and date of transfer.

      For non-casework samples, use a sequential OCME membrane number obtained from the OCME membrane logbook.

      For casework samples, use a membrane number of the form 93-0444A, where the first part is the year, the second part the FB case number, and the letter designating whether this is the first, second, etc. membrane of that case.

   B. Pre-wet the membrane in denaturation solution. Make sure the membrane wets evenly.

   C. Rinse sponges thoroughly in dH_20. Squeeze out excess water and saturate in Denaturation Solution. Squeeze out solution and saturate in fresh Denaturation Solution. Place 2-3 sponges in a tray. Place one GB004 and then one GB002 piece of filter paper, both saturated in Denaturation Solution, on top of the sponges.

4. Invert the gel onto the back of an empty gel bed. Slide the inverted gel directly onto the GB002 (the wells are now down in contact with the GB002). With gloved fingers, press down carefully on the gel to remove any air bubbles; do not distort or stretch gel. With a gloved hand, remove any excess buffer from upward side of gel.

5. Surround the gel with a mask cut from used pieces of X-ray film to help prevent "short circuiting" the transfer. **Make sure the mask covers the wells.**

Initials: RCJ Date: 2/6/95

6. Place pre-wet, labeled membrane on top of the gel so that the nick is in the upper left-hand corner of the gel. Remove any trapped air bubbles by carefully rolling with a disposable pipet.

7. Pre-wet 2 sheets of GB002 in Denaturation Solution and place directly on top of the membrane. Do not allow these sheets to be in contact with the sheets or sponges below the gel. Remove any trapped air bubbles by carefully rolling with a disposable pipet.

8. Place 5 sheets of dry GB004 on top of sheets of GB002. Dry off a gel bed and place on top of GB004 as a weight.

9. Carefully add Denaturation Solution to the tray so that there is a reservoir of solution which can be drawn up through the gel. Do not add solution above the bottom of the gel.

10. Allow transfer to proceed for 4 hours. Each hour during the transfer, remove wet GB004 and replace with 5 new sheets of GB004 and replenish reservoir with Denaturation Solution.

11. Disassemble the transfer apparatus and peel nylon membrane away from the gel.

12. With a gloved hand, gently rub membrane in 500 mL of Gel Neutralization Buffer. After rubbing the membrane, rinse in 500 mL of fresh Gel Neutralization Buffer.

13. Air dry the membrane on a sheet of GB002 for at least 10 minutes and then bake until dry in 80°C forced air oven (30-60 minutes) or dry the membrane overnight at room temperature.

14. Rinse sponges and x-ray mask in water.

Initials: RC Date: 6/21/95

HYBRIDIZATION

Blots should be hybridized to probes in the following order. D2S44, D10S28, D4S139, and D7Z2

1. Prewarm the Hybridization Solution, glass hybridization bottles, and oven to 65°C for at least 1 hour. **After warming, make sure the hybridization solution is well mixed. Mix by inverting the bottle several times.**

2. Pre-wet meshes and hybridization membranes in a suitable tray containing 2X SP. There should be 1 mesh per membrane.

3. Alternate meshes and membranes into an even stack; finish with one more mesh. Up to 5 membranes can be hybridized in a single bottle.

4. Roll the membrane/mesh stack away from you into a tight roll. When done rolling leave the stack in place and hold the rolled stack together with your left hand.

5. Grasp the hybridization bottle with your right hand and place the membranes into the open bottle from left to right.

6. Add 30 mL of 2X SP to the bottle.

7. Place the bottle on a flat surface. Rock the bottle backwards and forwards to attach the first part of the membranes to the bottle. Then roll the bottle in order to fully unwind the mesh and membranes. No fixed air bubbles should be visible between the membranes and the bottle. If fixed bubbles are present the membranes should be removed and re-rolled.

8. Pour off the 2X SP. Make sure it is completely poured off before pouring in 20 mL of Hybridization Solution.

9. Replace the cap and put the bottle in the hybridization oven with the cap pointing to the **left**. The rotisserie should be balanced in the same manner as a centrifuge.

10 Turn on the rotor and allow membranes to pre-hybridize for 15-180 minutes.

Initials: RC1 Date: 5/9/95

HYBRIDIZATION

Blots should be hybridized to probes in the following order. D2S44, D10S28, D17S26, D4S139, D7Z2, (DYZ1 if necessary).

1. Prewarm the Hybridization Solution, glass hybridization bottles, and oven to 65°C for at least 1 hour. **After warming, make sure the hybridization solution is well mixed. Mix by inverting the bottle several times.**

2. Pre-wet meshes and hybridization membranes in a suitable tray containing 2X SP. There should be 1 mesh per membrane.

3. Alternate meshes and membranes into an even stack; finish with one more mesh. Up to 5 membranes can be hybridized in a single bottle.

4. Roll the membrane/mesh stack away from you into a tight roll. When done rolling leave the stack in place and hold the rolled stack together with your left hand.

5. Grasp the hybridization bottle with your right hand and place the membranes into the open bottle from left to right.

6. Add 30 mL of 2X SP to the bottle.

7. Place the bottle on a flat surface. Rock the bottle backwards and forwards to attach the first part of the membranes to the bottle. Then roll the bottle in order to fully unwind the mesh and membranes. No fixed air bubbles should be visible between the membranes and the bottle. If fixed bubbles are present the membranes should be removed and re-rolled.

8. Pour off the 2X SP. Make sure it is completely poured off before pouring in 20 mL of Hybridization Solution.

9. Replace the cap and put the bottle in the hybridization oven with the cap pointing to the **left**. The rotisserie should be balanced in the same manner as a centrifuge.

10 Turn on the rotor and allow membranes to pre-hybridize for 15-180 minutes.

Initials: RD Date: 2/1/95

HYBRIDIZATION

Blots should be hybridized to probes in the following order. D2S44, D10S28, D17S26, D4S139, D7Z2, (DYZ1 if necessary).

1. Prewarm the Hybridization Solution, glass hybridization bottles, and oven to 65°C for at least 1 hour. **After warming, make sure the hybridization solution is well mixed. Mix by inverting the bottle several times.**

2. Pre-wet meshes and hybridization membranes in a suitable tray containing 2X SSC. There should be 1 mesh per membrane.

3. Alternate meshes and membranes into an even stack; finish with one more mesh. Up to 5 membranes can be hybridized in a single bottle.

4. Roll the membrane/mesh stack away from you into a tight roll. When done rolling leave the stack in place and hold the rolled stack together with your left hand.

5. Grasp the hybridization bottle with your right hand and place the membranes into the open bottle from left to right.

6. Add 30 mL of 2X SP to the bottle.

7. Place the bottle on a flat surface. Rock the bottle backwards and forwards to attach the first part of the membranes to the bottle. Then roll the bottle in order to fully unwind the mesh and membranes. No fixed air bubbles should be visible between the membranes and the bottle. If fixed bubbles are present the membranes should be removed and re-rolled.

8. Pour off the 2X SSC. Make sure it is completely poured off before pouring in 20 mL of Hybridization Solution.

9. Replace the cap and put the bottle in the hybridization oven with the cap pointing to the **left**. The rotisserie should be balanced in the same manner as a centrifuge.

10 Turn on the rotor and allow membranes to pre-hybridize for 15-180 minutes.

Initials: [illegible] Date: 2/6/95

11. If using commercially labeled probes:

    During the above pre-hybridization:

    A. Remove the labeled probes from the freezer and thaw.

    B. Add 200 μL heparin to each tube.

    C. Place the tubes in a 95°C heat block for 8-10 minutes to denature the probes. Make sure there is sufficient sand in the heat block.

    D. Transfer the tubes containing the probes from the heat block to an ice bath.

    E. Continue at step 13.

12. If using probes labeled in-house:

    During the above pre-hybridization:

    A. Remove the labeled probes from the freezer and thaw.

    B. For each probe, label a screw top microcentrifuge tube and add 250 μL of salmon sperm DNA and 200 μL of heparin.

    C. Aliquot out the amount of probe needed (see Appendix A) and 20 x 10^6 dpm of the labeled BRL Sizing Standard into each tube.

    D. Place the tubes in a 95°C heat block for 8-10 minutes to denature the probes.

    E. Transfer the tubes containing the probes from the heat block to an ice bath. Leave the probe on ice for at least 5 minutes.

13. Remove the bottle from the hybridization oven and pour the prehybridization solution into a 50 mL disposable centrifuge tube.

14. Add the probe to the prehybridization solution and **mix** gently.

15. Pour the prehybridization solution back into the hybridization bottles. Replace the cap.

16. Place the bottle back in the hybridization oven with the cap pointing to the **left** and rotate for 12-24 hours at 65°C.

Initials: RCJ Date: 2/1/95

WASHING OF MEMBRANES

1. Make sure there is enough Wash Solution #1 and Wash Solution #2 to wash the number of membranes hybridizing. Preheat the wash solutions and water baths to 65 °C. THIS NEEDS TO BE STARTED AT LEAST THE NIGHT BEFORE!

2. Before beginning washing, measure the temperature of the wash solutions and waterbaths. Do not proceed if the temperatures are below 63°C.

3. Remove a bottle from the 65°C incubator and pour the Hybridization Solution onto a plastic backed absorbent pad. Discard the pad in the radioactive waste.

4. Add 25 mL of Wash Solution #1 to the bottle. Return to the rotisserie for 1 minute and discard as above.

5. Repeat step 4 one more time.

6. Add 125 mL of Wash Solution #1 to the bottle. Cap and swirl. Discard down the "hot" sink.

7. Add 1500 mL of Wash Solution #1 to a rectangular container and place up to 10 radioactive membranes and mesh into the solution. Do not separate the membranes from the mesh.

8. Place the lid on the container and put into a 65°C shaking water bath for 25-30 minutes.

9. Carefully pour out the liquid, add 1500 mL of fresh Wash Solution #1 and put the container into a 65°C shaking water bath for 25-30 minutes.

10. Carefully pour out the liquid, add 2000 mL of Wash Solution #2, place the lid on the container and put it into a 65°C shaking water bath for 20-25 minutes.

11. Carefully pour out the liquid. Blot membranes dry between blotting paper. The membranes should not dry to completion, but rather remain slightly damp. If they are too dry, it is more difficult to remove (strip) the radioactive probes.

12. Proceed to autoradiography on the next page or place membranes in plastic and store at 4°C indefinitely.

Initials: RCJ Date: 2/1/95

AUTORADIOGRAPHY

1. Wrap the damp membranes in Glad or Saran wrap. Do not use Reynolds food wrap for this step!

2. Prepare RadTape labels with membrane number, probe used, date and analyst's initials. Stick the labels on the plastic wrap on the side without the pencilled membrane information.

3. In the darkroom under red light illumination, place up to 3 membranes onto XAR film. Tape the membranes to the film. Place another sheet of XAR film on top of the membranes and close the cassette.

4. Label the cassettes on the side with Analyst's initials, date, membrane numbers, and a develop date, if known.

5. Place the cassette at -70°C with the label showing.

6. The next day, remove the top film and develop. Use this overnight exposure as a guide to determine the length of time the bottom film needs to be left in place.

Initials: RCJ Date: 6/6/95

ASSESSMENT OF AUTORADIOGRAPHY DATA

Total casework assessment can only be made after all typing probes and the monomorphic probe have been completed.

There are four major steps in the assessment of autoradiographic (autorad) data: visual evaluation, computer-assisted sizing, match determination, and generation of statistical data.

1. Visual evaluation of autorads - each autorad must have the appropriate Cell Pellet Control and BRL sizing standards.

   A. Examine the lane containing the Cell Pellet Control. There must be either 1 or 2 dark bands, depending on which RFLP locus has been probed. If the Cell Pellet Control does not exhibit the expected number of bands for the locus being probed, the autorad cannot be assessed further.

   B. Examine the lane containing the Cell Pellet Control band(s) for their position relative to the adjacent size markers. The Cell Pellet Control band(s) should be located in their expected position on the autorad. If the Cell Pellet Control band(s) are not found in their visually expected position, the autorad cannot be assessed further.

   C. Visually inspect the lanes that contain the BRL Sizing Standard. Thirty bands should be present in each of those lanes; however, it is possible for the smallest bands to run off the gel during an extended run. The bands in these lanes must be of sufficient intensity to enable them to be used as size references for the Cell Pellet Control, the exemplars and the evidence samples bands. If regions of the size ladder lanes are not visible, specimen bands cannot be sized in these regions.

   D. Visually inspect the lanes that contain exemplar or evidence sample DNA to assess the quality of the bands. Determine if the bands in these lanes are extremely broad or exhibit pronounced band curvature. These band irregularities can signal potential mobility shifts.

   E. Based on the assessment of band quality and band position decide which of the bands are suitable for computer-assisted band sizing procedure.

Initials: RD Date: 2/1/95

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   B. Examine the lane containing the Cell Pellet Control band(s) for their position relative to the adjacent size markers. The Cell Pellet Control band(s) should be located in their expected position on the autorad. If the Cell Pellet Control band(s) are not found in their visually expected position, the autorad cannot be assessed further.

   C. Visually inspect the lanes that contain the BRL Sizing Standard. Thirty bands should be present in each of those lanes; however, it is possible for the smallest bands to run off the gel during an extended run. The bands in these lanes must be of sufficient intensity to enable them to be used as size references for the Cell Pellet Control, the exemplars and the evidence samples bands. If regions of the size ladder lanes are not visible, specimen bands cannot be sized in these regions.

   D. Visually inspect the lanes that contain exemplar or evidence sample DNA to assess to quality of the bands. Determine if the bands in these lanes are extremely broad or exhibit pronounced band curvature. These band irregularities can signal potential mobility shifts.

   E. Based on the assessment of band quality and band position decide which of the bands are suitable for computer-assisted band sizing procedure.

Initials: RS Date: 6/22/95

2. Computer-assisted band size determination is carried out using the BioImager. The sizing program enables an objective determination of the sizes of the DNA fragments in the Cell Pellet Control, exemplars, and evidence samples by comparing band positions to the BRL Sizing Standards.

A. The case analyst and a second person must scan, analyze and print out band sizes for each autorad (Appendix C). Each analyst must agree on which bands are suitable for sizing and the band sizes must be within ±2.5% of each other. If the band sizes are greater than ±2.5% of each other, the autorad must be re-sized by both analysits. If the new band sizes are within ±2.5% of each other than the new sizings are used. If the sizes are still greater than ±2.5%, than the sizings must be reviewed by a supervisor for a possible sizing error. If there is a disagreement in the number of bands than the autorad must be reviewed by a supervisor for another objective opinion and to mediate a discussion between the analysts. If both analysts still disagree on the number of bands, only those bands agreed upon by both analysts are reported. Once these requirements are met, the case analyst continues with his/her values. The values of the second person are just used for verification.

B. Compare the Cell Pellet Control band sizes for each probe to their published values (NIST). The Cell Pellet Control band sizes must be within ±2.5% of the published values. If not, the autorad is inconclusive.

Locus	High Band	Acceptable Range High Band	Low Band	Acceptable Range Low Band
D2S44	2907	2835-2979	1791	1747-1835
D4S139	6474	6313-6635	3438	3353-3523
D7Z2	2725	2657-2793	n/a	n/a
D10S28	1757	1714-1800	1182	1153-1211

C. Compare the monomorphic probe (D7Z2) band sizes for the exemplars and evidence samples to the published value. The band sizes must be within ±2.5% of the published value. If not, the exemplar or evidence sample can not be used for comparison purposes.

3. Matching Criteria

A. Visual Match - The analyst should visually inspect the autorad and decide whether exemplar and evidence samples may match. This judgement is based on the analyst's experience and is confirmed by an independent observer.

Initials: RCJ Date: 6/6/95

2. Computer-assisted band size determination is carried out using the BioImager. The sizing program enables an objective determination of the sizes of the DNA fragments in the Cell Pellet Control, exemplars, and evidence samples by comparing band positions to the BRL Sizing Standards.

   A. The case analyst and a second person must scan, analyze and print out band sizes for each autorad (Appendix C). Each analyst must agree on which bands are suitable for sizing and the band sizes must be within ±2.5% of each other. Any disagreements must be reviewed by a supervisor. Once these requirements are met, the case analyst continues with his/her values. The values of the second person are just used for verification.

   B. Compare the Cell Pellet Control band sizes for each probe to their published values (NIST). The Cell Pellet Control band sizes must be within ±2.5% of the published values. If not, the autorad is inconclusive.

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D10S28	1757	1714-1800	1182	1153-1211
D17S26	4866	4745-4987	1372	1338-1410

   C. Compare the monomorphic probe (D7Z2) band sizes for the exemplars and evidence samples to the published value. The band sizes must be within ±2.5% of the published value. If not, the exemplar or evidence sample can not be used for comparison purposes.

3. Matching Criteria

   A. Visual Match - The analyst should visually inspect the autorad and decide whether exemplar and evidence samples may match. This judgement is based on the analyst's experience and is confirmed by an independent observer.

   B. Computer Match - If there is a visual match, the exemplars and evidence samples should be subjected to a computer aided determination of a match. For two samples to match, they must be within ±2.5% of each other.

      δ= (hi size - lo size)/lo size

Initials: RO Date: 2/1/95

2. Computer-assisted band size determination is carried out using the BioImager. The sizing program enables an objective determination of the sizes of the DNA fragments in the Cell Pellet Control, exemplars, and evidence samples by comparing band positions to the BRL Sizing Standards.

A. The case analyst and a second person must scan, analyze and print out band sizes for each autorad (Appendix C). Each analyst must agree on which bands are suitable for sizing and the band sizes must be within ±2.5% of each other. Any disagreements must be reviewed by a supervisor. Once these requirements are met, the case analyst continues with his/her values. The values of the second person are just used for verification.

B. Compare the Cell Pellet Control band sizes for each probe to their published values (NIST). The Cell Pellet Control band sizes must be within ±2.5% of the published values. If not, the autorad is inconclusive.

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D7Z2	2725	2657-2793	n/a	n/a
D10S79	1757	1714-1800	1182	1153-1211
D17S28	4866	4745-4987	1372	1338-1410

C. Compare the monomorphic probe (D7Z2) band sizes for the exemplars and evidence samples to the published value. The band sizes must be within ±2.5% of the published value. If not, the exemplar or evidence sample can not be used for comparison purposes.

3. Matching Criteria

A. Visual Match - The analyst should visually inspect the autorad and decide whether exemplar and evidence samples may match. This judgement is based on the analyst's experience and is confirmed by an independent observer.

B. Computer Match - If there is a visual match, the exemplars and evidence samples should be subjected to a computer aided determination of a match. For two samples to match, they must be within ±2.5% of each other.

δ= (hi size - lo size)/lo size

Initials: RJ Date: 6/21/95

B. Computer Match - If there is a visual match, the exemplars and evidence samples should be subjected to a computer aided determination of a match. For two samples to match, they must be within ±2.5% of each other.

δ= (hi size - lo size)/lo size

4. Statistical information is generated for all confirmed matches. All racial databases are searched, using a fixed bin approach and population frequencies are determined (Appendix D).

Initials: RO Date: 2/6/95

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Initials: RCJ Date: 2/6/95

STRIPPING OF PROBE FOR RE-USE OF MEMBRANES

A. In plastic containers:

1. Prewarm freshly made stripping solution to 65°C. Measure the temperature of the stripping solution and make sure it is at least 63°C before proceeding.

2. Remove plastic wrap from membranes.

3. Add 80-100 mL of prewarmed stripping solution to a rectangular plastic container with a lid.

4. Place up to 10 nylon membranes into the stripping solution.

5. Place the container in a 65°C shaking water bath. Allow the membranes to shake for one hour.

6. After one hour, remove the container from the 65°C shaking water bath. Carefully pour out the liquid.

7. With a gloved hand, gently rub membranes, one at a time, in 500 mL Wash Solution #2. After rubbing membrane, soak in 500 mL of fresh Wash Solution #2 for 5 minutes.

8. Blot membranes dry between blotting paper. The membranes should not dry to completion, but rather remain slightly damp.

9. If necessary, to verify complete stripping, expose membranes to x-ray film overnight.

10. Membranes can be rehybridized at this point or placed in plastic and stored at 4° C indefinitely.

B. In Roller Bottles:

1. Prewarm freshly made stripping solution to 65°C. Measure the temperature of the stripping solution and make sure it is at least 63°C before proceeding.

2. Remove plastic wrap from membranes.

3. Follow steps 2-8 (page 30) to add the membranes to the bottles. Up to 5 membranes may be placed in each bottle.

Initials: RC Date: 2/1/95

4. Add 10 mL of Stripping solution per membrane to each bottle. Use a minimum of 30 mL of Stripping solution.

5. Rotate membranes for 90-120 minutes at 65°C.

6. Pour out the stripping solution and rinse the bottle twice with 100 mL Wash Solution #2.

7. Remove the membranes from the bottle and with a gloved hand, gently rub membranes, one at a time, in 500 mL Wash Solution #2. After rubbing membrane, soak in 500 mL of Wash Solution #2 for 5 minutes at room temperature.

8. Blot membranes dry between blotting paper. The membranes should not dry to completion, but rather remain slightly damp.

9. If necessary, to verify complete stripping, expose membranes to x-ray film overnight.

10. Membranes can be rehybridized at this point or placed in plastic and stored at 4° C indefinitely.

Initials: RC Date: 6/21/95

APPENDIX A- RADIOACTIVE LABELING OF PROBES

THE PRECAUTIONS AND PROCEDURES OF THE RADIATION SAFETY MANUAL MUST BE FOLLOWED WHEN LABELING PROBE

I. Labeling Insert Probes

Different quantities of insert are labeled depending on the locus as follows:

Probe Name	Locus probed	ng insert labeled
YNH24	D2S44	25
PH30	D4S139	50
Monomorph	D7Z2	25
TBQ7	D10S28	25

Promega's Prime-a-Gene Labeling System

1. Remove the ^{32}P-dCTP (3000 Ci/mmole) from the freezer and place behind a shield.

2. Allow the kit components to thaw on ice. Nevertheless keep the Klenow enzyme at -20°C and return it to the freezer immediately after using.

3. Place the appropriate quantity of probe in a screw-cap microcentrifuge tube (see Table above). Add water to achieve a final volume of 30 μL.

4. Place tube in a 95°C heat block for 8 minutes. Immediately after heating, place the tube into crushed ice for 5 minutes. Spin briefly.

Initials: RCJ Date: 2/1/95

APPENDIX A- RADIOACTIVE LABELING OF PROBES

THE PRECAUTIONS AND PROCEDURES OF THE RADIATION SAFETY MANUAL MUST BE FOLLOWED WHEN LABELING PROBE

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Probe Name	Locus probed	ng insert labeled
YNH24	D2S44	25
PH30	D4S139	50
Monomorph	D7Z2	25
TBQ7	D10S28	25
EFD52	D17S26	25

Promega's Prime-a-Gene Labeling System

1. Remove the ^{32}P-dCTP (3000 Ci/mmole) from the freezer and place behind a shield.

2. Allow the kit components to thaw on ice. Nevertheless keep the Klenow enzyme at -20°C and return it to the freezer immediately after using.

3. Place the appropriate quantity of probe in a screw-cap microcentrifuge tube (see Table above). Add water to achieve a final volume of 30 μL.

4. Place tube in a 95°C heat block for 8 minutes. Immediately after heating, place the tube into crushed ice for 5 minutes. Spin briefly.

Initials: RCJ Date: 2/1/95

5. While on ice add to the tube:

   10 μL 5X labeling buffer
   2 μL dNTP Mix (0.5 mM dATP, dGTP, dTTP)
   2 μL nuclease free BSA (10mg/mL)

   Mix and spin briefly

   1 μL Klenow fragment
   5 μL ^{32}P-dCTP (50 μCi at 3000 Ci/mmole)

   Mix and spin briefly

6. Incubate at room temperature for 1 hour.

   NOTE: It is very easy to contaminate the tube threads with radioactivity. Once the threads are contaminated, the radioactivity will be transferred to the worker's fingers and thence transferred to anything else that is touched. To avoid such a scenario, spin the tube each time its contents are mixed. Check fingers for radioactivity frequently.

7. Terminate the reaction by adding 25 μL of 50 mM EDTA.

8. Determine the nucleotide incorporation according to the protocol on page 45.

9. If the incorporation is $>50\%$, the probe may be used directly. Otherwise, continue with the probe purification protocol on page 43.

Initials: RCJ Date: 2/1/95

II. Labeling Oligo Probes

1. Remove the ^{32}P-dGTP (3000 Ci/mmole) from the freezer and place behind a shield.

2. Remove a marked, premixed oligo labeling tube from the freezer and spin briefly. Add the following:

   6 μL 25 mM $MgCl_2$
   3 μL sterile dH_2O
   1 μL Taq Polymerase (5 U/μL)

   Mix and spin briefly

   5 μL ^{32}P-dGTP (10 mCi/mL at 3000 Ci/mmole)

   Mix and spin briefly

3. Program the Gene Machine Temperature Cycler as follows:
   This should be stored as Program #1

   95°C 1 min
   37°C 1 min
   72°C 1 min

   25 cycles

   72°C 4 min
   4°C Hold

4. Incubate the tubes in the Temperature Cycler using the above program.

5. Terminate the reaction by adding 20 μL of 50 mM EDTA.

6. Determine the nucleotide incorporation according to the protocol on page 45.

7. If the incorporation is >50%, the probe may be used directly. Otherwise, continue with the probe purification protocol on page 43.

Initials: RCJ Date: 2/1/95

III. Labeling Molecular Sizing Standards

BRL Sizing Standard Cat # 4401SA

1. Remove the ^{32}P-dCTP (3000 Ci/mmole) from the freezer and place behind a shield.

2. In a clean microcentrifuge tube place:

   44 μL Solution C (Labeling Solution)
   5 μL α^{32}P-dCTP (10 mCi/mL, 3000 Ci/mmole)
   1 μL DNA Polymerase large Fragment (3 units)

   Mix and spin briefly

3. Incubate for 1 hr at 23°C-25°C.

4. Terminate the reaction by the addition of 50 μL Stop Buffer.

5. Determine the nucleotide incorporation according to the protocol on page 45.

6. If the incorporation is >40%, the probe may be used directly. Otherwise, continue with the probe purification protocol on page 43.

Initials: RCJ Date: 2/1/95

IV. Probe Purification

Probes are purified on Stratagene's push columns (nuc traps)

1. Insert a microcentrifuge tube into the hole in the beta shield base.

2. Remove the end caps from the push column.

3. Pipet 70 μL of 1 X STE directly on top of the column.

   Note: Some of the resin may be displaced during shipping. Do not remove the loose resin, since it is important that all the resin be used.

4. Extend the plunger on a 10 cc syringe. Attach the syringe to the push column. Press the plunger to force the liquid down the length of the push column until small drops exit the end of the push column. The resin should wet as the liquid travels the length of the push column. For best results use the pre-wetted column within 5-10 minutes.

   Note: The plunger of the syringe must be extended before the syringe is locked on the push column, otherwise the resin will be sucked up into the syringe as the plunger is extended.

5. While holding open the locking device on the column beta shield, insert the push column three-fourths of the way in the locking device in the column beta shield. Release the locking device to firmly fix the position of the column. Place the column beta shield with the attached push column on the beta shield base. Make sure there is a collection tube in the beta shield base.

6. Add the 70 μL of probe to the top of the push column.

7. Extend the plunger on the 10 cc syringe. Attach the syringe to the push column. While holding the column locking device push the column down until the syringe fits snugly against the column beta shield. (Note: The plunger must remain extended.) Make sure the end of the push column is in the top of the collection tube.

8. Place the syringe cover over the extended plunger of the syringe. Place a slow and constant pressure directly down on the syringe cover to force the sample through the push column in 25 - 35 seconds. The plunger should be forced down at a slow and constant rate, **NOT** all at once.

9. Remove the syringe cover and unscrew the syringe from the top of the push column.

Initials: RC Date: 2/1/95

10. Add 80 μL of 1 X STE to the top of the push column. This liquid should be added as the push column remains in the column beta shield. Push this wash through as before. If at the end of this second push there is a drop of liquid at the bottom of the push column, a bolus of air can be pushed through the column to dislodge the drop.

11. The probe is in the microcentrifuge tube and the unincorporated small molecules remain in the push column resin.

12. Add 340 μL of 1 X STE to the probe. Determine the quantity of the probe according to the procedure in the next section.

13. Monitor all parts of the apparatus for radioactivity. To remove the column, press the push column locking device and let the column drop out into the radioactive waste container.

Initials: RCJ Date: 2/1/95

V. Probe QA/QC- Incorporation and Usage Calculations

There are two different type of filters which can be used, DE-81 or GF/C.

1. Using a soft-lead pencil label the appropriate number of filters (2.4-cm diameter). The pencil markings may be washed off so also mark the filters with a series of scissor nicks.

2. Dilute probe 50-fold by adding 2 μL of probe to 98 μL 1 X STE.

3. Spot 2 μL of diluted probe on the center of each of two labeled filters.

4. Store the filters at room temperature until all of the fluid has evaporated.

5. Using blunt-end forceps, transfer one of each pair of filters to a plastic weigh boat containing approx. 100 mL (enough to cover the bottom) of ice-cold 0.5 M Na_2HPO_4 (pH 7.0) for DE-81 filters or 100 mL of ice-cold 5% TCA, 20 mM Sodium Pyrophosphate for GF/C filters. Using the forceps, swirl the filters in the solution for 2 minutes.

6. Transfer the filters to a fresh weigh boat containing the same volume of the solution used above and swirl as before.

7. Repeat the washing two more times for a total of 4 washings.

8. Transfer the washed filters to a weigh boat containing 70% ethanol and allow them to remain there briefly. Dry the filters briefly at room temperature on a piece of blotting paper.

9. Insert each of the filters (washed and unwashed) into a scintillation vial.

10. Add 3 mL of scintillation fluor to each vial. Measure the amount of radioactivity on each filter.

11. Compare the amount of radioactivity on the unwashed filter with the amount on the washed filter, and then calculate the proportion of the precursor that has been incorporated:

$$\frac{\text{cpm in washed filter}}{\text{cpm in unwashed filter}} = \text{proportion incorporated}$$

cpm in washed filter * dilution * 0.5 * .9 = dpm/μL

Initials: RC Date: 6/21/95

12. The concentration of radioactivity that must be placed in the hybridization solution is a function of the particular probe used. These concentrations are shown in the following table. Using this table and the calculations from the incorporation assay, calculate the amount of probe to be pipetted and used in a hybridization.

Probe	DPM/ 20 mL hybridization solution
D2S44	3.0×10^7
D4S139	1.5×10^7
D7Z2	1.5×10^7
D10S28	3.0×10^7
BRL Sizing Stds.	2×10^7

Initials: RCJ Date: 2/1/95

12. The concentration of radioactivity that must be placed in the hybridization solution is a function of the particular probe used. These concentrations are shown in the following table. Using this table and the calculations from the incorporation assay, calculate the amount of probe to be pipetted and used in a hybridization.

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D2S44	3.0×10^7
D4S139	1.5×10^7
D7Z2	1.5×10^7
D10S28	3.0×10^7
D17S26	3.0×10^7
BRL Sizing Stds.	2×10^7

Initials: RC Date: 2/1/95

APPENDIX B- MOLECULAR SIZE STANDARDS

The marker fragment bands can be easily identified by counting from the gap in the marker pattern at approximately 1 kilobase (kb).

Fragment Sizes (base pairs):

22,621	
15,004	
11,919	
9,416	
8,271	
7,421	
6,442	
5,861	
5,415	
4,716	
4,333	
3,812	
3,397	
3,101	
2,876	
2,650	
2,433	
2,213	
2,015	
1,861	
1,672	
1,568	
1,431	
1,287	
1,176	
	"1-kb" gap
993	
910	
784	
653	
526	

Initials: RC Date: 6/21/95

APPENDIX C - AUTORAD SCANNING AND ANALYSIS

Autorad processing can be done in three stages: scanning the image into the BioImager, analysis of the bands, and entering lane information needed for the database. These need not be done at the same time, but the status of each autorad MUST be kept track of.

POWERING UP

Turn on power switches for camera and light box, if not already on.

Turn on main power strip; it will take about 10 minutes for the system to boot up.

LOGGING IN

At login: type your last name (max 8 characters) and hit **return**. You will then be at the Main Menu.

TO SCAN

Be ready to scan lots of autorads at one time. If there are multiple exposures of the same membrane/probe combination, pick the best one (for example, a 4 day that shows all samples). DO NOT do more than one since that could result in the samples being represented twice in the database.

1. Begin at Main Menu. Click on "Scan". Click on "Camera"

2. Place an "average" autorad on the light box, surrounded by a mask. Adjust so that the entire ladder is visible. Focus if needed. Adjust exposure time (use "+" and "-") if needed.

3. Hit "e" to equalize. Remove autorad. Put the OD wedge on the light box and center it horizontally on the blue line. Adjust exposure time until the light meter peak at the bottom is on scale.

4. Press "c" to calibrate. Remove OD wedge. Return autorad to light box and re-adjust image.

5. Press "s" to scan. When prompted for image name, type it in using the format; **m123.d4.4** where the first part is the membrane number or case number (FB95-xxx), then the probe (d2, d4, d10, d7) and the last part is the number of days exposure.

   The BioImager software is case sensitive. Use upper and lower case letters exactly as shown.

Initials: RCJ Date: 2/1/95

APPENDIX C - AUTORAD SCANNING AND ANALYSIS

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1. Begin at Main Menu. Click on "Scan". Click on "Camera"

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3. Hit "e" to equalize. Remove autorad. Put the OD wedge on the light box and center it horizontally on the blue line. Adjust exposure time until the light meter peak at the bottom is on scale.

4. Press "c" to calibrate. Remove OD wedge. Return autorad to light box and re-adjust image.

5. Press "s" to scan. When prompted for image name, type it in using the format; **m123.d4.4** where the first part is the membrane number or case number (FB95-xxx), then the probe (d2, d4, d10, d17, d7) and the last part is the number of days exposure.

   The BioImager software is case sensitive. Use upper and lower case letters exactly as shown.

Initials: RCJ Date: 2/1/95

6. Click on "done". When you hear the camera click, the scan is complete and you can change autorads.

7. Click on "acknowledge" when prompted.

8. Continue scanning. Repeat steps 5-7 to scan additional autorads.

9. Hit "delete" when you are done with all scanning. Click "yes" to exit.

Bundle up the autorads and clearly mark them as "scanned, need WBA and lane info" if you are not continuing.

WHOLE BAND ANALYSIS (WBA)

Before you begin whole band analysis, have with you the autorads, analytical gel worksheets, and the MB logbook (if lane information is going to be entered). To use the time-saving lane configuration files and lane information files, analyze the autorads from each membrane set as follows.

Bring up image:

Click on "Whole band". Click on "Editor".

Type in image name. Click on "done".

When the image appears, compare to the autorad and analytical gel worksheet to make sure no images or membranes were misnamed.

Choose configuration file:

Go to "Utilities" and click on "use configuration file"; or use "J", shift-j.

Select the configuration file "16lane".

If lane information has been entered, select the corresponding info file; if no lane information files exist yet, select "off" for the lane info files.

Click on "done".

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Define lanes and pick bands automatically:

Use the cursor to pick top center of first lane; use "R1" to zoom in, "R2" or "R3" to zoom out. Drag and click to define lane curves, etc. Hit "e" to finish first lane. Do the same for the last lane. Hit "e" to end.

The BioImager will then automatically assign lane parameters and pick bands using the parameters previously defined as "16lane."

Check that each ladder lane has 30 bands picked. If there are more than 30, manually delete the extra "bands." If there are less, adjust parameters as described below and re-find bands. **If the ladders are too weak to scan, the entire autorad is inconclusive and there is no need to continue. If some of the ladder bands are missing or unable to be interpreted then those samples with bands in the area of the "bad" ladder bands are inconclusive.**

Check that the bands picked in each lane are the "true" bands seen on the autorad. Spots, spillover from adjacent lanes, etc., may be recognized as bands by the BioImager.

Do any necessary parameter changes, re-finding of bands, and/or deleting of bands as described below.

Assign Y-standards as described below.

Print a report, if needed, as described below.

Manual finding and editing of bands and parameters:

Click on "find bands in lanes". Click on "default" to choose all lanes or choose the lane(s) desired. Click on "done".

There are two ways to remove unwanted bands: use "delete" to manually remove them, or adjust parameters and re-find bands. In order to best use the lane configuration files, it is better to adjust parameters.

There is one acceptable way to add bands to non-ladder lanes, adjusting parameters and re-finding bands.

Initials: RC Date: 2/1/95

Go to "Utilities" and click on "Set parameters" or use shift-p then try the following:

Set "limit of darkest bands..." to 2 (or more) and click on "done" (if you are analyzing a D7Z2 autorad, set "limit of darkest bands..." to 1). Then re-find bands in all non-ladder lanes. This should solve most extra bands; manually delete any found in the negative lane.

For lanes where the true bands were not found, try adjusting other parameters; re-find bands **only in those lanes that need it.**

Adjusting individual lane boundaries can help center bands, exclude strong bands encroaching from other lanes, etc. Re-find bands **only in those lanes that need it.**

When all else fails, manually delete "bands" as needed.

NEVER MANUALLY ADD BANDS IN LANES OTHER THAN LADDER LANES.

Assign Y-standards:

Click on "Assign Y-standards trains". Click on "BRL". Click on "done".

Click on those lanes that have the ladder in them (usually 1, 4, 10, 16). Click on "done".

If there are more or less than 30 bands in the standard lanes, the BioImager will alert you. Click on "acknowledge". Delete the standards from the lane(s), fix, re-find bands, and re-assign Y-standards to that lane.

ADDING LANE INFORMATION

If you prefer, lane information can be added later by two people. The previously analyzed image needs to be recalled first.

Go to "Edit lanes" and click on "edit lane info"; or use "i".

Click on "default" to choose all lanes. Click on "done".

Initials: RCJ Date: 2/1/95

Type in lane descriptions, **using upper and lower case as shown:**

BRL for ladder lanes
CPC for cell pellet control lanes
NEG for negative control lanes
mb-xxxx for database samples

V(x) for victim	(x) for number of victim if multiple victims
S(x) for suspect	(x) for number of suspect if multiple suspects
Q(x)(y) for evidence	(x) for number of evidence if multiple items: (y) for m or f for male or female fractions, respectively, if applicable

Type in victim's and suspect's characteristics, **using upper case as shown:**

1: race B, W, H, I (India, Pakistani, etc.), O (Oriental)

Click on "done" and continue for all lanes. If there is no sample in a lane, skip it by clicking on "done".

Save lane information:

Go to "Utilities" and click on "save configuration file"; or use "j".

Select "off" for the lane configuration.

Name the lane information file "m000" to correspond to the membrane number. This file will be used with the rest of the autorads corresponding to that membrane.

If desired, print as described later.

To exit the WBA, hit "delete". Click on "yes" to save your work.

TO PRINT REPORTS

If needed, bring up image as previously described.

Click on "Report band list". Click on all non-ladder lanes.

Click on "print"; change template to "matrix". Turn off screen display of report by clicking on "show". Click on "done".

Initials: RCJ Date: 2/1/95

LOGGING OUT

Click on "exit" from any menu.

Click **outside** all windows. Click on "Exit Sunview". Click on "confirm".

DO NOT SHUT THE POWER OFF! THE POWER IS USUALLY LEFT ON.

Initials: RC Date: 5/9/95

APPENDIX D- STATISTICAL CALCULATIONS

Determination of Match Frequencies

Once it has been decided that an RFLP match exists, see sections, see Assessment of Autoradiography Data, page 35, it is necessary to determine the strength of that match. The statistical method used will be the Fixed Bin Ceiling Approach adapted by TWGDAM[1] from the "interim" ceiling principle described in the 1992 NRC report[2].

The "interim" ceiling principle defines each band as the maximum of the 95% upper confidence limit of the fixed bin frequency in each general database (Caucasians, Blacks, Hispanics or Asians) or a minimum of 0.100. In using this approach, the frequency of a single locus and/or multiple locus profile is obtained by the product of these "interim" ceiling frequencies.

A. Calculation Guidelines

1. Preliminary Tests for Population Databases

a. General Considerations

Before performing frequency estimate calculations, it is necessary to establish conformance to Hardy-Weinberg and pair-wise linkage equilibrium expectations (i.e., two-locus independence tests) using a global test[3,4,5] in three out of the four major population groups, i.e., Caucasians, Blacks, Hispanics and Asians. The Global test is performed on each population data set. If all loci meet Hardy-Weinberg and pair-wise linkage equilibrium expectations, calculation of the ceiling principle estimate can be performed.

If the global test for any locus is not in equilibrium, a local test[5] will be performed only on the alleles in the particular population sample. Global and Local tests can only be performed using a computer. If Hardy Weinberg and linkage equilibrium expectations at the local test level are met, calculation of the ceiling principle estimate can be performed.

If the criterion of the local test is not met, the counting method will be used for that locus; the observed number of genotypes with the particular combination of alleles will be used. When this method is used, if no alleles have been observed, the 95% upper confidence limit on no observations for those databases with no observed genotypes will be employed using the formula: $UCL = 1-0.05^{1/n}$, where 'n' is the size of the database.

When the alleles in one population are in disequilibrium based on a local test, yet the second and third populations meet equilibrium expectations, and the alleles used in the ceiling principle estimate derive from the second and third populations, the genotype counting method for the two

alleles in all databases with a minimum frequency of 0.02 will be used. This situation is not covered by the ceiling principle approach in the NRC Report.

When two loci are found to be in disequilibrium by the local test, the counting method will be used for both loci. The observed number of genotypes with the particular combination of alleles at the two loci will be used to determine a genotype frequency. For the situation where no alleles have been observed, the 95% upper confidence limit (UCL), determined for no observations, for each of the databases will be estimated. The highest frequency or minimum of 0.0004 (the minimum "interim" ceiling two-locus genotype frequency) will be used.

2. Calculation of DNA Profile Frequencies

a. Fixed Bin Method

(1). Calculate +/- 2.5% of Band Size

When determining DNA profile frequencies, the FBI method using Fixed Bins will be used. The bands from either the exemplar (known) or the evidentiary specimen can be used as long as the more common bin frequency is used. The appropriate bin for the putative band(s) will be determined by the measurement error window determined: +/- 2.5%.

(2). Use Bin with Highest Frequency

When the measurement error spans a bin boundary, the larger bin frequency will be used.

(3). Calculate 95% Upper Confidence Limit (UCL)

The 95% UCL will be calculated for each of the fixed bin frequencies in each population data set (Caucasians, Blacks, Hispanics and Asians) using the formula:

$$95\% \text{ Confidence Limit} = p + 1.96 \times \sqrt{p(1-p)/n}$$

p = bin frequency
n = number of alleles in the data set

The largest 95% upper confidence limits values across all data sets at each bin or a minimum of 0.100 will be selected for the "interim" ceiling allele frequency estimate.

Following the calculation of the 95% UCL in each of the databases for each probe being used, the product rule will be applied in order to obtain a frequency estimate for each probe's DNA profile.

Initials: RCS Date: 5/8/95

(4). Calculation of Locus Frequency

(a). Heterozygote (2-band) Pattern

The single-locus estimates from a two-band (heterozygote) pattern will be derived from the Hardy Weinberg equation,

$2p_1p_2$,

(b). Homozygote (1-band) Pattern

The single-locus estimates from a one-band (homozygote) pattern is derived from an adaptation of the Hardy Weinberg equation because on does not know whether the pattern is a true homozygote.

$2p$

where p_1 and p_2 are the respective allele frequencies obtained from the 95% upper confidence limit values or .10, which ever is larger.

(5). Calculate Pattern Frequency

After obtaining the frequency estimate for each probe's DNA profile, the multi-locus DNA profile frequency estimate is calculated as the product of the individual locus frequencies.

Pattern Frequency (F) = f1 x f2 x f3 x f4

Where f(x) = Frequency at each individual locus
x (1,2,3,4,etc.) = Number of locus

B. Calculation Example

1. Fix Bin Frequency

Fixed bins are constructed using the FBI bins. Each bin must have a minimum number of 5 (five) events. For bins that have fewer, they must be combined with other bins. The genotypic frequency for the first bin, p_1, is the number of occurrences divided by the number of chromosomes analyzed (number of individuals examined, n, times 2 (2n).

Example: For bin #1 having 5 occurrences and a database size of 100 individuals, the genotypic frequency of the bin is: p_1 = No. Occurrences/2n = 5/200 = .025

For bin #2 having 7 occurrences in the same database, the genotypic frequency of the bin is: p_2 = No. Occurrences/2n = 7/200 = .035

Alternative Example:

If the population has not met Hardy Weinberg expectations, that is, the pair of alleles are out of HW, the counting rule must be used. In this instance the number of occurrences in the total database would be used:

If this were the first occurrence in, say 100 individuals, the calculation would be according to the formula in section A1a where UCL = $1\text{-}0.05^{1/n}$.

UCL = $1 - .05^{1/100}$ or .03

2. Calculating the Hardy-Weinberg (HW) Frequencies of Two Bins

Example: Using the same values in the example from B1, the HW frequency is based upon the HW equation for heterozygotes, $2p_1p_2$.

$p_1 = .025$ and $p_2 = .035$
$2p_1p_2 = 2(.025 \times .035) = 0.00175$

3. Calculating the Upper 95% Confidence Limit (UCL) Used for the Ceiling Principle

The ceiling principle requires that the frequency used in DNA reports (using the example in B1) does not exceed the value obtained from the upper 95% confidence limit for the genotypic frequency $2p_1p_2$.

Bin #1 UCL: .0466
Bin #2 UCL: .0605

4. Selection of UCL or .10 for Bin Frequency for Genotype Frequency Calculation

Since the UCL for each bin is less than 0.10, the value 0.10 will used to calculate the frequency of that locus.

$2p_1p_2 = 2 \times .10 \times .10 = 0.02$ or 2%

Initials: CJ Date: 5/9/01

C. Calculation by Computer

1. Logon to the network and switch to the H:\users\fbiology directory.

2. Type "RFLP" and follow directions on the screen. The largest size band is by convention Band 1.

3. All lanes containing DNA profiles must be entered including the cell pellet control. Print outs must be generated for all lanes.

4. The most conservative (higher) of the evidence or exemplar frequencies are reported.

D. Mixture Calculations

1. If a lane contains an apparent simple mixture of two samples, and one component (contributing individual) of the mixture can reasonably be deduced (i.e. victim's band(s) from a vaginal swab in a sexual assault), then bands are assigned to the appropriate individual and the frequency of each individual's profile is calculated.

2. If a lane contains a mixture of samples, and each component (contributing individual) of the mixture can be accounted for based on the facts of the case, then bands are assigned to the appropriate individual and the frequency of each individual's profile is calculated. In the case report, it must be reported that the frequency calculations are based on the **assumption** that the stated individuals contribute to the mixture.

3. For all other mixtures, only inclusions and exclusions are reported.

Initials: RCJ Date: 2/1/95

APPENDIX D- STATISTICAL CALCULATIONS

Determination of Match Frequencies

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The "interim" ceiling principle defines each band as the maximum of the 95% upper confidence limit of the fixed bin frequency in each general database (Caucasians, Blacks, Hispanics or Asians) or a minimum of 0.100. In using this approach, the frequency of a single locus and/or multiple locus profile is obtained by the product of these "interim" ceiling frequencies.

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If the global test for any locus is not in equilibrium, a local test[5] will be performed only on the alleles in the particular population sample. Global and Local tests can only be performed using a computer. If Hardy Weinberg and linkage equilibrium expectations at the local test level are met, calculation of the ceiling principle estimate can be performed.

If the criterion of the local test is not met, the counting method will be used for that locus; the observed number of genotypes with the particular combination of alleles will be used. When this method is used, if no alleles have been observed, the 95% upper confidence limit on no observations for those databases with no observed genotypes will be employed using the formula: $UCL = 1-0.05^{1/n}$, where 'n' is the size of the database.

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alleles in all databases with a minimum frequency of 0.02 will be used. This situation is not covered by the ceiling principle approach in the NRC Report.

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2. Calculation of DNA Profile Frequencies

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(1). Calculate +/- 2.5% of Band Size

When determining DNA profile frequencies, the FBI method using Fixed Bins will be used. The bands from either the exemplar (known) or the evidentiary specimen can be used as long as the more common bin frequency is used. The appropriate bin for the putative band(s) will be determined by the measurement error window determined: +/- 2.5%.

(2). Use Bin with Highest Frequency

When the measurement error spans a bin boundary, the larger bin frequency will be used.

(3). Calculate 95% Upper Confidence Limit (UCL)

The 95% UCL will be calculated for each of the fixed bin frequencies in each population data set (Caucasians, Blacks, Hispanics and Asians) using the formula:

$$95\% \text{ Confidence Limit} = p + 1.645 \times \sqrt{p(1-p)/n}$$

p = bin frequency
n = number of alleles in the data set

The largest 95% upper confidence limits values across all data sets at each bin or a minimum of 0.100 will be selected for the "interim" ceiling allele frequency estimate.

Following the calculation of the 95% UCL in each of the databases for each probe being used, the product rule will be applied in order to obtain a frequency estimate for each probe's DNA profile.

Initials: RCJ Date: 2/1/95

(4). Calculation of Locus Frequency

(a). Heterozygote (2-band) Pattern

The single-locus estimates from a two-band (heterozygote) pattern will be derived from the Hardy Weinberg equation,

$2p_1p_2$,

(b). Homozygote (1-band) Pattern

The single-locus estimates from a one-band (homozygote) pattern is derived from an adaptation of the Hardy Weinberg equation because on does not know whether the pattern is a true homozygote.

$2p$

where p_1 and p_2 are the respective allele frequencies obtained from the 95% upper confidence limit values or .10, which ever is larger.

(5). Calculate Pattern Frequency

After obtaining the frequency estimate for each probe's DNA profile, the multi-locus DNA profile frequency estimate is calculated as the product of the individual locus frequencies.

Pattern Frequency (F) = f1 x f2 x f3 x f4

Where f(x) = Frequency at each individual locus
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Initials: RCJ Date: 2/1/95

Example: For bin #1 having 5 occurrences and a database size of 100 individuals, the genotypic frequency of the bin is: p_1 = No. Occurrences/2n = 5/200 = .025

For bin #2 having 7 occurrences in the same database, the genotypic frequency of the bin is: p_2 = No. Occurrences/2n = 7/200 = .035

Alternative Example:

If the population has not met Hardy Weinberg expectations, that is, the pair of alleles are out of HW, the counting rule must be used. In this instance the number of occurrences in the total database would be used:

If this were the first occurrence in, say 100 individuals, the calculation would be according to the formula in section A1a where UCL = $1\text{-}0.05_{1/n}$.

UCL = $1 - .05^{1/100}$ or .9995 or .99

2. Calculating the Hardy-Weinberg (HW) Frequencies of Two Bins

Example: Using the same values in the example from B1, the HW frequency is based upon the HW equation for heterozygotes, $2p_1p_2$.

p_1 = .025 and p_2 = .035
$2p_1p_2 = 2(.025 \times .035) = 0.00175$

3. Calculating the Upper 95% Confidence Limit (UCL) Used for the Ceiling Principle

The ceiling principle requires that the frequency used in DNA reports (using the example in B1) does not exceed the value obtained from the upper 95% confidence limit for the genotypic frequency $2p_1p_2$.

Bin #1 UCL: .0251
Bin #2 UCL: .0351

4. Selection of UCL or .10 for Bin Frequency for Genotype Frequency Calculation

Since the UCL for each bin is less than 0.10, the value 0.10 will used to calculate the frequency of that locus.

$2p_1p_2 = .10 \times .10 = 0.01$ or 1%

Initials: RCJ Date: 2/1/95

References

1. TWGDAM, The TWGDAM Consensus Approach for Applying the "Ceiling Principle" to Derive Conservative Estimates of DNA Profile Frequencies. Crime Laboratory Digest 21(2): 21-25, 1994.

2. National Research Council. DNA typing: Statistical basis for interpretation. In: DNA Technology in Forensic Science. National Academy Press, Washington, DC, 1992.

3. Chakraborty, R., Srinivasan, M.R., and Daiger, S.P. Evaluation of standard error and confidence interval of estimated multilocus genotype probabilities and their implications in DNA forensics, Am. J. Hum. Genet. 54: 60-70, 1993.

4. Devlin, B. and Risch, N. A note on Hardy-Weinberg equilibrium of VNTR data by using the Federal Bureau of Investigation's fixed-bin method. Am. J. Hum. Genet. 51: 549-548, 1992.

5. Weir, B.S. Independence of VNTR alleles defined as fixed bins. Genetics 130: 873-887, 1992.

Initials: RCJ Date: 2/1/95

APPENDIX E- REPORTING RESULTS

See report writing in the Forensic Biochemistry and Hematology Manual. Attached is a sample RFLP report.

Initials: RW Date: 2/1/95

DEPARTMENT OF HEALTH
OFFICE OF CHIEF MEDICAL EXAMINER
520 FIRST AVENUE, NEW YORK, N.Y. 10016
Telephone (212) 447-2618 Fax: (212) 447-2630

CHARLES S. HIRSCH, M.D., *Chief Medical Examiner*
Robert C. Shaler, Ph.D. *Director of Forensic Biology*

January 19, 1995

DEPARTMENT OF FORENSIC BIOLOGY
LABORATORY REPORT

DECEASED: **LAB NO:**

AUTOPSY: **M.E. NO:**

PRECINCT: **COMPLAINT NO:**

SUMMARY OF RESULTS:

DNA profiles were obtained from blood from the victim and the shirt from "suspect" using the Restriction Fragment Length Polymorphism (RFLP) technique. Based on these results, the blood found on the shirt from "suspect":

matches the blood from the victim.
has the same DNA profile as the victim
could have come from the victim

The blood on the shirt from "suspect" could not have come from the suspect.

This DNA profile is found in 1 of 1,000,000 Caucasians, 1 in 2,000,000 Blacks, and 1 of 500,000 Hispanics.

Initials: RCJ Date: 2/1/95

EXAMINATIONS:

Most of the cells within the body contain deoxyribonucleic acid (DNA); the DNA is identical in every cell of an individual. However, except for identical twins, no two individuals have identical DNA. DNA is inherited, that is, passes from generation to generation. It can be used to compare biological samples from different sources, using a technique called restriction length fragment polymorphism (RFLP) analysis or "DNA profiling".

DNA can be visualized as a very long twisted ladder. The basic unit of DNA is a base pair (bp) and can be thought of as a rung on the ladder. A human has about 3 billion base pairs of DNA in each nucleated cell, packaged into 46 chromosomes. There are 23 pairs of chromosomes, with one of each pair coming from the mother and the other from the father.

A portion of DNA which is responsible for a certain trait (such as eye color or ABO blood type) is called a gene. There are thousands of genes scattered among the chromosomes, with non-gene DNA making up the majority of the chromosomes. Each gene is located at a specific site (locus) on a specific chromosome. Alternate forms of DNA are called alleles; any locus which has multiple alleles is considered polymorphic ("many forms"). Although a locus might have tens or hundreds of alleles, an individual can have a maximum of two different alleles at that locus, one on each homologous chromosome. The alleles might be identified by observation (eye color), testing of the blood (ABO blood types), or by testing the DNA directly (DNA profiling).

In an RFLP test, several different polymorphic regions of a sample's DNA is examined; each region tested represents a different locus on the chromosomes. Generally, four loci are tested. Remembering that an individual can have a maximum of two alleles per locus, this leads to a maximum of eight alleles, visualized as fragments or "bands" of DNA on autoradiographs. A DNA profile is the overall pattern of these DNA bands.

Samples are compared by comparing the sizes of their bands, measured in base pairs. By examining the sizes of a sufficient number of bands at different sites on different chromosomes, statistical procedures can determine the approximate relative frequency of a sample's or individual's DNA profile.

Initials: RCJ Date: 6/21/95

DNA profiling was done with the following results:

Band Sized Determined, in base pairs*

ITEM	D2S44	D10S28	D4S139
(V) Smith	4567	3456	9876
	2345	1234	8901
(S) Jones	6543	5432	17802[1]
	5678	4321	8888
shirt from "suspect"	4612	3490	10073
	2368	1246	8990

The DNA profile developed on the bloodstain found on the shirt from "suspect" matches the DNA profile of the victim's blood (in all three typing probes)(at all three loci typed).

* = Allele sizes which fall within +/- 2.5% (5% range) of each other are considered indistinguishable and are therefore matches. All band sizes are determined from specimens which contain good quality DNA (high molecular weight or slightly degraded) unless noted in footnotes.

Footnotes:

1. Band sizes greater than 15004 bp are not used in the calculation of profile frequencies.

EVIDENCE RECEIVED:

ITEM NO. **VOUCHER NO.** **DESCRIPTION**

DISPOSITION:

Analyst:_____ **DATE:**

Forensic Analyst

Supervisor:_____ **DATE:**

Forensic Scientist

Initials: RCJ Date: 2/1/95

DNA profiling was done with the following results:

Band Sized Determined, in base pairs*

ITEM	D2S44	D10S28	D17S26	D4S139
(V) Smith	4567 2345	3456 1234	2456 1678	9876 8901
(S) Jones	6543 5678	5432 4321	6789 4567	17802[1] 8888
shirt from "suspect"	4612 2368	3490 1246	2480 1694	10073 8990

The DNA profile developed on the bloodstain found on the shirt from "suspect" matches the DNA profile of the victim's blood (in all four typing probes)(at all four loci typed).

* = Allele sizes which fall within +/- 2.5% (5% range) of each other are considered indistinguishable and are therefore matches. All band sizes are determined from specimens which contain good quality DNA (high molecular weight or slightly degraded) unless noted in footnotes.

Footnotes:

1. Band sizes greater than 15004 bp are not used in the calculation of profile frequencies.

EVIDENCE RECEIVED:

ITEM NO. **VOUCHER NO.** **DESCRIPTION**

DISPOSITION:

Analyst:_____ **DATE:**

Forensic Analyst

Supervisor:_____ **DATE:**

Forensic Scientist