Forensic Biology Section

Extraction of DNA with Phenol-Chloroform

1. Scope

1.1. Isolating DNA from nucleated cells using organic solvents, which consists of lysing cells with digest buffer coupled with protein digestion using proteinase-K enzyme (and DTT solution in the case of sperm heads).After digestion is complete, several phenol/chloroform/isoamyl alcohol washes are performed to remove any remaining proteins.

1.2. The extracted DNA (in aqueous solution) is then washed and concentrated in a DNA Ultra Filtration device.

1.3. Phenol-chloroform extractions are generally reserved for specimens with very high amounts of substrate (such as tissue samples or bone), but it can be utilized for any sample. The procedure is just more labor-intensive and requires labelling several sets of tubes.

2. Safety

2.1. CHEMICAL HAZARD. Wear chemical-resistant gloves and eye protection when handling phenol-chloroform solution. Work in well-ventilated area, preferably a total exhaust biosafety hood.

2.2. Treat all biological specimens as potentially infectious. Gloves and laboratory coat must be worn at all times. Follow Universal Precautions.

3. Specimens

- Tissue, muscle or organ (~1 - 3 cm^3)
- Bone (sample size varies)
- Liquid blood (~3 µl) or dried bloodstain (~3 mm^2 to 1½ cm^2)
- Sperm / epithelial cell pellet (entire pellet) or semen stain (~½ cm^2 to 1½ cm^2)
- Saliva stain (sample size varies) or Envelopes
- Cigarette butt (~5 mm of filter paper)
- Hair root (entire root)

3.1. NOTE: Sizes are only guidelines; evidentiary samples may be in limited supply. Add the maximum amount of evidentiary sample that can fit in a tube if very little biological material is present, trying to retain sufficient sample for replicate analysis when possible.

4. Reagents and Special Supplies

- DNA Ultra Filtration devices (Amicon Ultra 100k, Centricon-100 or Microcon-100)
- Digest Buffer (10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, 2% SDS, pH 7.5)
- Dithiothreitol (DTT), 1 M
- Molecular biology grade water
- Phenol/chloroform/isoamyl alcohol solution (25:24:1)
- Proteinase K (20 mg/ml)
- Sperm Wash Buffer
- Tris-EDTA, "TE^{-4}" (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)

5. Procedural Notes

5.1. This protocol is written for the use of 1.5 ml microcentrifuge tubes. If it is necessary to use larger tubes (such as 15 ml conical tubes), scale-up the proportions of reagents accordingly.

5.2. This protocol is written for the use of the Eppendorf model 5415C microcentrifuge (or equivalent). The "maximum speed" referenced in this protocol corresponds to approximately 15,000 rpm (approximately 14,000 to 15,000 g-force). When "2400 rpm" is referenced, the relative centrifugal force should be approximately 500 g.

5.3. If larger tubes are used (15 ml or 50 ml conical tubes), the rotor in the centrifuge will have to be changed to accommodate the larger tubes.

6. Extracting Tissue, Muscle or Organ

6.1. Using a clean cutting surface for each different sample, dissect a sample approximately 1 mm square (or larger) and place in a labeled microcentrifuge tube

6.2. Add 500 µl of Digest Buffer and 7.5 µl of 20 mg/ml Proteinase K solution.

6.3. Incubate at 56° C for at least one hour. Digestion may continue overnight, up to 24 hours.

6.4. Add 500 µl of phenol/chloroform/isoamyl alcohol. Vortex at least 15 seconds until an emulsion forms.

6.5. Spin in microcentrifuge for 3 minutes at maximum speed.

6.6. Transfer the upper (aqueous) phase to a new-labeled microcentrifuge tube. Do NOT transfer the white layer of protein that may be visible between the two layers.

6.7. Repeat steps 6.4 through 6.6 an additional two or three times until nothing is visible at the interface and the aqueous phase appears clear.

6.8. Transfer the entire upper (aqueous) phase containing extracted DNA to a DNA Ultra Filtration device (approximately 500 µl). If the aqueous phase is more than 500 µl, it can be concentrated using several DNA Ultra Filtration devices or up to 2 ml in a single Centricon-100. [Do not touch the membrane with the pipette tip or transfer any lower (organic) phase].

6.9. Spin the device at 2400 rpm for 15 minutes (the speed is the same whether using a microcentrifuge or a clinical centrifuge).

6.10. NOTE: almost all of the liquid in the upper chamber should pass through the membrane. If a large amount of liquid remains in the upper chamber, the filter may be clogged; transfer the remaining liquid to a new DNA Ultra Filtration device and repeat step 6.9.

6.11. Add 500 µl TE^{-4} to the retentate in the upper chamber and spin at 2400 rpm for 15 minutes (if using a Centricon-100, add 2 ml of TE^{-4}).

6.12. Place sample reservoir upside down in a new vial. Spin at maximum speed for 3 minutes (or 5000 rpm if using clinical centrifuge).

6.13. Bring the recovered retentate volume up to 50 µl with TE^{-4} if necessary.

6.14. Store in a refrigerator (1° C - 8° C) for short-term storage. Store in a freezer (-10° C or colder) for long-term storage.

7. Extracting Bone

7.1. Weigh and measure bone sample before extracting. Photo documentation is optional.

7.2. Using a clean surface for each different sample, scrape and sand the surface of the bone to remove debris and potential foreign contamination using Dremel-type tool with new, UV-irradiated sanding disks.

7.3. Cut a section of bone using Dremel-type tool with new, UV-irradiated cutting blades.

7.4. Attempt to quantify the bone section to be analyzed (weight, size, etc.).

7.5. Grind the bone sample in a blender cleaned with bleach and alcohol, or scrape the bone surface if possible.

7.6. Transfer the bone pieces to a 15 ml conical tube.

7.7. Add a measured amount of Digest Buffer sufficient to completely submerge the bone dust.

7.8. Add 15 µl of 20 mg/ml Proteinase K solution for every ml of digest buffer added in step 7.7.

7.9. Incubate at 56° C for at least one hour. Digestion may continue overnight, up to 24 hours.

7.10. Add 500 µl of phenol/chloroform/isoamyl alcohol. Vortex at least 15 seconds until an emulsion forms.

7.11. Spin in a clinical centrifuge for 3 minutes at 5000 rpm.

7.12. Transfer the upper (aqueous) phase to a new-labeled 15 ml tube. Do NOT transfer the white layer of protein that may be visible between the two layers.

7.13. Repeat steps 7.10 through 7.12 an additional two or three times until nothing is visible at the interface and the aqueous phase appears clear.

7.14. Transfer the entire upper (aqueous) phase containing extracted DNA to a DNA Ultra Filtration device (approximately 500 µl if extracting in microcentrifuge tubes). If the aqueous phase is more than 500 µl, it can be concentrated using several DNA Ultra Filtration devices or up to 2 ml in a single Centricon-100. [Do not touch the membrane with the pipette tip or transfer any lower (organic) phase].

7.15. Spin the device at 2400 rpm for 15 minutes (the speed is the same whether using a microcentrifuge or a clinical centrifuge).

7.16. NOTE: almost all of the liquid in the upper chamber should pass through the membrane. If a large amount of liquid remains in the upper chamber, the filter may be clogged; transfer the remaining liquid to a new DNA Ultra Filtration device and repeat step 7.15.

7.17. Add 500 µl TE^{-4} to the retentate in the upper chamber and spin at 2400 rpm for 15 minutes (if using a Centricon-100, add 2 ml of TE^{-4}).

7.18. Place sample reservoir upside down in a new vial. Spin at maximum speed for 3 minutes (or 5000 rpm if using clinical centrifuge).

7.19. Bring the recovered retentate volume up to 50 µl with TE^{-4} if necessary.

7.20. Store in a refrigerator (1° C - 8° C) for short-term storage. Store in a freezer (-10° C or colder) for long-term storage.

8. Extracting Dried Bloodstains

8.1. Dissect swab or fabric into small pieces and place in a labeled tube.

8.2. NOTE: The sample size will be dependent upon the quantity of material available and whether the sample appears to be heavily stained or not. Generally, take approximately a 1/8" diameter punch or a ½ cm^2 if the stain is very heavy, and up to approximately 1 ½ cm^2 if the stain is light.

8.3. OPTION: If it is necessary to verify the presence of human hemoglobin using the HemaTrace test, extract the substrate in 1 ml of TE^{-4} for 15 minutes (or until the TE buffer becomes colored), centrifuge for 5 minutes at maximum speed, test approximately 150 µl of the supernatant [See "HemaTrace Human Blood Detection" method], discard the remainder of the supernatant without disturbing the cell pellet, and continue with next step.

8.4. Add 500 µl of Digest Buffer to the tube.

8.5. Add 7.5 µl of 20 mg/ml Proteinase K solution. Vortex.

8.6. Incubate at 56°C for at least one hour. Digestion may continue overnight, up to 24 hours.

8.7. Add 500 µl of phenol/chloroform/isoamyl alcohol. Vortex at least 15 seconds until an emulsion forms.

8.8. Spin in microcentrifuge for 3 minutes at maximum speed.

8.9. Transfer the upper (aqueous) phase to a new-labeled microcentrifuge tube. Do NOT transfer the white layer of protein that may be visible between the two layers.

8.10. Repeat steps 8.7 through 8.9 an additional two or three times until nothing is visible at the interface and the aqueous phase appears clear.

8.11. Transfer the entire upper (aqueous) phase containing extracted DNA to a DNA Ultra Filtration device (approximately 500 µl if extracting in microcentrifuge tubes). If the aqueous phase is more than 500 µl, it can be concentrated using several DNA Ultra Filtration tubes or up to 2 ml in a single Centricon-100. [Do not touch the membrane with the pipette tip or transfer any lower (organic) phase].

8.12. Spin the device at 2400 rpm for 15 minutes (the speed is the same whether using a microcentrifuge or a clinical centrifuge).

8.13. NOTE: almost all of the liquid in the upper chamber should pass through the membrane. If a large amount of liquid remains in the upper chamber, the filter may be clogged; transfer the remaining liquid to a new DNA Ultra Filtration device and repeat.

8.14. Add 500 µl TE^{-4} to the retentate in the upper chamber and spin at 2400 rpm for 15 minutes (if using a Centricon-100, add 2 ml of TE^{-4}).

8.15. Place sample reservoir upside down in a new vial. Spin at maximum speed for 3 minutes (or 5000 rpm if using clinical centrifuge).

8.16. Bring the recovered retentate volume up to 50 µl with TE^{-4} if necessary.

8.17. NOTE: If the color of the extracted sample is a dark red brown color, the potential for inhibition must be considered.

9. Extracting Liquid Blood

9.1. Add 500 µl of Digest Buffer to a labeled tube.

9.2. Add 3-5 µl of well-mixed whole blood.

9.3. Add 7.5 µl of 20 mg/ml Proteinase K solution for every 500 µl of Digest Buffer. Vortex.

9.4. Incubate at 56ºC for at least one hour. Digestion may continue overnight, up to 24 hours.

9.5. Add 500 µl of phenol/chloroform/isoamyl alcohol. Vortex at least 15 seconds until an emulsion forms.

9.6. Spin in microcentrifuge for 3 minutes at maximum speed, or in clinical centrifuge for 5 minutes at 5000 rpm, depending on size of tube.

9.7. Transfer the upper (aqueous) phase to a new-labeled microcentrifuge tube (or 15 ml tube). Do NOT transfer the white layer of protein that may be visible between the two layers.

9.8. Repeat steps 9.5 through 9.7 an additional two or three times until nothing is visible at the interface and the aqueous phase appears clear.

9.9. Transfer the entire upper (aqueous) phase containing extracted DNA to a DNA Ultra Filtration device (approximately 500 µl if extracting in microcentrifuge tubes). If the aqueous phase is more than 500 µl, it can be concentrated using several DNA Ultra Filtration devices or up to 2 ml in a single Centricon-100. [Do not touch the membrane with the pipette tip or transfer any lower (organic) phase].

9.10. Spin the device at 2400 rpm for 15 minutes (the speed is the same whether using a microcentrifuge or a clinical centrifuge).

9.11. NOTE: almost all of the liquid in the upper chamber should pass through the membrane. If a large amount of liquid remains in the upper chamber, the filter may be clogged; transfer the remaining liquid to a new DNA Ultra Filtration device and repeat step 9.10.

9.12. Add 500 µl TE^{-4} to the retentate in the upper chamber and spin at 2400 rpm for 15 minutes (if using a Centricon-100, add 2 ml of TE^{-4}).

9.13. Place sample reservoir upside down in a new vial. Spin at maximum speed for 3 minutes (or 5000 rpm if using clinical centrifuge).

9.14. Bring the recovered retentate volume up to 50 µl with TE^{-4} if necessary.

9.15. NOTE: If the color of the extracted sample is a dark red brown color, the potential for inhibition must be considered.

10. Extracting sperm cell cutting or swab

10.1. Dissect swab or fabric into small pieces and place in a labeled Spin tube.

10.2. NOTE: The sample size will be dependent upon the quantity of material available, whether the sample appears to be heavily stained or not, and whether the material is light-weight or heavy-weight. Generally, use ½ of a swab if there is only one swab, or use an entire swab head if there is more than one swab; for light-weight fabric or gauze, use approximately 1 ½ cm^2 cuttings; for heavy-weight fabric (such as jeans or upholstery), use as much material as can easily fit in a tube and leave sufficient room for mechanically agitating the substrate.

10.3. Add 800 µl of TE^{-4} to the tube. Vortex for at least 10 seconds (and/or agitate with a sterile pipette tip) to aid in releasing cells from the substrate.

10.4. Incubate at room temperature for 30 minutes, or longer in a refrigerator (1^o – 8^o C).

10.5. Vortex for at least 10 seconds (and/or agitate with a sterile pipette tip) again.

10.6. Transfer the substrate with a sterile pipette tip or forceps into a Spin Basket and place the basket in the top of the corresponding tube.

10.7. Spin for 5 minutes at maximum speed to elute the liquid and cells from the substrate in the basket.

10.8. Remove and discard the Spin Basket and substrate.

10.9. Without disturbing the pellet, remove all but approximately 50 µl of the supernatant (or leave behind twice the volume of the pellet, whichever is greater).

10.10. Resuspend the cell debris pellet (potentially containing both sperm and epithelial cells) thoroughly in the remaining supernatant (by pipetting up and down vigorously), and then remove an aliquot equal to one-tenth of the volume in the tube for quantification of the sperm/epithelial cell content. Follow SOP "Microscopic Examination of Cell Debris."

10.11. Add 500 µl of phenol/chloroform/isoamyl alcohol to both the sperm-fraction ("S" tube) and the epithelial-fraction ("E" tube). Vortex at least 15 seconds until an emulsion forms.

10.12. Spin for 3 minutes at maximum speed, or in clinical centrifuge for 5 minutes at 5000 rpm, depending on size of tube.

10.13. Transfer the upper (aqueous) phase to a new-labeled microcentrifuge tube (or 15 ml tube). Do NOT transfer the white layer of protein that may be visible between the two layers.

10.14. Repeat steps 10.11 through 10.13 an additional two or three times until nothing is visible at the interface and the aqueous phase appears clear.

10.15. Transfer the entire upper (aqueous) phase containing extracted DNA to a DNA Ultra Filtration device (approximately 500 µl if extracting in microcentrifuge tubes). If the aqueous phase is more than 500 µl, it can be concentrated using several DNA Ultra Filtration devices or up to 2

ml in a single Centricon-100. [Do not touch the membrane with the pipette tip or transfer any lower (organic) phase].

10.16. Spin the device at 2400 rpm for 15 minutes (the speed is the same whether using a microcentrifuge or a clinical centrifuge).

10.17. NOTE: almost all of the liquid in the upper chamber should pass through the membrane. If a large amount of liquid remains in the upper chamber, the filter may be clogged; transfer the remaining liquid to a new DNA Ultra Filtration device and repeat step 10.16.

10.18. Add 500 µl TE^{-4} to the retentate in the upper chamber and spin at 2400 rpm for 15 minutes (if using a Centricon-100, add 2 ml of TE^{-4}).

10.19. Place sample reservoir upside down in a new vial. Spin at maximum speed for 1 minutes (or 5000 rpm if using clinical centrifuge).

10.20. Bring the recovered retentate volume up to 50 µl with TE^{-4} if necessary.

10.21. Store in a refrigerator (1° C - 8° C) for short-term storage. Store in a freezer (-10° C or colder) for long-term storage.

11. Extracting sperm cell/epithelial cell pellet

11.1. Add approximately 450 µl molecular biology grade water to the cell debris pellet (final volume should be approximately 500 µl).

11.2. Add 6 µl of proteinase-K (20 mg/ml). Vortex gently.

11.3. Incubate at 37°C for approximately 1 hour to lyse epithelial cells, but for no more than 2 hours to minimize the lysis of sperm cells.

11.4. Spin for 5 minutes at maximum speed. This original tube is now labeled with an "S", "SP", or "sperm" (will contain the resulting sperm-pellet).

11.5. Without disturbing the sperm pellet, transfer all but approximately 50 µl of the supernatant (or leave behind twice the volume of the pellet, whichever is greater) to a new tube, labeled with an "E", "Ep", or "Epi" (contains the aqueous solution of epithelial cell DNA).

11.6. Wash the sperm-pellet ("S" tube) with Sperm Wash Buffer as follows: Resuspend the pellet in 500 µl Sperm Wash Buffer. Vortex briefly. Spin in a microcentrifuge for 5 minutes at maximum speed. Remove and discard as much of the supernatant as possible without disturbing the pellet.

11.7. Repeat step 11.6 an additional 2-3 times, for a maximum of 4 washes.

11.8. Wash sperm-pellet ("S" tube) once with molecular biology grade water as follows: Resuspend the pellet in 1 ml water. Vortex briefly. Spin in a microcentrifuge for 5 minutes at maximum speed. Remove and discard as much of the supernatant as possible without disturbing the pellet.

11.9. OPTION: Resuspend the pellet and remove a one-tenth aliquot for a microscopic examination to assess the quantity of sperm present and the efficiency of removing epithelial cells. Follow SOP "Microscopic Examination of Cell Debris."

11.10. Add 450 µl of molecular biology grade water to the sperm-pellet ("S" tube, for a final volume of approximately 500 µl). Add 7.5 µl of 20 mg/ml Proteinase K solution and 21 µl of DTT.

11.11. Vortex and incubate at 56ºC for 30 – 60 minutes.

11.12. Add 500 µl of phenol/chloroform/isoamyl alcohol to both the sperm-fraction ("S" tube) and the epithelial-fraction ("E" tube). Vortex at least 15 seconds until an emulsion forms.

11.13. Spin for 3 minutes at maximum speed, or in clinical centrifuge for 5 minutes at 5000 rpm, depending on size of tube.

11.14. Transfer the upper (aqueous) phase to a new-labeled microcentrifuge tube (or 15ml tube). Do NOT transfer the white layer of protein that may be visible between the two layers.

11.15. Repeat steps 11.12 through 11.14 an additional two or three times until nothing is visible at the interface and the aqueous phase appears clear.

11.16. Transfer the entire upper (aqueous) phase containing extracted DNA to a DNA Ultra Filtration device (approximately 500 µl if extracting in microcentrifuge tubes). If the aqueous phase is more than 500 µl, it can be concentrated using several DNA Ultra Filtration devices or up to 2 ml in a single Centricon-100. [Do not touch the membrane with the pipette tip or transfer any lower (organic) phase].

11.17. Spin the device at 2400 rpm for 15 minutes (the speed is the same whether using a microcentrifuge or a clinical centrifuge).

11.18. NOTE: almost all of the liquid in the upper chamber should pass through the membrane. If a large amount of liquid remains in the upper chamber, the filter may be clogged; transfer the remaining liquid to a new DNA Ultra Filtration device and repeat step 11.17.

11.19. Add 500 µl TE^{-4} to the retentate in the upper chamber and spin at 2400 rpm for 15 minutes (if using a Centricon-100, add 2 ml of TE^{-4}).

11.20. Place sample reservoir upside down in a new vial. Spin at maximum speed for 1 minutes (or 5000 rpm if using clinical centrifuge).

11.21. Bring the recovered retentate volume up to 50 µl with TE^{-4} if necessary.

11.22. Store in a refrigerator (1º C - 8º C) for short-term storage. Store in a freezer (-10º C or colder) for long-term storage.

12. Extracting Saliva stains or Envelope Flaps

12.1. For envelopes, use steam to carefully open the flap on the envelope, wet a cotton-tipped swab with molecular biology grade water and swab the adhesive area.

12.2. Cut the swab head (or stain) and place into a labeled microcentrifuge tube.

12.3. Add 500 µl of Digest Buffer to the tube.

12.4. Add 7.5 µl of 20 mg/ml Proteinase K solution. Vortex.

12.5. Incubate at 56ºC for at least one hour. Digestion may continue overnight, up to 24 hours.

12.6. Add 500 µl of phenol/chloroform/isoamyl alcohol. Vortex at least 15 seconds until an emulsion forms.

12.7. Spin in microcentrifuge for 3 minutes at maximum speed.

12.8. Transfer the upper (aqueous) phase to a new-labeled microcentrifuge tube. Do NOT transfer the white layer of protein that may be visible between the two layers.

12.9. Repeat steps 12.6 through 12.8 an additional two or three times until nothing is visible at the interface and the aqueous phase appears clear.

12.10. Transfer the entire upper (aqueous) phase containing extracted DNA to a DNA Ultra Filtration device (approximately 500 µl). [Do not touch the membrane with the pipette tip or transfer any lower (organic) phase].

12.11. Spin the device at 2400 rpm for 15 minutes.

12.12. NOTE: almost all of the liquid in the upper chamber should pass through the membrane. If a large amount of liquid remains in the upper chamber, the filter may be clogged; transfer the remaining liquid to a new DNA Ultra Filtration device and repeat step 12.11.

12.13. Add 500 µl TE^{-4} to the retentate in the upper chamber and spin at 2400 rpm for 15 minutes.

12.14. Place sample reservoir upside down in a new vial. Spin at maximum speed for 3 minutes.

12.15. Bring the recovered retentate volume up to 50 µl with TE^{-4} if necessary.

12.16. Store in a refrigerator (1° C - 8° C) for short-term storage. Store in a freezer (-10° C or colder) for long-term storage.

13. Extracting Cigarette Butts

13.1. Slice a strip approximately 5 mm wide from the paper covering the cigarette butt filter in the area that would have been in contact with the mouth (optional to cut up into smaller pieces) and place in a microcentrifuge tube.

13.2. Add 500 µl of Digest Buffer to the tube.

13.3. Add 7.5 µl of 20 mg/ml Proteinase K solution. Vortex.

13.4. Incubate at 56°C for at least one hour. Digestion may continue overnight, up to 24 hours.

13.5. Add 500 µl of phenol/chloroform/isoamyl alcohol. Vortex at least 15 seconds until an emulsion forms.

13.6. Spin in microcentrifuge for 3 minutes at maximum speed.

13.7. Transfer the upper (aqueous) phase to a new-labeled microcentrifuge tube. Do NOT transfer the white layer of protein that may be visible between the two layers.

13.8. Repeat steps 13.5 through 13.7 an additional two or three times until nothing is visible at the interface and the aqueous phase appears clear.

13.9. Transfer the entire upper (aqueous) phase containing extracted DNA to a DNA Ultra Filtration device (approximately 500 µl). [Do not touch the membrane with the pipette tip or transfer any lower (organic) phase].

13.10. Spin the device at 2400 rpm for 15 minutes.

13.11. NOTE: almost all of the liquid in the upper chamber should pass through the membrane. If a large amount of liquid remains in the upper chamber, the filter may be clogged; transfer the remaining liquid to a new DNA Ultra Filtration device and repeat step 13.10.

13.12. Add 500 µl TE^{-4} to the retentate in the upper chamber and spin at 2400 rpm for 15 minutes.

13.13. Place sample reservoir upside down in a new vial. Spin at maximum speed for 3 minutes.

13.14. Bring the recovered retentate volume up to 50 µl with TE^{-4} if necessary.

13.15. Store in a refrigerator (1° C - 8° C) for short-term storage. Store in a freezer (-10° C or colder) for long-term storage.

14. Extracting Hair Roots

14.1. Handling hair with clean forceps, examine hair under a microscope for the presence of skin cells or sheath material attached at the root end of the hair.

14.2. Use a clean scalpel to cut approximately 0.5 cm from the root end of the hair and place in a microcentrifuge tube labeled “root” or “rt”.

14.3. If the hair has not been washed for microscopic analysis, immerse the hair root cutting in molecular biology grade water to reduce surface dirt and contaminants, then vortex at high speed for 10 seconds. Decant or pipet the water out of the tube, being careful not to draw the hair up into the pipet tip. Repeat if necessary.

14.4. Add 500 µl of Digest Buffer to the tube.

14.5. Add 7.5 µl of 20 mg/ml Proteinase K solution. Vortex.

14.6. Incubate at 56°C for at least one hour. Digestion may continue overnight, up to 24 hours.

14.7. Add 500 µl of phenol/chloroform/isoamyl alcohol. Vortex at least 15 seconds until an emulsion forms.

14.8. Spin in microcentrifuge for 3 minutes at maximum speed.

14.9. Transfer the upper (aqueous) phase to a new-labeled microcentrifuge tube. Do NOT transfer the white layer of protein that may be visible between the two layers.

14.10. Repeat steps 14.7 through 14.9 an additional two or three times until nothing is visible at the interface and the aqueous phase appears clear.

14.11. Transfer the entire upper (aqueous) phase containing extracted DNA to a DNA Ultra Filtration device (approximately 500 µl). [Do not touch the membrane with the pipette tip or transfer any lower (organic) phase].

14.12. Spin the device at 2400 rpm for 15 minutes.

14.13. NOTE: almost all of the liquid in the upper chamber should pass through the membrane. If a large amount of liquid remains in the upper chamber, the filter may be clogged; transfer the remaining liquid to a new DNA Ultra Filtration device and repeat step 14.12.

14.14. Add 500 µl TE^{-4} to the retentate in the upper chamber and spin at 2400 rpm for 15 minutes.

14.15. Place sample reservoir upside down in a new vial. Spin at maximum speed for 3 minutes.

14.16. Bring the recovered retentate volume up to 50 µl with TE^{-4} if necessary.

14.17. Store in a refrigerator (1° C - 8° C) for short-term storage. Store in a freezer (-10° C or colder) for long-term storage.