

FBS20- EZ1 Advanced XL – DNA Extraction

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1. Scope

- 1.1. This procedure describes the isolation of deoxyribonucleic acid (DNA) from biological specimens recovered from evidentiary items for nuclear DNA typing using the EZ1 Advanced XL Robots.

2. Background

- 2.1. To establish the practices for documenting the examination of evidence to conform to the requirements of the Department of Forensic Sciences (DFS) Forensic Science Laboratory (FSL) *Quality Assurance Manual*, the accreditation standards under ISO/IEC 17025:2005, and any supplemental standards
- 2.2. The EZ1 Advanced XL and the EZ1 DNA Investigator Kit can purify genomic DNA from 1 to 14 samples at a time, in lysis volumes of 200 μ l or 500 μ l, depending on amount and condition of starting material. DNA is isolated from lysates through its binding to the silica surface of the magnetic bead particles in the presence of a chaotropic salt. The magnetic bead particles are then separated from the lysates using a magnet. The DNA is then washed and eluted in TE buffer or water.
- 2.3. Read Material Safety Data Sheets to determine the safety hazards for chemicals and reagents used in the standard operating procedures.

The following protocols are validated for use:

Trace – 200 μ l lysis volume, no substrate in tube

Tip Dance – 200 μ l lysis volume, small cutting of substrate in tube

Large Volume – 500 µl lysis volume

3. Safety

- 3.1. Wear personal protective equipment (e.g., lab coat, gloves, mask, eye protection), when carrying out standard operating procedures.
- 3.2. Read Material Safety Data Sheets to determine the safety hazards for chemicals and reagents used in the standard operating procedures.
- 3.3. Bleach should not be directly used to clean-up spills with the Qiagen EZ1 DNA Investigator Kit. All spills should first be thoroughly washed with soap and water.

4. Materials Required

4.1. Qiagen EZ1 DNA Investigator Kit:

- 4.1.1. Reagent cartridges (DNA Investigator)
- 4.1.2. Disposable filter-tips
- 4.1.3. Disposable tip-holders
- 4.1.4. Sample tubes (2.0 ml)
- 4.1.5. Elution tubes (1.5ml)
- 4.1.6. Buffer G2
- 4.1.7. Proteinase K (20 mg/ml)
- 4.1.8. Carrier RNA

4.2. Additional Reagents:

- 4.2.1. Qiagen Buffer MTL (for 500 ml lysis volume protocol only)
- 4.2.2. Low TE buffer (for cRNA hydration)
- 4.2.3. Digest Buffer (FBR35)
- 4.2.4. Proteinase K (FBR36) (10 mg/ml)
- 4.2.5. 1.0 M DTT (FBR38)
- 4.2.6. TE Buffer (FBR14)
- 4.2.7. Sterile water

4.2.7.1. NOTE: Never use solutions directly from the stock bottles. Use Reagent SOPs for preparation and labeling instructions.

5. Standards and Controls

- 5.1. At least one reagent blank (i.e., extraction control) must be prepared and processed in parallel with each set of evidentiary specimens processed for DNA typing purposes. The reagent blank(s) is comprised of all the reagents used in the analytical process and is carried through the same extraction, quantitation, amplification and electrophoretic typing procedures as the evidence samples. If more than one extraction method is used then at least one reagent blank must be processed for each type of procedure. For extraction sets exceeding fourteen samples, the extraction procedure may be divided into sequential runs on the same robot.
- 5.2. For differentials, the reagent blank created in conjunction with the isolation of the female fraction is designated as the non-sperm fraction (RB#EF). The reagent blank created in conjunction with the isolation of the male fraction is designated as the sperm fraction (RB#SF).
- 5.3. To maintain a separation in time and space between questioned and known samples, all questioned samples must be processed through incubation and extraction before the known samples are processed. At no time should questioned and known samples be simultaneously incubating in the same heat block. The RB should always be the last sample processed in a set. **Any RBs created must be matching the volume of the smallest sample.**
- 5.4. Any utensils used to cut or manipulate the substrate must be cleaned with 10% bleach followed by 70% ethanol or sterile water after each use.
- 5.5. All work surfaces, utensils, and instruments used must be cleaned as necessary with 10% bleach. A 70% ethanol or sterile water rinse must follow for all metal items. Alternatively, if using razor blades, a new blade should be used for each cutting.
- 5.6. If processing forensic material samples, the Carrier RNA reagent must be resuspended prior to use. Add 310 µl of Low TE Buffer to the lyophilized Carrier RNA and vortex to mix.
- 5.7. Perform all appropriate instrument maintenance (**daily**, weekly) before beginning any EZ1 Advanced XL instrument run.
- 5.8. **After completion of any EZ1 Advanced XL instrument run remove and clean the tray, tube and cartridge rack with 70% ethanol followed by dH₂O. Replace them into the instrument.**

- 5.9 Sample Tubes must be witnessed prior to loading on the EZ1 Advanced XL robot and the analyst must be witnessed while loading samples onto the robot.

6. Calibration

- 6.1. See FBQ35 EZ1 Advanced XL – Maintenance for Maintenance and calibration.

7. Procedures

- 7.1. Non-Differential Samples: This procedure may be performed in either 200 µl or 500 µl lysis volumes. The 200 µl lysis is recommended for most samples; however, larger volumes may be needed for large substrates or to remove inhibitors. 200 µl Lysis protocol steps start at 7.1.1. 500 µl Lysis protocol steps start at 7.1.21.

200 µl Lysis:

7.1.1. Preheat a thermomixer to 56°C.

7.1.2. Place each sample into a labeled, sterile tube.

7.1.2.1. If using the trace protocol and cutting or swab, add samples to a non-QIAGEN 2.0 ml microcentrifuge tube.

7.1.2.2. If using the tip dance protocol, ensure that only a small cutting is added directly to the 2.0 ml skirted EZ1 tube.

7.1.3. Add 190µl of Buffer G2 and 10 µl Qiagen Proteinase K (20mg/ml) to each tube containing sample. Vortex well and spin down.

Note: The Buffer G2 and Proteinase K may be aliquoted as a master mix. If the master mix method is used, this aliquot expires at the end of the prepared date.

7.1.4. Incubate/shake on the thermomixer at 56°C and 900 rpm for a minimum of 2 hours. After digestion, vortex and spin-down the sample tubes.

7.1.5. **Trace Protocol Only:** Transfer the substrate to a filterless basket in a 2.0 ml microcentrifuge tube and spin at maximum speed for 2 minutes. Remove the spin basket and retain the substrate.

7.1.5.1. Transfer each lysate (approximately 200 µl) to a labeled 2.0 ml skirted EZ1 tube

7.1.6. Add 1.0 µl (1.0 µg/µl) carrier RNA to each EZ1 tube. Vortex well and spin down.

7.1.7. Turn on the robot (the switch is located on the back left hand side of the robot, just above the electrical cord) and then press “Start” to go to the main menu. “Investigator Protocols” should appear on the screen.

- 7.1.7.1. Turn on the robot (the switch is located on the back left hand side of the robot, just above the electrical cord) and then press “Start” to go to the main menu. “Investigator Protocols” should appear on the screen.
 - 7.1.7.1.1. Select “Start” to begin run.
 - 7.1.7.1.2. Select “ESC” to opt out of generating a QC report.
- 7.1.8. Select the “Trace” or “Trace TD” protocol.
- 7.1.9. Select “TE” as the elution solution.
- 7.1.10. Select “50 µl” as the elution volume.
- 7.1.11. The screen prompts that follow will summarize the selections and prompt a verification of positions of reagents, samples and plastics on the worktable.
- 7.1.12. Gently lift the front metal piece (tube rack) and place to the side. Gently lift the second metal piece (cartridge rack) located closer to the back of the robot and place to the side.
- 7.1.13. Insert the appropriate number of EZ1 Reagent Cartridges, corresponding to the number of samples to be extracted into the cartridge rack After sliding each EZ1 cartridge all the way in, gently push down on the front plastic tab of the cartridge so that it makes a slight clicking sound. Once all EZ1 Reagent Cartridges are loaded, return the cartridge rack back to the original position in the robot.
- 7.1.14. Return the front metal piece (tube rack) into the robot. Remove and discard all caps from the EZ1 tubes containing the sample lysates. Place each of these tubes into Row 4.
- 7.1.15. Place the plastic EZ1 tip into Row 2. The total number of tip holders should equal to the number of samples to be extracted.
- 7.1.16. Place a filter tip into each of the tip holders from 7.1.15.
- 7.1.17. Place clean, sterile 1.5 ml elution tubes (with sample name on the side of the tube) into Row 1. The total number of elution tubes should equal the number of samples to be extracted. Retain the labeled sterile caps on a Kimwipe in front of the robot in the same orientation as the corresponding tube (these will be used once the extraction is completed by the robot). See image of work table in Appendix 14.2.

NOTE: Ensure that 1.5 ml and *not* 2.0 ml elution tubes are used.
- 7.1.18. Follow the prompts on the screen to close the instrument door and begin the run.
- 7.1.19. Refer to Appendix 14.1 for a list of messages displayed on the LCD during the robotic extraction.

- 7.1.20. After completion of the “Trace” or “Trace TD” protocol (~18 minutes), remove each tube from the EZ1 and add the correctly labeled screw cap to each tube.

500 µl Lysis:

- 7.1.21. Preheat a thermomixer to 56°C.
- 7.1.22. Place each sample into a labeled, sterile 2.0 ml microcentrifuge tube.
- 7.1.23. Add 480µl of Buffer G2 and 20 µl Qiagen Proteinase K (20mg/ml) to each tube. Vortex well and spin down.

Note: The Buffer G2 and Proteinase K may be aliquoted as a master mix. If the master mix method is used, this aliquot expires at the end of the prepared date.
- 7.1.24. Incubate/shake on the thermomixer at 56°C and 900 rpm for a minimum of 2 hours. After digestion, vortex and spin-down the sample tubes.
- 7.1.25. **Swabs or Cuttings Only:** Transfer the substrate to a filterless basket in a 2.0 ml microcentrifuge tube and spin at maximum speed for 2 min. Remove the spin basket and retain the substrate.
- 7.1.26. Add 400µl Buffer MTL and 1.0µl (1.0 µg/µl) carrier RNA to each EZ1 tube.

Note: The Buffer MTL and carrier RNA may be aliquoted as a master mix. If the master mix method is used, this aliquot expires at the end of the prepared date.
- 7.1.27. Transfer each lysate (approximately 500 µl) to a 2.0 ml skirted EZ1 tube. Vortex briefly and spin down.
- 7.1.28. Turn on the robot (the switch is located on the back left hand side of the robot, just above the electrical cord) and then press “Start” to go to the main menu. “Investigator Protocols” should appear on the screen.
 - 7.1.28.1. When using the EZ1 Advanced workstation three prompts will occur prior to reaching the main menu.
 - 7.1.28.1.1. Select “Start” to begin run
 - 7.1.28.1.2. Select “ESC” to opt out of generating a QC report
- 7.1.29. Select the “Large Volume” protocol.
- 7.1.30. Select “TE” as the elution solution.
- 7.1.31. Select “50 µl” as the elution volume.
- 7.1.32. The screen prompts that follow will summarize the selections and prompt a verification of positions of reagents, samples and plastics on the worktable.

- 7.1.33. Gently lift the front metal piece (tube rack) and place to the side. Gently lift the second metal piece (cartridge rack) located closer to the back of the robot and place to the side.
- 7.1.34. Insert the appropriate number of EZ1 Reagent Cartridges, corresponding to the number of samples to be extracted into the cartridge rack. After sliding each EZ1 cartridge all the way in, gently push down on the front plastic tab of the cartridge so that it makes a slight clicking sound. Once all EZ1 Reagent Cartridges are loaded, return the cartridge rack back to the original position in the robot.
- 7.1.35. Return the front metal piece (tube rack) into the robot. Remove the caps from the EZ1 tubes containing the sample lysates. Place each of these tubes into Row 4. Save the caps for disposal of tubes after run is completed.
- 7.1.36. Place the plastic EZ1 tip into Row 2. The total number of tip holders should equal to the number of samples to be extracted.
- 7.1.37. Place a filter tip into each of the tip holders from 7.1.36.
- 7.1.38. Place clean, sterile 1.5 ml elution tubes (with sample name on the side of the tube) into Row 1. The total number of elution tubes should equal the number of samples to be extracted. Retain the labeled sterile caps on a Kimwipe in front of the robot in the same orientation as the corresponding tube (these will be used once the extraction is completed by the robot). See image of work table in Appendix 14.2.

NOTE: Ensure that 1.5 ml and *not* 2.0 ml elution tubes are used

- 7.1.39. Follow the prompts on the screen to close the instrument door and begin the run.
- 7.1.40. Refer to Appendix 14.1 for a list of messages displayed on the LCD during the robotic extraction.
- 7.1.41. After completion of the “Large Volume” protocol (~18 minutes), remove each tube from the EZ1 and add the correctly labeled screw cap to each tube.

7.2 Differential Samples:

- 7.2.1. To each sample tube, pipette 400 µl Digest Buffer and 12 µl of Proteinase K (10 mg/ml) solution. Vortex and quick-spin in a microcentrifuge.

NOTE: The Digest Buffer and Proteinase K may be aliquoted as a master mix. If the master mix method is used, this aliquot expires at the end of the prepared date

- 7.2.2. Incubate the samples either at 37°C for 2 hours to overnight or at 56°C for 1 hour.

- 7.2.3. After digestion, vortex and spin-down the sample tubes. Transfer the substrate to a filterless basket in a 2.0ml microcentrifuge tube and spin for 3 – 5 minutes at maximum speed. Save the substrate in a new sterile tube labeled with the case number, sample number, and “SR” (for substrate remains).
- 7.2.4. Remove the supernatant from the samples, being careful not to disturb the sperm pellet (approximately 50µL remaining in the tube). Place the supernatant (non-sperm fraction [EF]) into a new appropriately labeled microcentrifuge tube.

NOTE: EF tubes can be processed immediately on the EZ1 (see step 7.2.12) or can be stored at 4°C overnight for simultaneous processing with SF.
- 7.2.5. Wash the sperm pellet by re-suspending in 500-1000 µl of TE Buffer or diH₂O. Vortex and spin the samples in a microcentrifuge for 3-5 minutes at maximum velocity. Remove and discard the supernatant fluid, being careful not to disturb the cell pellet (up to 50µL may be left in the tube).
- 7.2.6. Repeat Steps 4 and 5 an additional 2 times for a total of 3 washes of the sperm pellet.

NOTE: The wash step can be repeated an additional 1 to 5 times depending upon the nature of the sample.
- 7.2.7. After the final spin, remove and discard all but approximately 50 µl of the supernatant.
- 7.2.8. OPTIONAL: Resuspend the pellet within the remaining 50 µl of supernatant by gently mixing the sample with a pipette. Remove approximately 3-5 µl of the sample and spot it on a glass microscope slide. Perform a Christmas Tree Stain using the technique described in (FBS07) Proceed to Step 7.2.9 if no epithelial cells are observed. However, if any intact epithelial cells remain, redigest the sperm pellet by following these additional steps.
 - 7.2.8.1. Add 400 µl of Digest Buffer to re-suspend the sperm pellet.
 - 7.2.8.2. Add 12 µl of Proteinase K (10 mg/ml). Mix gently.
 - 7.2.8.3. Incubate at 37°C for 1 hour.
 - 7.2.8.4. Spin at a maximum velocity for 3-5 minutes in a microcentrifuge. Remove and discard all but approximately 50 µl of the supernatant.
 - 7.2.8.5. Re-suspend the pellet in 500 µl of Digest Buffer and vortex. Spin the sample 3-5 minutes in the microcentrifuge at maximum velocity. Remove and discard all but approximately 50 µl of the supernatant. Proceed to Step 7.2.9.
- 7.2.9. To the sperm pellet add 400 µl Buffer G2, 12 µl 1M DTT, and 15 µl **Qiagen** Proteinase K (20mg/ml). The Digest Buffer and Proteinase K may be added to the sample and incubated at 37°C or 56°C while

performing the Christmas Tree Stain. Once a slide is confirmed as containing no epithelial cells, the DTT may be added and the incubation continued.

Note: The Buffer G2, **Qiagen** Proteinase K and DTT may be aliquoted as a master mix. If the master mix method is used, this aliquot expires at the end of the day.

- 7.2.10. Preheat a thermomixer to 56°C.
- 7.2.11. Mix the samples gently and incubate at 56°C for 2 hours to overnight.
- 7.2.12. Add 400µl Buffer MTL and 1.0µl (1.0 µg/µl) carrier RNA to both the EF and SF tubes.

NOTE: If EF tubes were stored overnight at 4°C, allow lysates to equilibrate to room temperature prior to processing.

NOTE: The Buffer MTL and carrier RNA may be aliquoted as a master mix. If the master mix method is used, this aliquot expires at the end of the prepared date.
- 7.2.13. Transfer each lysate (approximately 900 µl) to a 2.0 ml skirted EZ1 tube. Vortex briefly and spin down.
- 7.2.14. Turn on the robot (the switch is located on the back left hand side of the robot, just above the electrical cord) and then press “Start” to go to the main menu. “Investigator Protocols” should appear on the screen.
 - 7.2.14.1 When using the EZ1 Advanced workstation three prompts will occur prior to reaching the main menu.
 - 7.2.14.1.1 Select “Start” to begin run.
 - 7.2.14.1.2 Select “ESC” to opt out of generating a QC report.
- 7.2.15. Select the “Large Volume” protocol.
- 7.2.16. Select “TE” as the elution solution.
- 7.2.17. Select “50 µl” as the elution volume.
- 7.2.18. The screen prompts that follow will summarize the selections and prompt a verification of positions of reagents, samples and plastics on the worktable.
- 7.2.19. Gently lift the front metal piece (tube rack) and place to the side. Gently lift the second metal piece (cartridge rack) located closer to the back of the robot and place to the side.
- 7.2.20. Insert the appropriate number of EZ1 Reagent Cartridges, corresponding to the number of samples to be extracted into the cartridge rack After sliding each EZ1 cartridge all the way in, gently push down on the front plastic tab of the cartridge so that it makes a slight clicking sound. Once

all EZ1 Reagent Cartridges are loaded, return the cartridge rack back to the original position in the robot.

- 7.2.21. Return the front metal piece (tube rack) into the robot. Remove the caps from the EZ1 tubes containing the sample lysates. Place each of these tubes into Row 4. Save the caps for disposal of tubes after run is completed.
- 7.2.22. Place the plastic EZ1 tip holders into Row 2. The total number of tip holders should equal to the number of samples to be extracted.
- 7.2.23. Place a filter tip into each of the tip holders from 7.2.22.
- 7.2.24. Place clean, sterile 1.5 ml elution tubes (with sample name on the side of the tube) into Row 1. The total number of elution tubes should equal the number of samples to be extracted. Retain the labeled sterile caps on a Kimwipe in front of the robot in the same orientation as the corresponding tube (these will be used once the extraction is completed by the robot). See image of work table in Appendix 14.2.

NOTE: Ensure that 1.5 ml and *not* 2.0 ml elution tubes are used.

- 7.2.25. Follow the prompts on the screen to close the instrument door and begin the run.
- 7.2.26. Refer to Appendix 14.1 for a list of messages displayed on the LCD during the robotic extraction.
- 7.2.27. After completion of the “Large Volume” protocol (~18 minutes), remove each tube from the EZ1 and add the correctly labeled screw cap to each tube.

OPTIONAL: Spin tubes at maximum speed for 1 minute to pellet any remaining magnetic material and transfer the 50 µl extract to a new labeled 1.5 ml tube for storage, avoiding any pelleted material at the bottom.

NOTE: If optional step is not performed, spin tubes prior to amplification to avoid introduction of magnetic material into the amplification reaction.

8. Sampling

- 8.1. Not applicable

9. Calculations

- 9.1. Not applicable

10. Uncertainty of Measurement

- 10.1. When quantitative results are obtained, and the significance of the value may impact the report, the uncertainty of measurement must be determined. The method used to determine the estimation of uncertainty can be found in the *FSL Quality Assurance Manual – Estimation of Uncertainty of Measurement (Section 5.4.6)*.

11. Limitations

- 11.1. The quantity and quality of the DNA present within any biological material ultimately determines if a nuclear DNA isolation is successful.
- 11.2. The separation of non-sperm and sperm cell DNA into the female and male fractions is not always a complete division. It is not unusual for male DNA to be observed in the female fraction and vice versa. The number of intact cells recovered in a sample and their capacity to endure the abrasive conditions of the differential extraction method is dependent upon the quality of the biological material being tested and the environmental conditions to which it has been subjected. The detection of residual DNA within a given fraction does not inhibit the use of the DNA typing results from that fraction.
- 11.3. The presence of nuclear DNA in the male fraction of a differential extraction is not a dependable method of determining whether semen or spermatozoa in a particular biological specimen is present.

12. Documentation

- 12.1. FBU EZ1 Advanced XL – DNA Extraction Worksheet

13. References

- 13.1. Comey, C.T., Koons, B.W., Presley, K.W., Smerick, J.B., Sobieralski, C.A., Stanley, D.M., and Baechtel, F.S. DNA extraction strategies for amplified fragment length polymorphism analysis. *Journal of Forensic Sciences* (1994) 39: 1254-1269.
13.1.1.
- 13.2. EZ1 DNA Investigator Handbook (Revised 04/2009)
- 13.3. Developmental Validation of QIAGEN EZ1[®] Robotic Platform And DNA Investigator[®] Silica-based Extraction Chemistry Version

- 13.4. Butler, John. 2012. Forensic DNA Typing: Methodology. Elsevier Academic Press. 34-35
- 13.5. Melzak, KA. et. al. (1996) Driving forces for DNA adsorption to silica in perchlorate solutions. Journal of Colloid and Interface Science Volume 181, Number 2, 635-644.
- 13.6. Kishore, R. et. al. (2006) Optimization of DNA extraction from low-yield and degraded samples using the BioRobot EZ1 and BioRobot M48. Journal of Forensic Science Volume 51, Number 5, 1055.
- 13.7. *Forensic Science Laboratory Quality Assurance Manual (Current Version)*
- 13.8. *FSL Departmental Operations Manuals (Current Versions)*
- 13.9. *FSL Laboratory Operations Manuals (Current Versions)*
- 13.10. FBR35 - Digest Buffer (Current Version)
- 13.11. FBR36 - 10 mg/ml Proteinase K in 10mM Tris pH8.0 (Current Version)
- 13.12. FBR38 - 1.0 M Dithiothreitol (DTT) (Current Version)
- 13.13. FBR14 - TE Buffer (Current Version)
- 13.14. FBS07 - Microscopic Examination of Spermatozoa by Christmas Tree Stain (Current Version)

14. Appendix

14.1 The following steps are displayed and performed by the EZ1 Advanced XL during the DNA Investigator Kit runs (times are approximate):

| | | |
|--------|---------------------------|---------------------|
| 14.1.1 | Protocol started | |
| 14.1.2 | Piercing foil | 17 of 18 min left |
| 14.1.3 | Collecting elution buffer | |
| 14.1.4 | Collecting beads | 16 of 18 min left |
| 14.1.5 | Resuspending beads | |
| 14.1.6 | Collecting lysate | 15 of 18 min left |
| 14.1.7 | Binding | 14 of 18 min left – |

| | | |
|---------|------------------------------|-------------------------------------|
| | | 9 of 18 min left |
| 14.1.8 | Binding: magnetic separation | 9 of 18 min left |
| 14.1.9 | Wash 1 | 8 of 18 min left |
| 14.1.10 | Wash 1: magnetic separation | 7 of 18 min left |
| 14.1.11 | Wash 2 | 6 of 18 min left |
| 14.1.12 | Wash 2: magnetic separation | |
| 14.1.13 | Wash 3 | 5 of 18 min left |
| 14.1.14 | Wash 3: magnetic separation | |
| 14.1.15 | Rinse | 4 of 18 min left |
| 14.1.16 | Checking temperature | |
| 14.1.17 | Elution | 3 of 18 min left – 1 of 18 min left |
| 14.1.18 | Protocol finished | |

14.2 Image of Worktable (Image from DNA Investigator Handbook)



Figure 4. Typical EZ1 worktable.

1. First row: Elution tubes (1.5 ml) are loaded here.
2. Second row: Tip holders containing filter-tips are loaded here.
3. Third row: Tip holders containing filter-tips are loaded here. (In some protocols, this row is empty or loaded with 2 ml Sarstedt tubes.)
4. Fourth row: Sample tubes (2 ml) are loaded here.
5. Reagent cartridges are loaded into the cartridge rack.
6. Heating block with 2 ml tubes in the reagent cartridges for lysis.