Differential Extraction from Semen Stains or Swabs utilizing the QIAcube and EZ1

This extraction is applicable for casework samples suspected of containing semen.

Follow all relevant processes in the General Guidelines for Forensic Biology and DNA Casework procedure.

Follow all relevant processes in the BEAST DNA Worksheet Setup Manual for creating and adding to worksheets and BEAST DNA Worksheet Processing Manual for how to record all relevant information while processing the worksheets.

1 Tube setup

1.1 Retrieve sample cuttings in 1.5 mL tubes and take each sample into your custody.

1.2 Add all samples to the Differential Worksheet.

1.3 Obtain two 1.5 mL Eppendorf tubes for the extraction negatives and label them with the associated extraction negative labels.

1.4 Retrieve reagents for epithelial cell digestion and record the lot numbers. Consult the Mixture Information table for the exact amount of Diluted ATL and Proteinase K needed to prepare the Master Mix.

Epithelial Cell Digestion master mix (per sample)		
Diluted ATL (µL)	Proteinase K (Qiagen) (µL)	Total
480	20	500

1.5 Add 500µL master mix to each sample and negative controls.

1.6 Incubate all samples in a Thermomixer set to 56°C (+/- 3°C) for 1.5 hours (and no longer than 1.75 hours) at 900rpm. Record the thermomixer and the temperature reading on the temperature probe.

1.7 Record the 'Incubation Run By' review task.

NOTE: The 'epithelial cell' fraction will be denoted as F1 and the 'sperm cell' fraction will be denoted as F2

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1.8 During the incubation, prepare the sample tubes:

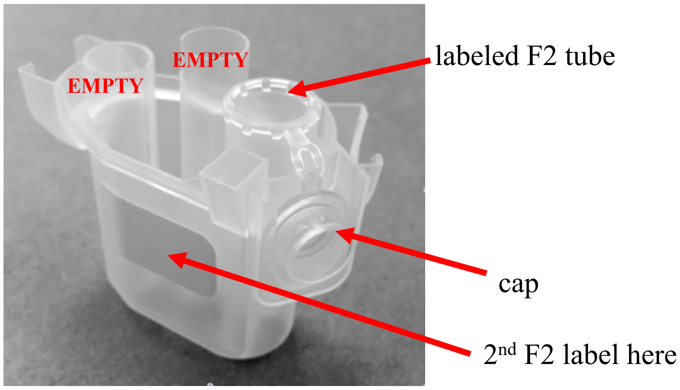
1.8.1 Print your labels. For the entire protocol you will need:

- Three copies of the **F1** sample labels
- Three copies of the **F2** sample labels

1.8.2 Obtain tubes and label them:

- Spin baskets and 1.5 mL collection tubes – **F1** labels
- 2.0 mL Qiagen skirted screw cap tubes – **F1** labels (place the label below the ridges on the side of the tube)
- Rotor adapters – **F2** labels
- 1.5 mL Qiagen snap cap tubes – **F2** labels
- 1.5ml Qiagen screw cap elution tubes – **F1** sample. (place the label below the ridges on the side of the tube)
- 1.5ml Qiagen screw cap elution tubes – **F2** sample. (place the label below the ridges on the side of the tube)

1.8.3 The 1.5 mL Qiagen snap cap tubes with the F2 labels will go inside of the rotor adapters. Bend the cap of each tube back and insert it into the cap holder in position L3 (see diagram below).



Rotor

1.8.4 If the batch size is 11 samples, an additional blank (rotor adapter set-up and 2.0 mL Qiagen skirted screw cap tube) must be prepared.

2 QIAcube setup and Sample Preparation:

2.1 Record the QIAcube instrument.

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2.2 Check that the waste drawer is empty, and the liner is clean (can be wiped with 70% ethanol if needed).

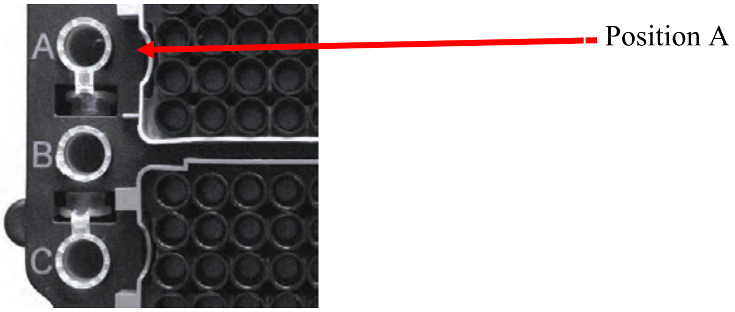
2.3 Fill both tip racks with Disposable Filter-Tips, 1000µL , wide bore.

2.4 Retrieve reagents for the sperm lysis buffer and record lot numbers. Prepare buffer in a 2 mL screw cap tube – see the table below for minimum volumes required for this protocol:

NOTE: G2 buffer_AutoDiff reagent comes in 2 sizes: 13mL and 260mL bottles. Either size may be used for the sperm lysis buffer and the 30mL reagent bottle as described below.

Sperm Lysis Buffer reagents			
# samples	**G2 buffer_AutoDiff (µL)**	**Qiagen ProK (µL)**	**1M DTT (µL)**
3	398	27	106
4	506	34	135
5	623	42	166
6	750	50	200
7	863	58	230
8	975	65	260
9	1106	74	295
10	1219	81	325
(11 or) 12	1470	98	392

2.5 Once the sperm lysis buffer is prepared, place the tube on the QIAcube worktable in Position A.



2.6 Aliquot G2 buffer into a 30ml reagent bottle and place in Reagent Bottle Rack position 1. The minimum amount of buffer needed based on the number of samples is listed on the table below. Do not fill the bottle above the fill line on the side of the bottle. Make sure to pop any bubbles that may have formed covering the mouth of the reagent bottle using a clean pipette tip.

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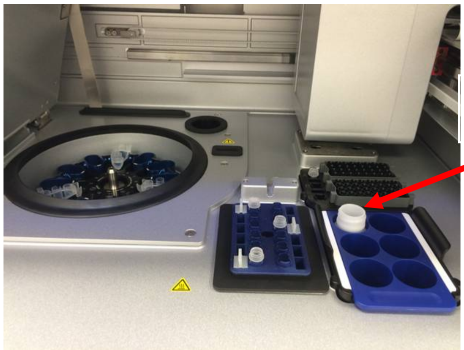
G2 minimum buffer amounts	
# samples	**G2 buffer (mL)**
3	8.68
4	10.74
5	12.8
6	14.86
7	16.92
8	18.98
9	21.04
10	23.1
(11 or) 12	27.22



Reagent bottle rack position 1

2.7 Referring to the ‘QIAcube Loading chart’, (Appendix A) place sample plugs into the QIAcube shaker for each Extraction Negative and F1 fraction. **ONLY PLACE PLUGS IN APPROPRIATE LOCATIONS BASED ON THE NUMBER OF SAMPLES.**

sample plugs placed in these slots

2.8 After the incubation from step 1.6 is done, spin down the original samples briefly to remove condensation from the tube caps.

2.9 **Tube Setup WITNESS:** Have another analyst verify three sets of tubes:

- Original incubation tube (sample labels)
- Spin basket tube (**F1** sample labels)
- Rotor setup (**F2** sample labels on outside of rotor adapter)

2.9.1 Record the 'Tube Setup Witness'.

2.10 Prepare 10% bleach, distilled water, and 70% alcohol in three 50ml conical tubes. Clean a pair of forceps by dipping the forceps in each of the three tubes briefly and then drying with a fresh Lint free wipe.

2.11 For each sample, transfer the substrate using the forceps to its associated spin basket tube. Pipette mix the sample lysate within the incubation tube to disturb any sperm pellet that may have formed and then pipette the entire lysate volume (~500µL) over the top of the substrate in the spin basket. Close the tube top over the spin basket. Repeat this step for each sample, cleaning and drying the forceps between each sample.

2.12 Spin the substrates in spin baskets at 13,200 rpm to 15,000 rpm for 2 minutes.

2.13 Using clean and dry forceps, remove and discard the spin baskets containing the swab remains taking care to avoid bubbles at the rim of the open tube. Clean and dry forceps between each sample.

NOTE: If it is necessary for a particular case or sample to retain the substrate remains for potential future testing, please see the Lab Manager and/or the LIMS team.

2.14 Transfer the lysate from the spin basket collection tube, pipette mixing as needed, to its corresponding F2 sample tube within the QIAcube rotor adapter.

NOTE: If the batch size is 11 samples, add 500µL of water to a 'Blank' sample tube and rotor adapter, to be placed in the 12th rotor position.

2.15 Record the 'Extraction Run By' review task 'Response' dropdown for the Separation and Lysis step.

2.16 **QIAcube Setup WITNESS**: Have another analyst verify the samples and loading pattern on the QIAcube. Refer to 'QIAcube Loading Chart' (Appendix A) for correct tube positioning based on sample number.

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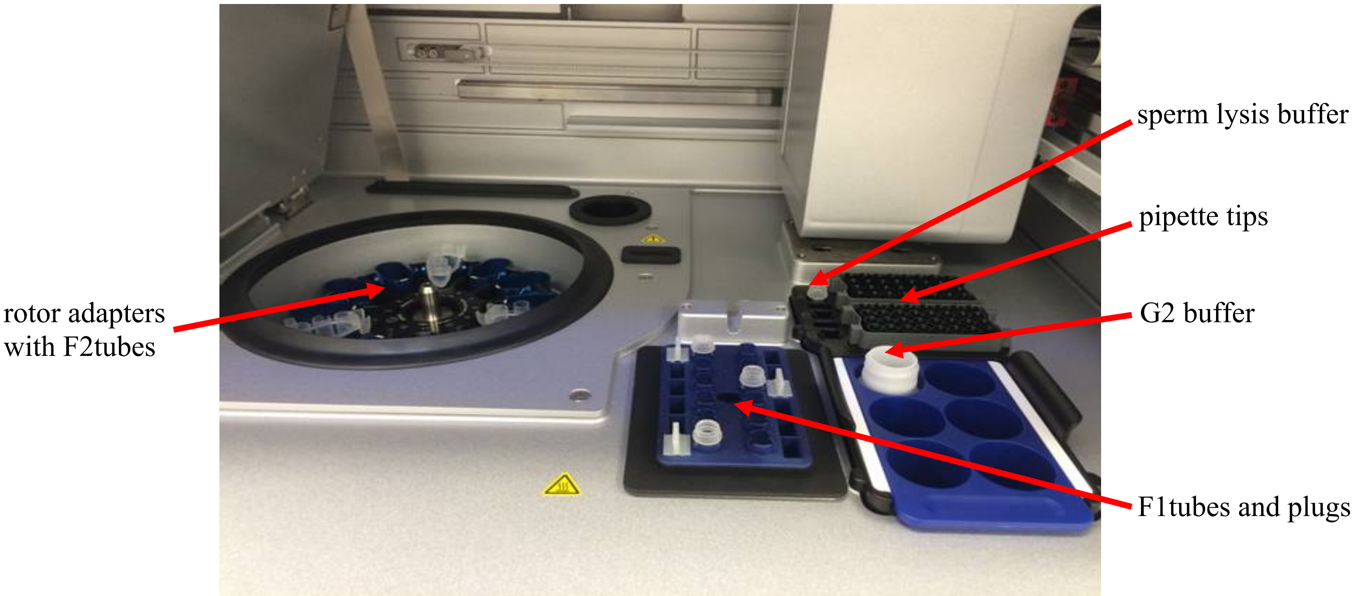
2.16.1 Load the rotor adapters containing the samples into the QIAcube centrifuge, reading the F2 label and rotor position for that sample.

2.16.2 Load the empty 2.0 mL Qiagen skirted screw cap tubes into the QIAcube shaker, reading the F1 label and shaker position for each sample. Check to make sure that the tubes are pressed all the way down. If the batch size is 11 samples, an additional blank tube must be placed in the shaker in the 12th slot.

2.16.3 The witness should also check that all sample and reagent bottle caps are removed, tips are full, sample plugs are in place in the shaker, and robot setup is correct for the number of samples being processed.



QIAcube worktable

2.17 Close the instrument door and start the separation protocol on the QIAcube:

2.17.1 From the main menu, select ‘Pipetting Separation and Lysis 12A’

2.17.1.1 This protocol may be chosen from ‘Last’, if available. Otherwise, choose ‘DNA’ → ‘Pipetting’ -→ ‘Epithelial and Sperm Cell’ → ‘Separation and Lysis 12A’

2.17.2 Press ‘Start’

2.17.3 The screen will display instructions to ensure the QIAcube has been loaded properly. After checking each step, press ‘Next’ until the final step, and then press ‘Start’.

2.17.4 Run time for protocol 12A is ~52 minutes for a full set of 12 samples.

2.18 While QIAcube protocol 12A is running, setup the EZ1 for the F1s.

3 EZ1 setup for F1s:

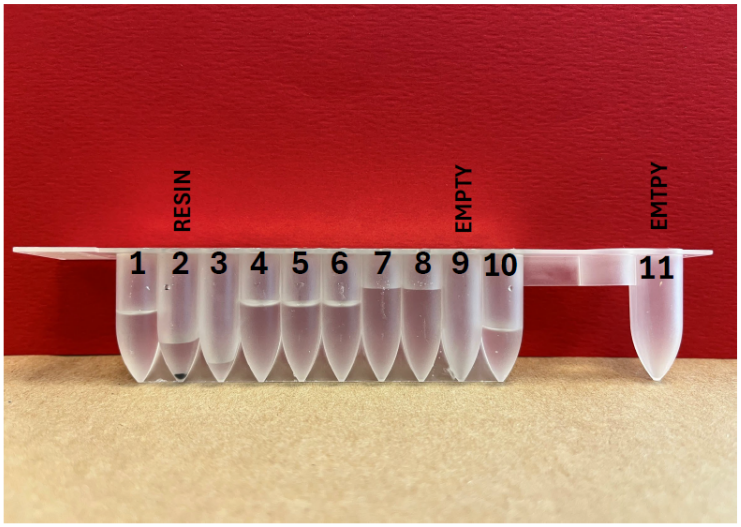
3.1 Remove both the tube rack and cartridge rack from the EZ1.

3.2 Obtain one reagent cartridge for each F1 sample and extraction negative and record the lot number.

NOTE: More than one lot may be used if there are not enough individual cartridges available for your full batch. Please record the additional lot number and samples it was used for in the Extraction Run By review task comments box.

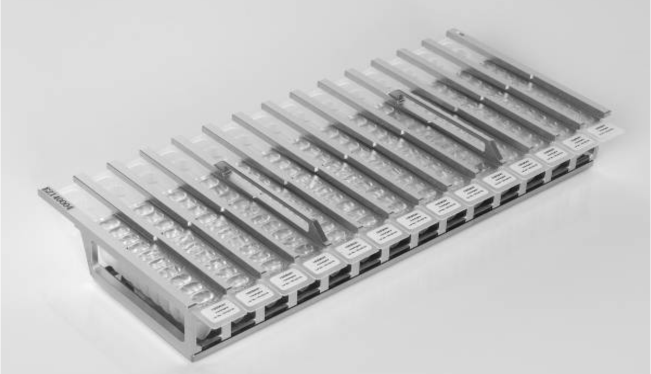
3.3 Invert reagent cartridges twice to mix the magnetic particles. Then tap the cartridges to deposit the reagents at the bottoms of their wells. Check that the magnetic particles are re-suspended. Also, visually inspect each reagent well. All wells except wells 9 and 11 must contain liquid.

EZ1/2 CARTRIDGES: Well volumes should appear as below:



Set aside any cartridges that do not appear to be filled like the above and alert QA.

3.4 Slide the EZ1 reagent cartridges into the cartridge rack. The cartridge label should be at the blunt end of the rack, closest to the tube rack when loaded onto the instrument. Place the rack in EZ1.



Reagent cartridge rack with cartridges loaded

3.5 Assemble EZ1 tips and tip holders. Place them in row 2 of the EZ1 tube rack.

3.6 Record the EZ1 instrument.

3.7 At the conclusion of protocol ‘Separation and Lysis 12A’ on the QIAcube, the screen will then give instructions on how to clean the QIAcube. Press ‘Next’ after each step until the main menu appears on the screen. Open the QIAcube instrument door and take out the F1 fraction samples from the QIAcube shaker, capping each sample as it is removed.

4 QIAcube set up for F2s:

4.1 Refill the tip racks on the QIAcube with disposable Filter-Tips and refill the G2 buffer; ensure the sperm lysis buffer is in place and uncapped. Empty the waste drawer.

4.2 Close the instrument door and start the sperm washing protocol on the QIAcube:

4.2.1 From the main menu, select ‘Pipetting Separation and Lysis 12B’

4.2.1.1 This protocol may be chosen from ‘Last’, if available. Otherwise, choose ‘DNA’ → ‘Pipetting’ -→ ‘Epithelial and Sperm Cell’ → ‘Separation and Lysis 12B’.

4.2.2 Press ‘Start’

4.2.3 The screen will display instructions to ensure the QIAcube has been loaded properly. After checking each step, press ‘Next’ until the final step, and then press ‘Start’.

4.2.4 Run time for this protocol is ~48 minutes for a set of 12 samples.

5 EZ1 F1 Sample preparation and purification:

5.1 While the QIAcube is running, prepare the F1 samples for the EZ1.

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5.2 Record the 'Extraction Run By' review task 'Response' dropdown for the F1 Purification step.

5.3 Retrieve MTL and carrier RNA and record the lot numbers.

5.4 Add 400µL of MTL buffer, and 1µL carrier RNA (1µg/µL) to each F1 fraction Extraction Negative and sample obtained from the QIAcube. Precipitate may form in the tube with the addition of the MTL buffer.

5.5 EZ1 F1 Setup WITNESS: Have another analyst verify the instrument setup and loading of all samples and elution tubes for the F1 fraction onto the EZ1:

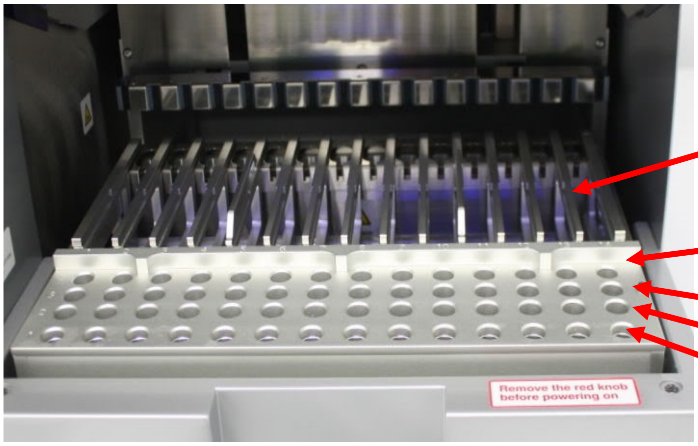
5.5.1 Load the 2mL F1 sample tubes into row 4 of the EZ1 tube rack, reading the label and discarding the screw cap as you load each sample.

5.5.2 Load the labeled F1 elution tubes into row 1 of the EZ1 tube rack, reading the label and removing and retaining the screw cap as you load each sample.

5.5.3 Ensure the tube rack is loaded onto the instrument after the cartridge rack.

5.5.4 Verify that all samples, reagents, and racks are loaded appropriately on the instrument.

5.5.5 Record the 'EZ1 F1 Setup Witness'.



Reagent cartridge rack – place into instrument **first**

Tube rack:
Row 4 – sample tubes from QIAcube (2.0mL screw top)
Row 3 – empty
Row 2 – pipette tips
Row 1 – elution tubes (1.5ml Screw top)

EZ1 Worktable

5.6 Run the EZ1 protocol for purification of the **F1 fractions**:

5.6.1 If needed, press 'ESC' to get to the main menu.

5.6.2 From the main menu press 'Start' to begin a run.

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5.6.3 When asked if you would like to create a run report, press ‘ESC’ to select no.

5.6.4 Press ‘3’ to select the large volume protocol

5.6.5 Press ‘2’ to select elution in TE.

5.6.6 Press ‘1’ to select the 40µL elution volume.

5.6.7 The screen will display instructions to ensure the EZ1 has been loaded properly. After checking each step, press ‘ENT’ until the final step, and then press ‘Start’.

5.6.8 The protocol run time is ~18 minutes.

5.7 Create a reference tube with 36µL of 0.1X TE^{-4}.

5.8 After the protocol is completed, press ‘ENT’ to continue.

5.9 Open the instrument door and remove the tube rack. Remove the 1.5mL F1 elution tubes, capping each sample.

5.10 Compare each extract to the reference tube containing 36µL of 0.1X TE^{-4}. If the volume appears lower than 36µL, measure the extract.

5.10.1 Measured volumes below 36µL should be documented in the Pre/Post Issues Log and in the sample comments field on the worksheet.

5.10.2 Samples with measured volumes below 27µL must not proceed for further testing.

5.11 Discard all the used cartridges, lysate tubes, tip-holders, and tips.

5.12 The EZ1 instrument can now be set up for the F2 purification.

6 EZ1 F2 Sample preparation and purification:

6.1 Remove both the tube rack and cartridge rack from the EZ1.

6.2 Obtain one reagent cartridge for each F2 sample and extraction negative and record the lot number, if different from the lot number used for the F1 samples.

6.3 Invert reagent cartridges twice to mix the magnetic particles. Then tap the cartridges to deposit the reagents at the bottoms of their wells. Check that the magnetic particles are re-suspended. Visually inspect each reagent well and ensure that all wells except wells 9 and 11 contain liquid.

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6.4 Slide the EZ1 reagent cartridges into the cartridge rack. The cartridge label should be at the blunt end of the rack, closest to the tube rack when loaded onto the instrument. Place the rack in EZ1.

6.5 Assemble EZ1 tips and tip holders. Place them in row 2 of the EZ1 tube rack.

6.6 After the 'Separation and Lysis 12B' protocol is complete on the QIAcube, the F2 fractions will be found in the 1.5ml tubes in the rotor adapters. Remove the tubes from each rotor adapter, closing the cap as you remove each sample. Discard the rotor adapters.

6.7 Record the 'Extraction Run By' review task 'Response' dropdown for the F2 Purification step.

6.8 Vortex the F2 fraction samples for 5 seconds.

6.9 Incubate all samples in a Thermomixer set to 70°C (+/- 3°C) for 10 minutes at 900rpm. Record the thermomixer and the temperature reading on the temperature probe.

NOTE: The QIAcube may be cleaned during this incubation, or after the F2 fractions are loaded onto the EZ1 – refer to step 6.15.

6.10 Vortex the samples vigorously for 10 seconds after the incubation. Briefly spin down the samples in a centrifuge.

6.11 Add 1µL of carrier RNA to each sample.

6.12 Holding the lid closed with your thumb, cut through the tube cap connector for each F2 tube.

6.13 **EZ1 F2 Setup WITNESS**: Have another analyst verify the instrument setup and loading of all samples and elution tubes for the F2 fraction onto the EZ1:

6.13.1 Load the F2 sample tubes into row 4 of the EZ1 tube rack, reading the label and discarding the flip cap as you load each sample

6.13.2 Load the labeled F2 elution tubes into row 1 of the EZ1 tube rack, reading the label and removing and retaining the screw cap as you load each sample.

6.13.3 Ensure the tube rack is loaded onto the instrument after the cartridge rack.

6.13.4 Verify that all samples, reagents, and racks are loaded appropriately on the instrument.

6.13.5 Record the 'EZ1 F2 Setup Witness'.

6.14 Close the instrument door and start the protocol for **F2 purification**.

6.14.1 From the main menu, press 'Start' to begin a run.

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6.14.2 When asked if you would like to create a run report, press ‘ESC’ to select no.

6.14.3 Press ‘1’ to select the trace protocol.

6.14.4 Press ‘2’ to select elution in TE.

6.14.5 Press ‘1’ to select the 40µL elution volume.

6.14.6 The screen will display instructions to ensure the EZ1 has been loaded properly. After checking each step, press ‘ENT’ until the final step, and then press ‘Start’.

6.14.7 The run time for this protocol is ~16 minutes.

6.15 Clean the QIAcube instrument:

6.15.1 DO NOT USE BLEACH ON THE INSTRUMENT, ONLY 70% ETOH WITH LINT FREE WIPES. NEVER SPRAY ETHANOL DIRECTLY ON THE INSTRUMENT.

6.15.2 Empty the waste drawer and check that the liner is clean.

6.15.3 Remove but DO NOT DISCARD THE SAMPLE PLUGS.

6.15.4 Discard the sperm lysis buffer tube. Remove pipette tip racks and consolidate any partial racks of tips for future use.

6.15.5 Wipe down the entire inside of the QIAcube instrument using a lint-free wipe with 70% EtOH, including the waste drawer.

6.16 After the F2 run is complete on the EZ1, press ‘ENT’ to continue.

6.17 Open the instrument door and remove the tube rack. Remove 1.5mL F2 elution tubes, capping each sample.

6.18 Compare each extract to the reference tube containing 36µL of 0.1X TE^{-4}. If the volume appears lower than 36µL, measure the extract.

6.18.1 Measured volumes below 36µL should be documented in the Pre/Post Issues Log and in the sample comments field on the worksheet.

6.18.2 Samples with measured volumes below 27µL must not proceed for further testing.

6.19 Record the ‘Extraction Run By’ review task and ‘Response’ dropdown for the ‘Pass or Fail?’ step.

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6.20 Transfer custody of all extracts to a cryobox and store in a refrigerator or freezer.

6.21 Assign samples to the next process step. Quant is not needed for Eneg2 F1 and F2.

7 Clean the EZ1 instrument:

7.1 DO NOT USE BLEACH ON THE INSTRUMENT, ONLY 70% ETOH WITH LINT FREE WIPES. NEVER SPRAY ETHANOL DIRECTLY ON THE INSTRUMENT.

7.2 Remove reagent cartridges, lysate tubes, tip-holders and tips, and any waste and discard appropriately.

7.3 Wipe down the inside of the instrument using a lint free wipe and 70% EtOH.

7.4 Close the EZ1 door.

7.5 Follow the prompts on the screen to start a UV run, setting the time to 20 minutes.

NOTE: The UV lamps need a minimum switch-on time of 20 minutes. Do not interrupt a UV light cycle before 20 minutes have passed since it will reduce the lamp's lifetime. Do not touch UV lamp with your fingers. Notify QA when a UV lamp needs to be replaced.

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7.1 Appendix A: QIAcube Loading Chart

The chart below describes how to correctly load the QIAcube centrifuge and shaker if fewer than 12 samples are to be processed in one run. Please note, if only 11 samples are on the batch, a blank must be added to fill the 12th slot.