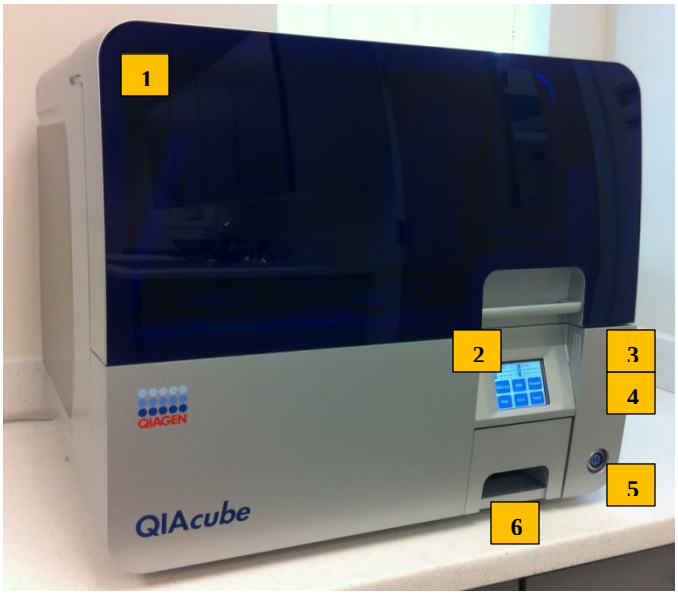
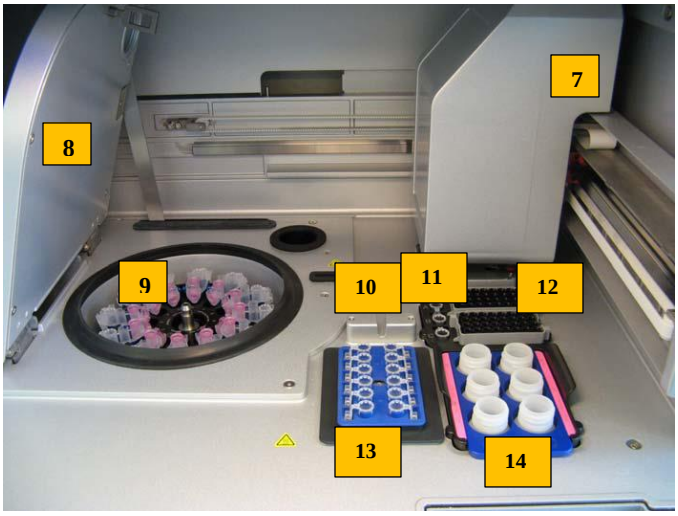
EZ1® DIFFERENTIAL EXTRACTION ON THE QIACUBE®

Background

Differential extraction is a procedure used for samples containing a mixture of sperm cells and non-sperm cells from different donors. The goal is to separate cell types into fractions (a sperm fraction and epithelial fraction) prior to purification, thereby preventing a mixture of DNA, which can complicate downstream interpretation. The differential extraction procedure can be partially automated using the QIAcube® and EZ1® Advanced XL instruments from QIAGEN.

The QIAcube® performs automated processing of up to 12 samples. The instrument controls integrated components including a centrifuge, heated shaker, pipetting system, and robotic gripper. The QIAcube® is preinstalled with various protocols, including differential separation and sperm lysis. An automated load check helps to ensure correct loading of the worktable.





1. Door
2. Touchscreen
3. RS232 serial port (behind panel)
4. USB port (behind panel)
5. Power switch
6. Waste drawer
7. Robotic arm
8. Centrifuge lid
9. Centrifuge
10. Tip sensor
11. Microcentrifuge tube slots
12. Tip racks
13. Shaker
14. Reagent bottle rack

The EZ1® Advanced XL performs automated purification of DNA from 1-14 samples using components of the EZ1® DNA Investigator Kit. Purification is accomplished through silica-coated magnetic particles. DNA is isolated from

lysates in one step through its binding to the silica surface of the particles in the presence of a chaotropic salt. The particles are separated from the lysates using a magnet. The DNA is then washed and eluted in water or TE buffer.

This procedure is fundamentally the same as **02.06.02 EZ1® DNA Extraction - Differential Samples**. Sample handling using the QIAcube® follows the same steps as the manual procedure (i.e., centrifugation of sperm, aspirating and transferring the epithelial lysate, washing of sperm cells and addition of sperm digestion buffer). No change in digestion, washing or purification chemistry is introduced in this procedure.

Summary of Procedure

Samples are exposed to a digestion buffer composed of Buffer G2, Proteinase K and Carrier RNA. This buffer will lyse non-sperm cells, but leave spermatozoa intact. On the QIAcube®, centrifugation will concentrate spermatozoa into a pellet and allow the epithelial fraction lysate to be aspirated into a pipette tip and transferred to a separate tube located on the shaker. Spermatozoa are washed four times, and then lysed using a digestion buffer composed of Buffer G2, Proteinase K, Carrier RNA and dithiothreitol (DTT). Following digestion, both fractions are purified and concentrated on the EZ1® Advanced XL instrument. Using this protocol, up to 12 samples (producing 12 sperm fractions and 12 epithelial fractions) can be extracted concurrently.

Sample Handling

All biological samples and DNA must be treated as potentially infectious. Appropriate sample handling and disposal techniques should be followed. See:

- **Safety Manual**, *Universal Precautions*
- **Quality Assurance Manual**, *General Sample Control and Forensic Sample Preservation Policy*
- **Analytical Procedures Manual**, *Forensic Evidence Handling*

Wear suitable personal protective equipment including a lab coat, gloves and eye/face protection while performing this procedure.

Warnings and Precautions

Buffers in the EZ1® DNA Investigator Kit reagent cartridge contain guanidine salts, which can form highly reactive compounds when combined with bleach. **DO NOT add bleach or acidic solutions directly to the sample preparation waste.**

Reagent cartridges contain ethanol, isopropanol and guanidine thiocyanate. These substances are flammable, harmful, and irritants.

QIAGEN Proteinase K is a sensitizer and irritant.

The QIAcube® door must remain closed during operation due to mechanical hazards. Only open the door when prompted by the software.

Reagents and Materials

QIAcube®
Rotor Adapters
1.5mL Elution tubes (flip-cap, V-bottom)
Filter tips, 1000µL wide-bore
30mL Reagent Bottles
Sample Tubes CB, 2mL
Shaker rack plugs

Reagent bottle rack labeling strips
EZ1® Advanced XL
EZ1® DNA Investigator Kit (48 reactions):
- 48 Reagent Cartridges
- 50 Tip Holders
- 50 Filter-Tips
- 50 2mL Sample Tubes (screw-cap, flat bottom)
- 50 1.5mL Elution Tubes (screw-cap, V-bottom)
- 11mL Buffer G2
- 2x250µL Proteinase K
- 310µg Carrier RNA

Buffer G2 (260mL bottle, available separately from the EZ1® DNA Investigator Kit)
QIAGEN Proteinase K (10mL bottle, available separately from the EZ1® DNA Investigator Kit)
1M Dithiothreitol (DTT)
Pipettes (1000µL, 200µL, 40µL, and 10µL) with associated tips
Serological pipettes (10mL, 25mL)
Heat block
Thermomixer
1.7mL or 2mL micro-centrifuge tubes
Spin baskets compatible with micro-centrifuge tubes
Permanent marker
Kimwipes
Vortex
Centrifuge

Reagents and Materials – Storage and Handling

All reagents and materials are to be kept under sterile conditions. Store all reagents according to the manufacturers' recommendations.

Do not use reagents beyond the listed expiration dates. Date and initial all reagents when put in use. Record in the **Reagent Log**.

Quality Control

Completion of the **QIAcube® Differential Extraction Workbook** is required for documentation.

Appropriate maintenance of the QIAcube® and EZ1® Advanced XL must be performed. See QAM protocols for QIAcube Maintenance Procedures and EZ1 Advanced XL Maintenance Procedures.

Negative Control

Extraction Negative Control: Reagent negative control(s) are processed and run with each set of extractions and consists of all reagents used in the procedure but contains no DNA sample.

Positive Control (Optional)

Extraction Positive Control: Positive control(s) are processed and run with each set of extractions and used to ensure the extraction, amplification and typing procedures are working as expected.

Procedure

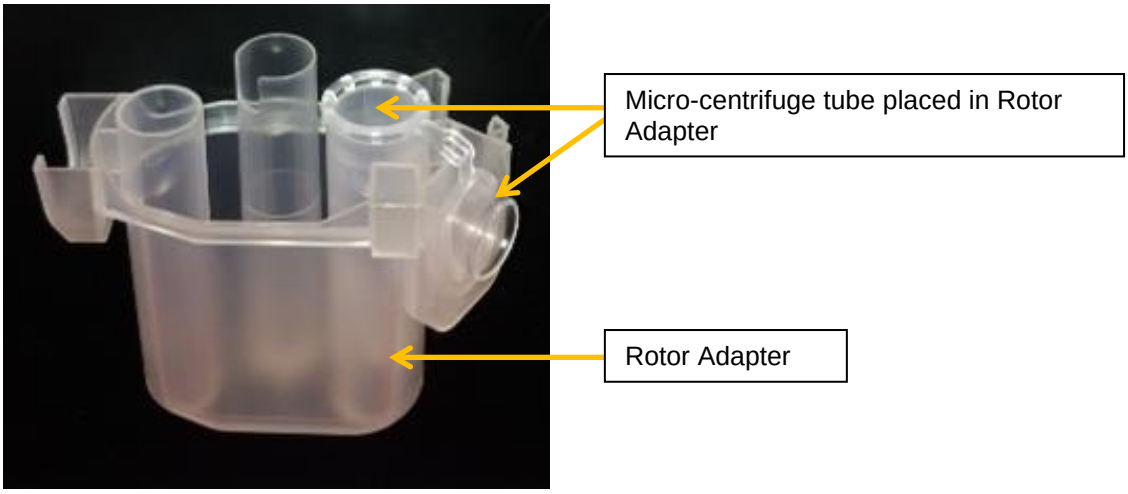
Before starting:

- EZ1 tip holders and filter-tips, sample tubes, elution tubes, reagent bottles.
- Molecular biology grade water must be added to lyophilized carrier RNA. Dispense carrier RNA into 20-50µL aliquots, and store frozen. Before using aliquots, allow carrier RNA to equilibrate to room temperature.
- Pre-warm Thermomixer to 56°C and Thermomixer to 70°C.
- Label all tubes with a unique identifier, initials and date using permanent marker or printed labels.

Epithelial Digestion:

1. Place a sampled portion of the evidence into a 1.7mL or 2mL micro-centrifuge tube.
2. Add 500uL Buffer G2, 20µL QIAGEN proteinase K and 1µL carrier RNA to each sample. For multiple samples extracted concurrently, it is recommended to prepare a master mix of these reagents in the same proportion and add 520µL of the master mix to each sample.
3. Vortex for 10 seconds. Incubate on Thermomixer at 56°C for 1.5-2 hours at 900 RPM. Centrifuge briefly.

   NOTE: Do not incubate sample(s) more than 2 hours.
4. Transfer the substrate and lysate to a micro-centrifuge tube containing a spin basket. Centrifuge at 13,200 rpm for 5 min. A witness must observe this transfer and initial the appropriate space on the worksheet.
5. The substrate may be retained and stored, if required. Remove spin basket from micro-centrifuge tube. A witness must observe the transfer of the spin basket to the final substrate storage tube if the substrate is being retained and initial the appropriate space on the worksheet.
6. Place micro-centrifuge tube (flip-cap, V-bottom) into a labeled Rotor Adapter. See picture below:

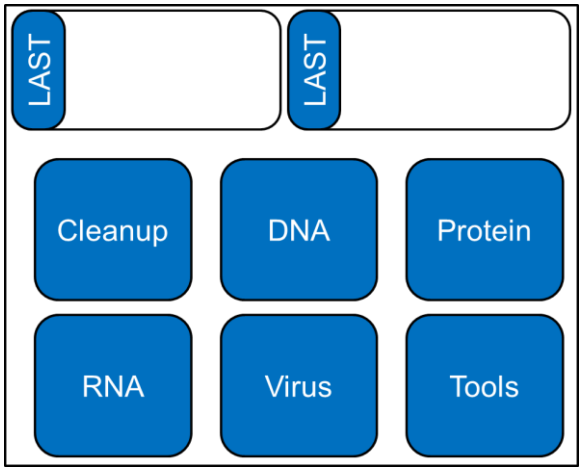


   A witness must observe this transfer of the tube to the rotor adapter and initial the appropriate space on the worksheet.

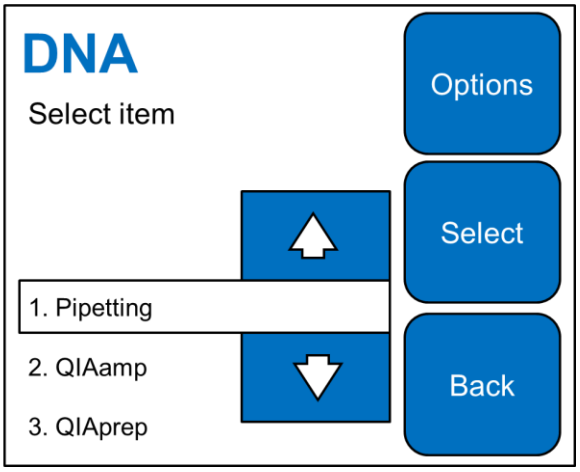
Selecting a QIAcube® Protocol

7. Turn on the QIAcube® by pressing the power switch. Ensure that the instrument door is closed before powering on.

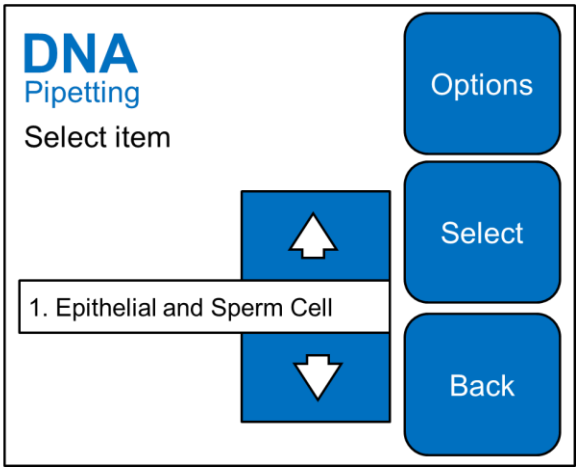
8. The main menu screen will display on the touchscreen.



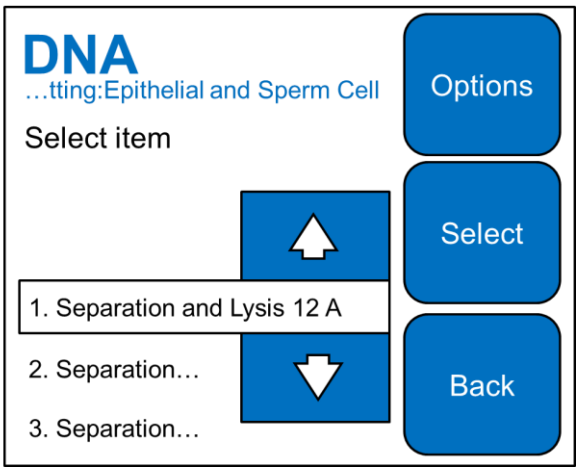
The two boxes labeled “LAST” indicate the last two protocols run (the names of the protocols will be displayed in the boxes). These are shortcut boxes to bypass navigation through the software. If the needed protocol is not displayed in either of these boxes, press “DNA” to select DNA protocols.



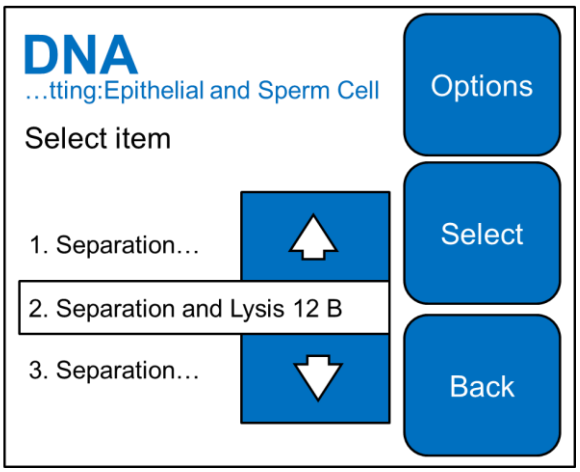
If necessary, press the up or down arrows until “Pipetting” is highlighted. Press “Select.”



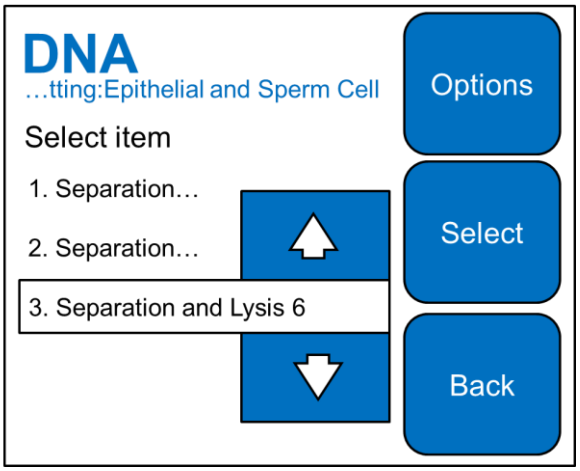
Currently, the only Pipetting protocol is "Epithelial and Sperm Cell." Press "Select." Three protocol options appear in the Epithelial and Sperm Cell menu. If 2-6 samples are being extracted, highlight and select "Separation and Lysis 6." This protocol performs all steps in a single run. If 7-12 samples are being extracted, two separate protocols are needed – "Separation and Lysis 12 A" and "Separation and Lysis 12 B."



Protocol "Separation and Lysis 12 A": First of two protocols used when extracting 7-12 samples. Sperm are centrifuged into a pellet; Epithelial fraction is removed and transferred to a separate tube on the shaker; Sperm fraction is washed twice with Buffer G2; Instrument stops.

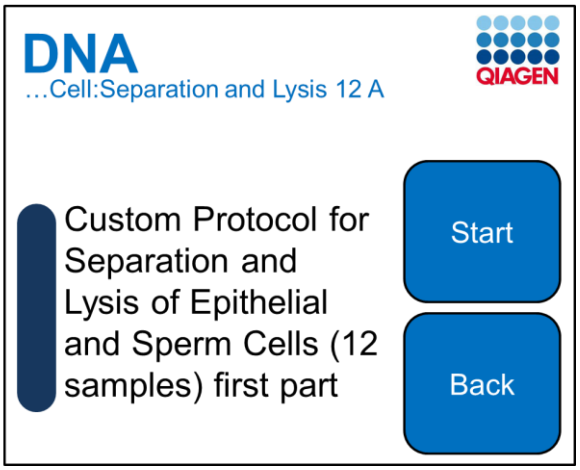


Protocol "Separation and Lysis 12 B": Second of two protocols used when extracting 7-12 samples. Sperm fraction is washed two additional times; 145µL sperm lysis buffer is added; Instrument stops.



Protocol "Separation and Lysis 6": For extraction of 2-6 samples. Sperm are centrifuged into a pellet; Epithelial fraction is removed and transferred to a separate tube on the shaker; Sperm fraction is washed four times with Buffer G2; 200µL sperm lysis buffer is added; Instrument stops.

Highlight and select the required protocol. The next screen will be a description of the protocol selected (12 A in the image below). Press "Start." The screens that follow will guide you through worktable setup.



Protocol "Separation and Lysis 6" or "Separation and Lysis 12 A"

9. Fill the tip rack with 2 cartridges of 1000µL wide-bore filter tips. If necessary, add tips to a partially empty cartridge to fill it up. When doing this (as always), take great care to avoid contamination of the tips. If adding tips to a cartridge by hand, make sure to change gloves immediately before handling tips. Another suggestion is to use a pair of sterile tweezers to handle tips. During the load check, the QIAcube® checks that the correct tip racks have been placed on the worktable and that there are sufficient tips for the protocol run.

10. Ensure the reagent bottle rack is in place on the worktable and labeling strips are attached to the sides.

Reagent bottle rack with attached labeling strips (white) on either side.

11. Add Buffer G2 to a sterile 30mL reagent bottle and place in position 1 of the reagent bottle rack (i.e. the top left position). This buffer will be used for sperm washing. See the table below for minimum volumes required for various sample numbers. If the QIAcube® is or will be used multiple times in succession, this reagent bottle may simply be "topped off" with Buffer G2 at this step rather that measuring a specific volume. Do not exceed the maximum fill line. The 30mL reagent bottles are designed with a V-bottom. Ensure that a blue ring is in place at the bottom of the reagent bottle to create a flat-bottom, allowing the bottle to sit properly in the rack. During the load check, the QIAcube® checks that a sufficient volume is present prior to the protocol run.

Number of Samples	Minimum Volume Buffer G2 (uL)
2	6620
3	8680
4	10740
5	12800
6	14860
7	16920
8	18980
9	21040
10	23100
12	27220

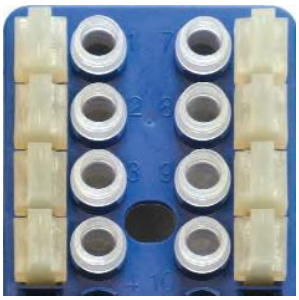
Reagent Bottle in Position 1 (sperm wash buffer)

12. **(NOTE: If extracting 7-12 samples, the lysis buffer described in this step can either be prepared now or delayed until protocol "Separation and Lysis 12 B" is started.)** Prepare sperm lysis buffer for the appropriate QIAcube protocol and number of samples as indicated in the table below. The proportion of reagents per sample is 75.83% Buffer G2, 4.74% QIAGEN proteinase K, 18.96% 1M DTT and 0.47% carrier RNA. Volumes can be rounded to the nearest whole number. Add buffer to a sterile 2.0 mL Sample Tube CB, mix with pipette and place in microcentrifuge tube slot A. Unlike sperm wash buffer, sperm lysis buffer must be prepared fresh for every run and cannot be re-used or "topped-off" for multiple runs in succession.

Formulation of sperm lysis buffer

Sample Number	QIAcube Protocol	Total Sperm Digest Buffer Volume	Buffer G2 (75.83%)	Proteinase K (4.74%)	1M DTT (18.96%)	Carrier RNA (0.47%)
2	S&L 12B	354	268.4	16.8	67.1	1.7
	S&L 6	532	403.4	25.2	100.9	2.5
3	S&L 12B	510	386.7	24.2	96.7	2.4
	S&L 6	768	582.4	36.4	145.6	3.6
4	S&L 12B	667	505.8	31.6	126.5	3.1
	S&L 6	1004	761.3	47.6	190.4	4.7
5	S&L 12B	823	624.1	39.0	156.0	3.9
	S&L 6	1240	940.3	58.8	235.1	5.8
6	S&L 12B	980	743.1	46.5	185.8	4.6
	S&L 6	1476	1119.3	70.0	279.8	6.9
7	S&L 12B	1137	862.2	53.9	215.6	5.3
8	S&L 12B	1293	980.5	61.3	245.2	6.1
9	S&L 12B	1450	1099.5	68.7	274.9	6.8
10	S&L 12B	1606	1217.8	76.1	304.5	7.5
12	S&L 12B	1920	1455.9	91.0	364.0	9.0

13. Place sterile, labeled 2mL EZ1® sample tubes (screw-cap, flat-bottom) into the appropriate positions of the shaker. Epithelial fractions will be dispensed into these tubes. Refer to **Appendix 1: Loading Guide** for appropriate positioning. Place shaker rack plugs into the slots at the edge of the shaker rack. This is only necessary for the positions in which a 2mL tube has been placed, and empty tube slots must not have a rack plug. The QIAcube® checks loading of the shaker, but cannot detect the actual 2mL tube. Instead, the instrument detects the presence or absence of a plug. A witness must check the setup of the sample tubes against the worksheet and initial the appropriate space on the worksheet.



2mL EZ1® sample tubes placed into the shaker with associated shaker rack plugs

14. In Step 6, micro-centrifuge tubes with digested samples were placed into rotor adapters. Now place the rotor adapters into appropriate centrifuge buckets on the centrifuge. Refer to **Appendix 1: Loading Guide** for appropriate positioning. A witness must check the setup of the rotor adapters in the centrifuge against the worksheet and initial the appropriate space on the worksheet.

15. Ensure all tubes and reagent bottles are open/un-capped. The worktable setup is now complete and protocol "Separation and Lysis 12 A" or "Separation and Lysis 6" can be started. At completion of the protocol, cap, then remove epithelial fraction tubes from the shaker and proceed to EZ1 purification (Steps 24-38). If ending protocol "Separation and Lysis 6," proceed to Step 21 for necessary disposal of consumables. At the end of the respective protocol, a witness must check the removal of the epithelial fraction sample tubes against the worksheet and initial the appropriate space on the worksheet.

Protocol "Separation and Lysis 12 B"

16. If extracting 7-12 samples, (after epithelial fraction tubes are removed) the protocol "Separation and Lysis 12 B" will now be initiated. Refer to Step 8 for instructions on selecting this protocol in the software.

17. If not completed previously, prepare sperm lysis buffer and place on the worktable as described in Step 12.

18. It may be necessary to add more Buffer G2 to reagent bottle 1. The software will prompt you during the load check if this is necessary.

19. Empty the waste drawer containing used tips.

20. Refill tip rack with 2 cartridges of 1000µL wide-bore filter tips (see Step 9).

21. Protocol "Separation and Lysis 12 B" will now run. At completion, empty the waste drawer containing used tips. Remove rotor adapters from centrifuge buckets.

Sperm Lysis

22. Remove and cap micro-centrifuge tubes from rotor adapters. At the end of the respective protocol, a witness must check the removal of the sperm fraction microcentrifuge tubes against the worksheet and initial the appropriate space on the worksheet. Dispose of rotor adapters. Include a sufficient amount of paper absorbent in the bag, as waste buffer in the rotor adapter will spill out. Double bag if necessary. Perform sperm lysis using one of the following methods:

    OPTION 1: Place tubes in a Thermomixer pre-heated to 70°C. Turn on the orbital shaker of the Thermomixer to 900 RPM for 10 minutes. Remove samples and vortex vigorously (i.e. on maximum speed) for 10 seconds. Centrifuge briefly to remove liquid from the caps.

    OPTION 2: Place samples in a 70°C stationary heat block; start a timer for 10 minutes; after 60 seconds, pause the timer and remove samples from heat block; vortex samples vigorously (i.e. on maximum speed) for 10 seconds; return samples to heat block and resume the timer; repeat every 60 seconds. Centrifuge briefly to remove liquid from the caps.

23. When ready to proceed with EZ1® purification, remove and discard caps from micro-centrifuge tubes containing digested sperm fractions. The cap can be removed by cutting through the hinge while the tube is closed.

Cut hinge along the marked area.

EZ1® Purification

24. Switch on the EZ1® workstation.
25. Press “START” to start the protocol setup.
26. For epithelial fraction, press “3” to select Large Volume protocol. For sperm fraction, press “1” to select Trace protocol.
27. Select water or TE as the elution buffer.
28. Select elution volume of 40µL, 50µL, 100µL or 200µL. Routinely, both fractions are eluted in 40uL to maximize DNA concentration. Larger elution volumes may be used at the discretion of the analyst.
29. Press any key to start the worktable setup.
30. Open the workstation door and invert 1-14 reagent cartridges at least twice to mix the magnetic particles. Tap cartridges to deposit reagents at the bottom of the well. Load reagent cartridges into the cartridge rack, (Make sure they click in place.)
31. Load 1-12 labeled 1.5mL elution tubes (screw-cap, V-bottom) into the first row of the tip rack. A witness must check the setup of the elution tubes against the worksheet and initial the appropriate space on the worksheet.
32. Load 1-12 tip holders containing filter-tips into the second row of the tip rack.
33. Load 1-12 sample tubes containing digested samples into the back row of the tip rack. For epithelial fractions, these will be 2mL EZ1® sample tubes (screw-cap, flat-bottom). For sperm fractions, these will be micro-centrifuge tubes (flip-cap removed, V-bottom). A witness must check the setup of the sample tubes against the worksheet and initial the appropriate space on the worksheet.
34. Make sure all tubes are open. Close the workstation door and press “START” to begin the purification procedure.
35. “Protocol Finished” should display when it is finished.
36. Open the workstation door and remove the elution tubes from the first row containing purified DNA. Replace tube caps and store samples appropriately (see **Comments on Storage** below).
37. Discard spent cartridges and tubes. Discard tip holders and filter-tips.
38. Perform cleanup and maintenance of the EZ1® Advanced XL and QIAcube instruments as required.

Comments on Storage

Non-amplified DNA must be stored separately from amplified product. Store DNA samples at 2-8°C short-term or -20°C long-term.

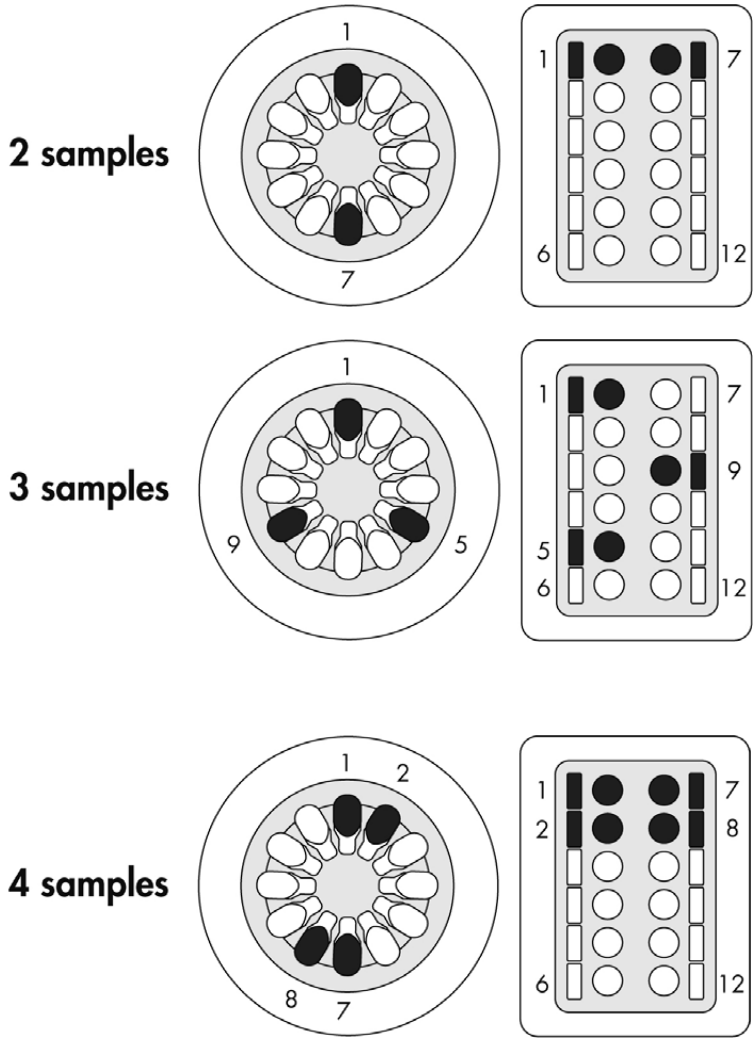
Technical Assistance

For information and assistance regarding the performance or applications, contact the Qiagen Technical Service Department 1-800-362-7737.

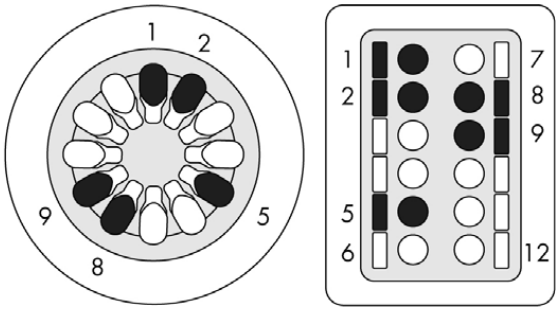
References

QIAcube® User Manual, Version 1.1, June 2008. QIAGEN
EZ1® Investigator Handbook, Fourth Edition, April 2009. QIAGEN

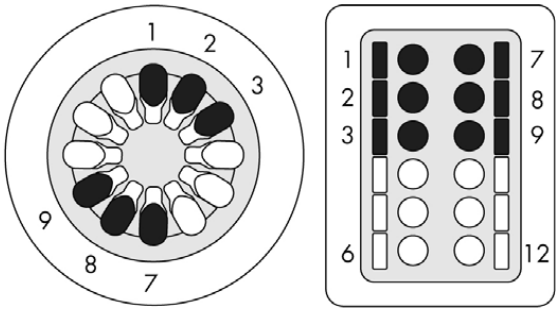
Appendix 1: Loading Guide



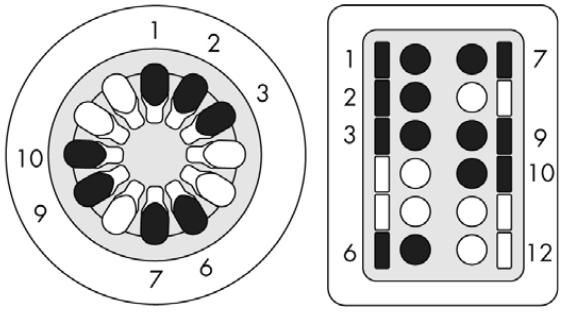
5 samples



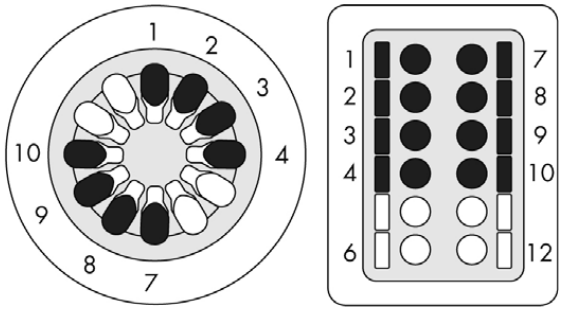
6 samples



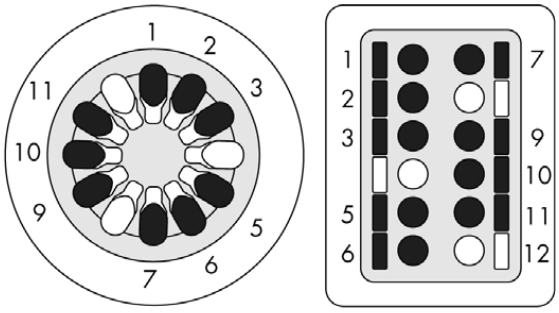
7 samples



8 samples



9 samples



10 samples