20.1 PURPOSE

Procedure for the purification, isolation and concentration of DNA in unknown samples using a silica-coated magnetic bead technique.

20.2 RESPONSIBILITY

DNA Section personnel

20.2.1 Document the extraction on DNA QR-26 (non-differential & hair root), DNA QR-27 (differential samples) or DNA QR-349 (QIAcube), as applicable.

20.3 GENERAL NOTES

20.3.1 If not previously prepared, cut out a sample of the stain. The size of the cutting depends upon the quantity and quality of the sample (~0.5cm^2). As needed, cut the stain into smaller pieces and place these into a labeled SPIN tube. Samples are not limited to stains. Samples could be swabs, cuttings, etc.

20.3.2 If a sample is split into two tubes, refer to section 20.10 for proper processing.

20.3.3 If debris is present in the tube prior to EZ 2 loading, the liquid may need to be transferred to another tube (minimizing the amount of debris transferred). This will prevent the debris from potentially clogging tips during the extraction process.

20.3.4 Semen stains thought to be single source in origin (i.e. non-intimate samples), minute semen samples, or semen samples from putative aspermic males may be extracted without a differential where appropriate but with the addition of 1M DTT.

20.4 NON-DIFFERENTIAL EXTRACTION - Body Fluid and Touch Samples

(Non-Hair and most Non-Semen Samples)

20.4.1 Tube Verification

20.4.1.1 Have a verifier check the labeling and order of the samples and controls to be extracted. Document on extraction worksheet.

20.4.2 Sample Pre-treatment

20.4.2.1 Make a master mix of n+1 (n = number of samples and corresponding controls):

480µl	Buffer G2 or Extraction Buffer
20µl	Proteinase K
1µl	cRNA (1µg/µl)

*20µl 1M DTT will be added to any sample containing human tissue.
**20µl 1M DTT may be added to any suspected semen sample (p30 or male screen positive) not undergoing a differential extraction.
Add DTT to the corresponding reagent blank(s).

20.4.2.2 Add 500µl master mix to each tube. Mix and centrifuge as needed to force samples into extraction buffer.

20.4.3 Lysis

20.4.3.1 Incubate sample tubes for 15 minutes to 18 hours on a thermal shaker set to 56°C and ~850 rpm.

20.4.3.2 After incubation, pulse spin tubes as needed to collect sample condensation to bottom of the tube.

20.4.3.3 Transfer the sample substrate into a spin basket and centrifuge for 2 minutes at 15,000 rpm. Discard the spin basket containing the substrate.

20.4.3.4 To purify the samples and corresponding controls, go to step 20.8.1.

20.5 NON-DIFFERENTIAL – Hair Root Samples

20.5.1 Tube Verification

20.5.1.1 Have a verifier check the labeling and order of the samples and controls to be extracted. Document on extraction worksheet.

20.5.2 Sample Pretreatment

20.5.2.1 Make a master mix of n+1 (n = number of samples and corresponding controls):

180µl	Buffer G2 or Extraction Buffer
10µl	Proteinase K
20µl	1M DTT
1µl	cRNA (1µg/µl)

20.5.2.2 Add 210µl master mix to each tube. Mix and centrifuge as needed to force samples into extraction buffer.

20.5.3 Lysis

20.5.3.1 Incubate sample tubes for 1-18 hour(s) on thermal shaker set to 56°C and ~850 rpm.

20.5.3.2 After incubation, pulse spin tubes as needed to collect sample condensation to the bottom of the tube.

20.5.3.3 Remove and discard any remaining hair sample.

20.5.3.4 To purify the samples and corresponding controls, go to step 20.8.2.

20.6 DIFFERENTIAL EXTRACTION – Using QIAcube Connect

20.6.1 Control Setup

20.6.1.1 When utilizing the QIAcube Connect for differential extractions, a saliva/semen mixture extraction positive (EP2) will be utilized.

20.6.1.2 Two reagent blanks (RBs) will be processed for each fraction ("A" and "B"). Analysts may choose to utilize one RB per fraction for batches with limited chances of RB consumption (i.e., proficiency).

20.6.2 Tube Verification

20.6.2.1 Sample tube caps **must** be labeled to correspond with the position number on QR-349. A verifier will check the labeling and order of the samples and controls to be extracted. Document (date and initials) on worksheet QR-349, **Verification Line #1**.

20.6.3 Sample Pretreatment

20.6.3.1 Make a master mix of (n+1) for samples and corresponding controls:

160µl	Qiagen ATL Buffer
320µl	Qiagen ATE Buffer
20µl	Qiagen Proteinase K
1µl	cRNA (1µg/µl)

20.6.3.2 Add 500µl of master mix to each tube.

20.6.3.3 Mix and centrifuge tubes as needed to force samples into extraction buffer.

20.6.4 Lysis

20.6.4.1 Incubate sample tubes for ~1.5 to 1.75 hours on a thermal shaker set to 56°C and ~900 rpm.

20.6.4.2 After incubation, pulse spin tubes as needed to collect sample condensation to bottom of the tube.

20.6.4.3 Transfer the solid substrate to a spin basket and centrifuge samples at ~15,000 rpm for two minutes to remove excess liquid from substrate. Discard the spin basket containing the substrate.

20.6.5 Sperm Lysis Buffer (SLB) Preparation

20.6.5.1 In a 2.0 mL screw cap tube, prepare a master mix of SLB using Buffer G2, Qiagen Proteinase K and 1M DTT in accordance with Appendix A. Exact volumes required will be listed in QR-349.

20.6.6 QIAcube Connect & "Separation and Lysis 12A" Protocol Setup

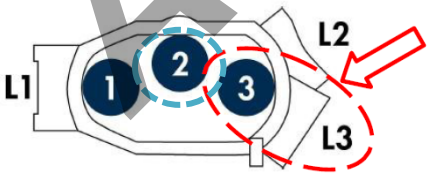
20.6.6.1 Prior to each run, wipe the surfaces and inside the centrifuge with ethanol. Wipe the QIAcube Connect door and rubber gaskets with dH_2O. Do not use ethanol on the door or gaskets.
UV the instrument interior: Select the maintenance tab from the tools menu. Select UV run and keep the cycle number at the defaulted "1" for a 12-minute decontamination. Press "Start".
Document this maintenance on QR-351 (QCC Maintenance Log).

20.6.6.2 On the instrument screen, select 'DNA' → 'Pipetting' → 'Epithelial and Sperm' → 'Separation and Lysis 12A'. Select the total number of samples, including controls.

20.6.6.3 Prior to sample loading, set up the worktable with the appropriate amounts of:

- Sample plugs (Shaker). Refer to Appendix D for positioning (Loading Scheme). Remove any plugs that are not required.
- 1000µl wide-bore pipette tips (tip racks)
- A 30mL reagent bottle filled with Buffer G2 (Position 1 of Reagent Bottle Rack). See Appendix B for minimum required volumes. ***The Buffer G2 lot number and expiration date must be clearly written on the bottle.***

Figure 1. QIAcube Rotor Adapter



20.6.6.4 Obtain a QIAcube rotor adapter (Figure 1) for each sample and control. Label column 2 (blue circle) of each rotor adapter with the appropriate position number according to QR-349. Place these rotor adaptors into the corresponding position numbered slots in a rotor adapter set-up tray.

20.6.6.5 Label 1.5mL flip cap Qiagen Elution tubes and caps with the appropriate position number found on QR-349. Place closed tubes in Position 3 (red oval) of the corresponding rotor

adapter in the set-up tray. Sample IDs may be added to the tubes along with the position number. **These tubes will become the "B" (sperm-rich) fraction sample tubes.**

20.6.6.6 Label 2.0mL EZ2 samples tubes and caps with the appropriate position number found on QR-349. Place capped tubes firmly into the shaker. Sample IDs may be added to the tubes along with the position number. **These tubes will become the "A" (epithelial-rich) fraction sample tubes.**

20.6.6.7 A verifier will ensure the position numbers of the consumables in 20.6.6.3 – 20.6.6.5 are correctly labeled and the placement properly corresponds across each setup. Verification will be documented (date and initials) on worksheet QR-349, **Verification Line #2**.

20.6.6.8 Opening one set up at a time, uncap the 1.5mL ("B" fraction) sample tube and place the cap in position L3 (Figure 1). Discard the spin basket and mix pipette lysate to re-suspend the pellet and pipette transfer the lysate to the sample tube.

Transfer the rotor adapter to the appropriate centrifuge position (Appendix D – Loading Scheme).

20.6.6.9 Remove all caps from the 2.0mL ("A" fraction) EZ2 tubes and the G2 Buffer bottle.

20.6.6.10 Close the instrument door and run the "Separation and Lysis 12A" protocol on the instrument. At the completion of the run, the "A" fractions may be removed from the shaker and proceed to step 20.8.1.

20.6.7 "Separation and Lysis 12B" Protocol Setup

20.6.7.1 Uncap and place the prepared SLB (20.6.5) in Position A of the Microcentrifuge Tube Slots.

20.6.7.2 On the instrument screen, select 'DNA' → 'Pipetting' → 'Epithelial and Sperm' → 'Separation and Lysis 12B' and follow prompts.

20.6.7.3 Replenish the Buffer G2 and tips and empty the waste drawer. Remove partial tips racks and replenish with full racks. Partial tip racks will be merged as necessary, but only full racks can be placed on the QIAcube Connect. Close the instrument door and start run.

At the completion of the run, remove and cap "B" fractions.

20.6.8 "B" Fraction Processing – Quick Sperm Lysis

20.6.8.1 Vigorously vortex the sperm fractions.

20.6.8.2 Incubate the samples for 10 minutes on a thermal mixer set to 70°C and ~900 rpm.

20.6.8.3 Vigorously vortex the fractions again and pulse spin to collect sample condensate. Proceed to step 20.8.2.

20.6.9 ***QIAcube Post-Run Cleanup***

20.6.9.1 After each run, perform the 'Daily Maintenance' protocol. Select 'Tools' menu → 'Daily' tab → 'Start'. Wait for the instrument arm to finish moving.

Discard the SLB tube. Return the unused sample plugs. Empty liquid from the rotor adapters into the sink and discard used adapters into the garbage. Cap the Buffer G2 reagent bottle and store off-deck.

Select 'Next' on the menu screen. Empty the tips from the waste drawer and wipe the liner with ethanol if needed. Wipe the surfaces and inside of the centrifuge with ethanol. Wipe the door and rubber gaskets with dH_2O. Do not use ethanol on the door or gaskets.

20.6.9.2 UV the instrument interior: Select the maintenance tab from the tools menu. Select UV run and keep the cycle number at the defaulted '1' for a 12 minute decontamination. Press 'Start'.

20.6.9.3 Document this maintenance on QR-351 (QCC Maintenance Log).

20.7 DIFFERENTIAL EXTRACTION – Manual Wash

20.7.1 Control Setup

20.7.1.1 An EP1 (blood) will be used for manual differential extractions. The "B" fraction EP1 is introduced at step 20.7.3.4.4.

20.7.1.2 Two reagent blanks (RBs) will be processed for each fraction ("A" and "B"). Analysts may choose to utilize one RB per fraction for batches with limited chances of RB consumption (i.e. proficiency).

20.7.2 Tube verification

20.7.2.1 Have a verifier check the labeling of the samples and controls to be extracted. Document verification on DNA QR-27.

20.7.3 Sample Pretreatment

20.7.3.1.1 Make a master mix of (n+1) for samples and corresponding controls:

480µl	Extraction Buffer
20µl	Proteinase K
1µl	cRNA (1µg/µl)

20.7.3.1.2 Add 500µl of master mix to each tube. Mix and centrifuge tubes as needed to force samples into extraction buffer.

20.7.3.2 ***Lysis***

20.7.3.2.1 Incubate sample tubes for 15 minutes to 1 hour on a thermal shaker set to 56°C and ~850 rpm.

20.7.3.2.2 After incubation, pulse spin tubes as needed to collect sample condensation to bottom of the tube.

20.7.3.3 Transfer the sample substrate into a spin basket and centrifuge for 2 minutes at 15,000 rpm. Discard the spin basket containing the substrate.

20.7.3.3.1 Carefully transfer the supernatant to an EZ2 Sample Tube or SPIN tube (labelled "A" fraction), without disturbing the sperm cell pellet. The "A" fraction samples can either be processed immediately (step 20.8.1) or incubated on the thermal shaker with the "B" fractions.

20.7.3.4 ***"B" Fraction Processing***

20.7.3.4.1 Wash the sperm cell pellet by re-suspending the pellet in 500µl Extraction Buffer.

20.7.3.4.2 Centrifuge the tube for 2 minutes at ~15,000 rpm and carefully remove and discard the supernatant, without disturbing the sperm cell pellet.

20.7.3.4.3 Repeat wash steps two to three times.

20.7.3.4.4 Make a master mix of (n+1) for samples and corresponding controls:

460µl	Extraction Buffer
20µl	Proteinase K
20µl	1M DTT
1µl	cRNA (1µg/µl)

*Extraction Positive – "B" Fraction is introduced at this step.

20.7.3.4.5 Add 500µl of master mix to each tube. Mix and centrifuge tubes as needed to force samples into extraction buffer.

20.7.3.4.6 Re-suspend the sperm pellet and incubate each tube at 56°C for 2 to 18 hours on a thermal shaker set to 56°C and at ~850 rpm.

20.7.3.5 To process the samples and corresponding controls, proceed to step 20.8.1.

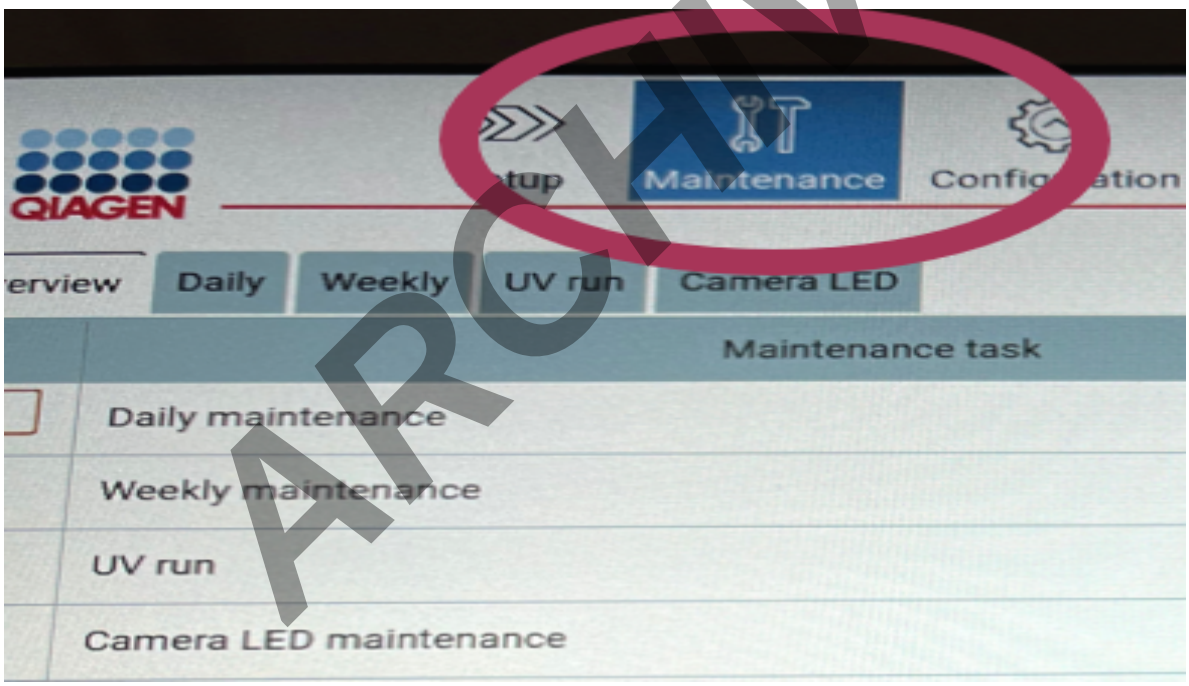
20.8 DNA PURIFICATION ON THE EZ2 CONNECT FX

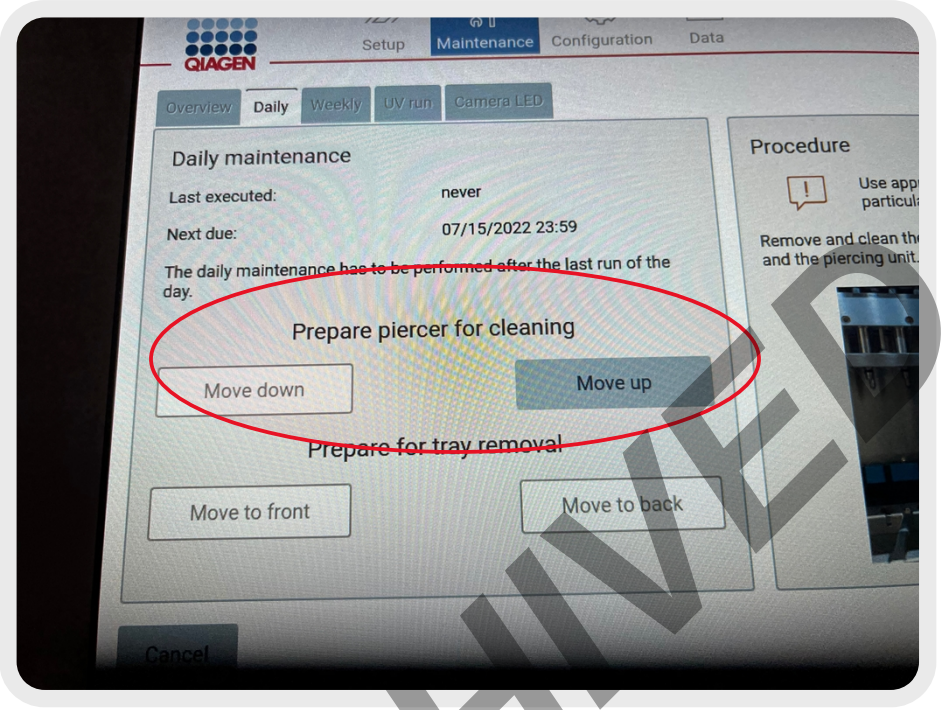
20.8.1 Add 400µl of Buffer MTL to the lysate.

20.8.1.1 To help prevent a precipitant from forming, the sample tubes containing the MTL buffer may be temporarily stored at 56°C until use or Buffer MTL can be pre-heated to 56°C.

20.8.2 Pre-Run Setup

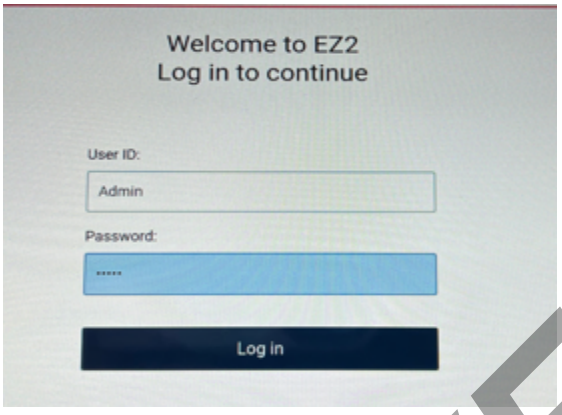
20.8.2.1 Select Maintenance to run the UV run, Daily Maintenance of cleaning the piercing Unit after the run. UV run is done before and after runs. Document cleaning on DNA QR-281 EZ1/EZ2 Maintenance Log.



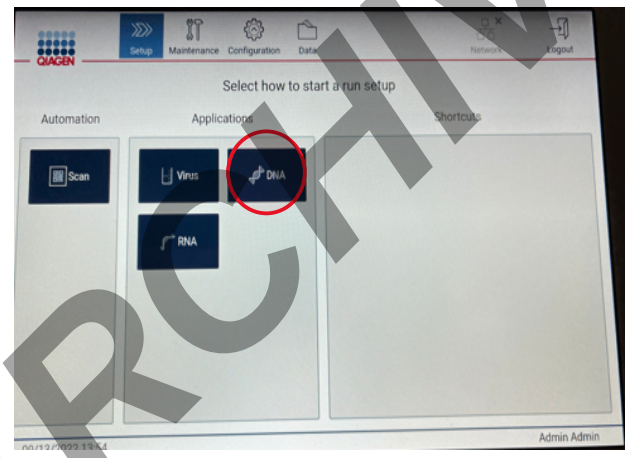


20.8.2.2 The reagent cartridges contain guanidine salts, which can form highly reactive compounds when combined with bleach. **Do not use bleach on the EZ2 or to clean up EZ2 reagent spills.** If reagents spill, completely soak up liquid with paper towel, then clean area with water, followed by bleach.

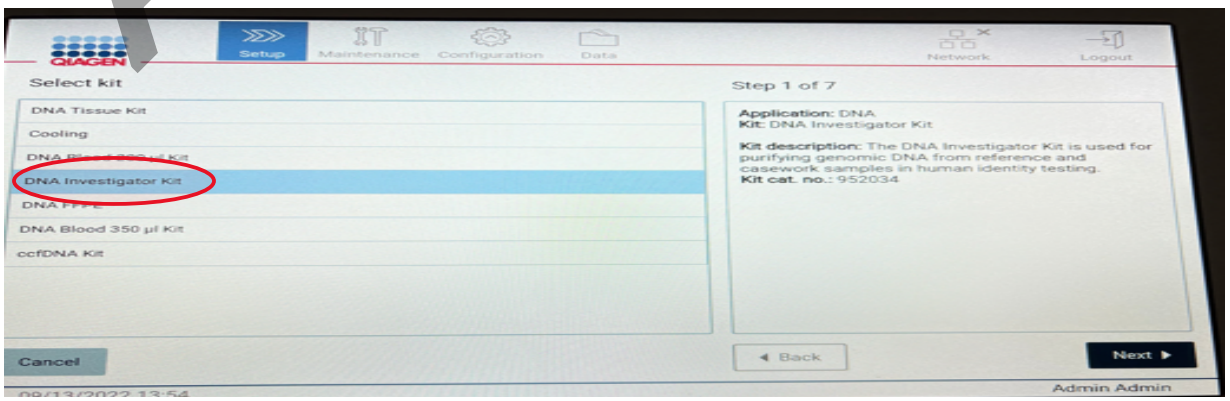
20.8.2.3 To log into the instrument use Admin, Admin for username and password.



20.8.2.4 On the next screen, select "Setup" and then, "DNA"



20.8.2.5 Next hit DNA Investigator Kit and press Next.

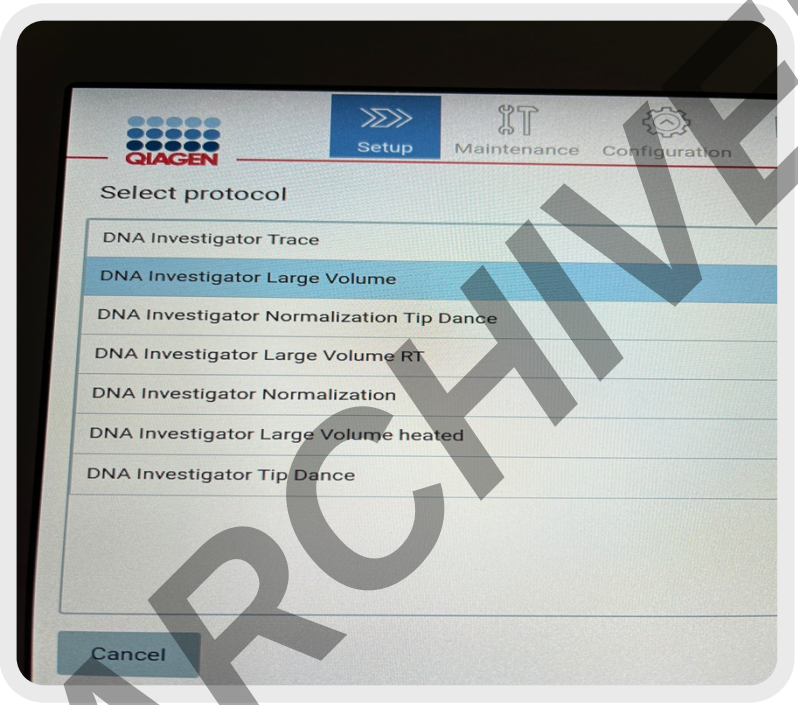


20.8.2.5 The DNA Investigator Large Volume, DNA Investigator Trace, and DNA Investigator Normalization Protocols are on the next screen to select. Based on the samples being extracted, select the appropriate protocol.

20.8.2.6 Large Volume protocol – used for most non-differential and manual differential.

20.8.2.7 Trace protocol - Hair roots and "B" fractions from QIAcube Connect.

20.8.2.8 Normalization Protocol for Known Samples.

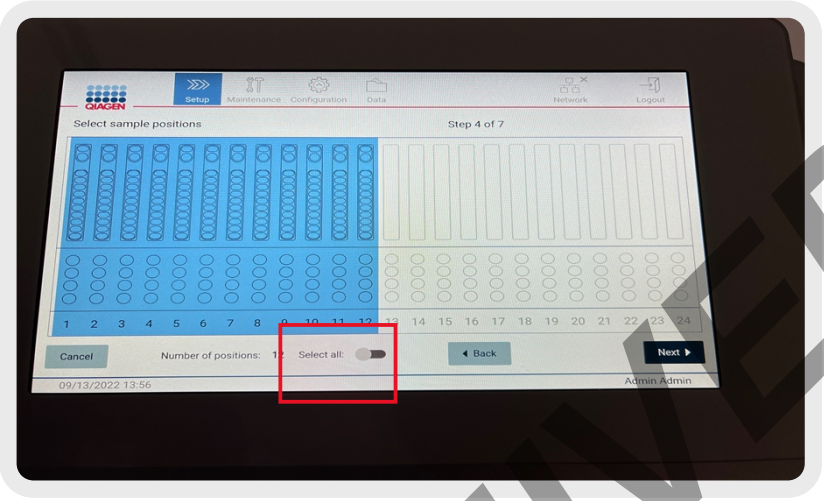


20.8.3 Instrument Setup

20.8.3.1 Under Define Parameters, select Water, Flip cap rack, and 50µL elution volume.

20.8.3.2 Cartridge and Tube Racks are labeled 1-12 and 13-24.

20.8.3.3 Select the sample positions on the next screen. There is a "select all" button shown below in the red square that will automatically select all samples.



20.8.4 Next just select "Generate Missing Sample IDs". The dates and times of the run will show on the screen, but the DNA QR extraction worksheet will have sample names.

20.8.5 Obtain a DNA Investigator Reagent Cartridge for each sample to be purified. Visually inspect each cartridge before use. If a precipitate is observed, do not use the cartridge, and notify the Equipment/QC Coordinators. Where appropriate, the cartridges have been pre-heated to minimize the formation of precipitates during the extraction process.

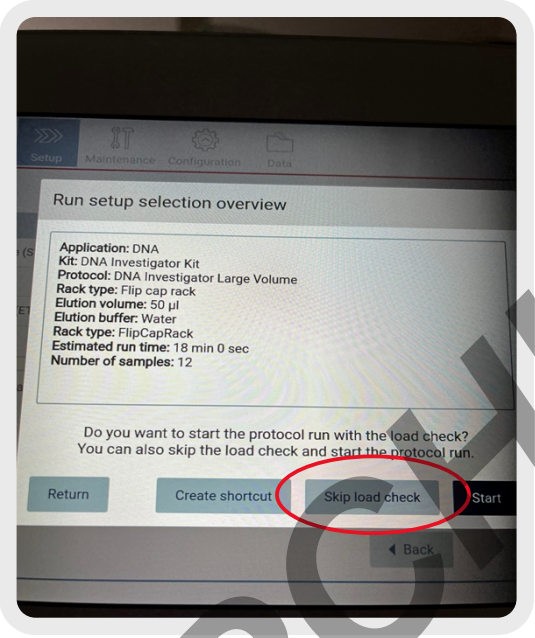
20.8.6 Invert the reagent cartridges to mix the magnetic particles. Load the reagent cartridges into the cartridge rack ensuring the cartridge clicks into place after you slide it into the rack. Prior to extraction, if cartridges are left in the rack for an extended period, remove cartridges and repeat this step.

20.8.7 EZ2 Setup

20.8.7.1 Load the EZ2 flip-cap rack as follows:

- Front row: opened 1.5mL flip or screw cap elution tubes
- Middle row: Tip holders containing filter tips
- Back row: opened sample tubes containing the digested sample.

20.8.7.2 Close the instrument door, review the screen, and press 'Skip Load Check' on the instrument screen. Note: if "start' is hit instead of "skip load check" and screw top tubes are used, an error will occur. This is due to the use of a flip cap rack and the instrument "expects" a "cap". When there is no cap present, the instrument will show an error. No detrimental effect to the samples will occur.



20.8.8 Post-Run Check

20.8.8.1 Check the pipette tips. Make sure the filters are not wet, crystalized, or discolored.

20.8.8.2 Replace/close elution tube caps and remove samples from the instrument.

20.8.8.3 Visually check the volumes in the elution tubes. They should have the same volume (~50µL), with the RB being at the most stringent volume.

20.8.8.3.1 Notify the Equipment Coordinator when elution issues occur. If necessary, consult a lead, the DNA Technical Leader/Assistant Technical Leader, or Assistant Director/Deputy Director.

20.8.8.3.2 If a volume discrepancy is observed, manually check the volumes of all tubes with a pipette to ensure that the RB is at the most stringent volume. Record each volume on the QR worksheet.

20.8.8.3.3 If the RB is not the most stringent volume, adjust sample volumes with dH_2O. Record adjustments on the QR worksheet.

20.8.8.3.4 If an elution volume is significantly increased (>60µL), concentrate the impacted sample and associated RB(s) using a Microcon-100, eluting to the most stringent volume of all the tubes in the set/batch. No approvals are needed for this concentration.

20.8.8.3.5 When the EZ2 elution tube volume is significantly reduced (e.g., 0-20µl), and the DNA resides either somewhere in the cartridge or in the sample tube, the following approach may be employed to recover additional DNA:

A. Make a master mix of extraction buffer, ProK, and cRNA as described above for an additional RB and EP1, if applicable. Add master mix to the control(s). If applicable, MTL buffer is added to the controls, but not to the sample tube.
B. Re-run the impacted sample in the same EZ2 run position, using the same cartridge and sample tube.
C. Run the EZ2 using the standard protocol discussed above.

20.8.9 Post-Run Cleanup

20.8.9.1 Discard waste into the designated receptacle.

20.8.9.2 Clean the cartridge rack and tip rack with ethanol.

20.8.9.3 Document cleaning on DNA QR-281 EZ2 Maintenance Log.

20.8.9.4 Weekly (+/- 3 days) O-ring wiping/greasing will be documented on DNA QR-223.

20.9 CONCENTRATION AND/OR PURIFICATION OF DNA SAMPLES

20.9.1 Microcon filters may be employed to either concentrate a low-level DNA sample, purify inhibited samples or to combine multiple extracts.

20.9.2 The following is general guidance for sample concentration and/or purification. Other approaches may be employed as appropriate with TL approval.

20.9.3 The analyst must evaluate the potential impact of sample concentration on the complete set of samples in the extraction set. Consider the number/volume of RBs, the number of different amplifications anticipated for the set and which amplification systems would be more critical/appropriate for the cases. In general, no-suspect case samples should not be concentrated unless sample concentration is expected to lead to sufficient profile results for CODIS entry (SDIS or NDIS).

20.9.4 The amount of sample to be concentrated and the eluate volume after concentration is based on the quant results and the amplification(s) required.

20.9.5 A reagent blank must be processed in the same fashion/at the same time as the evidentiary sample(s) to be concentrated, i.e., concurrent, and parallel; the RB must have the same or greater stringency compared to the evidentiary sample.

20.9.5.1 If another sample in an extraction set has previously been concentrated (e.g., to maximum stringency such that the RB has been consumed and amplified using the same STR system as the current concentration/amplification event), a manipulation blank must be created to account for the processing steps associated with the current concentration.

20.9.5.2 The sample shall not be concentrated if the RB is consumed (except as stated above) or missing. If the target volume of the sample to concentrate is greater than the amount of RB remaining, the sample may be concentrated using up to the same volume as the RB. If it can be determined that evaporation/sublimation caused the volume of the RB to be less than the sample volume, the sample may still be concentrated. For this determination, the analyst must review the other cases in the extraction set to determine how much RB was consumed.

20.9.6 Document the sample concentration on DNA QR-28 Concentration Worksheet. QR-28 must be set up or checked by a qualified DNA analyst.

20.9.7 Assemble a Microcon filter into a labeled Microcon 1.5 mL flip-cap tube.

20.9.8 Add ~ 400µl of dH_2O to each Microcon. For extracts that need to be combined, add ~300µl to account for the Microcon's maximum capacity.

20.9.9 Add the appropriate volume of sample/RB to the Microcon assembly. For extracts being combined, add the entire volume of each extract into a single Microcon assembly.

20.9.10 Centrifuge for ~ 10 minutes at 2300-4000 rpm (500g-1500g). If excess liquid remains, the centrifuge time may be extended as needed.

20.9.11 If PCR inhibitors are a concern, additional dH_2O washes through the Microcon may be performed.

20.9.12 To recover the DNA from the Microcon, add the appropriate elution volume of dH_2O to the Microcon membrane and invert the filter into a new labeled Microcon 1.5 mL flip-cap tube. Centrifuge for ~2-3 minutes at 2300 rmp or 500g.

20.9.12.1 When Microcon is performed to purify, the elution volume should be the same as the starting volume.

20.9.12.2 When Microcon is performed to concentrate, the elution volume should be appropriate for the subsequent amplification (i.e., 10 µl for YFP; 15 µl for GF).

20.9.12.3 When Microcon is performed to combine extracts, the volume should be ~50µl.

20.10 SWAB MATERIALS FROM ONE SAMPLE SPLIT INTO TWO TUBES

20.10.1 For swab materials to be properly incubated in extraction buffer and to fit into a spin basket, evidentiary swab materials can be processed in two separate tubes. The sample tubes are extracted separately as described above.

20.10.2 The pair of elutes is combined by adding both elutes to a single Microcon-100 and processing as described in 20.9. The combined elutes are eluted in 50µl. This combination is documented on DNA QR-26 or QR-27.

20.10.3 The reagent blank within the batch is processed with the exact same additional manipulation steps, parallel and concurrent with the sample 2 reagent blanks are processed separately as described above and the elutes are combined. The extracts are combined using a Microcon as described in 20.9.

20.10.4 The reagent blank which will go through the Microcon step also serves as the reagent blank for the other samples in the same set/batch which will not go through the Microcon step, since it is more stringent than the other samples.

APPENDIX A: SPERM LYSIS BUFFER TABLE

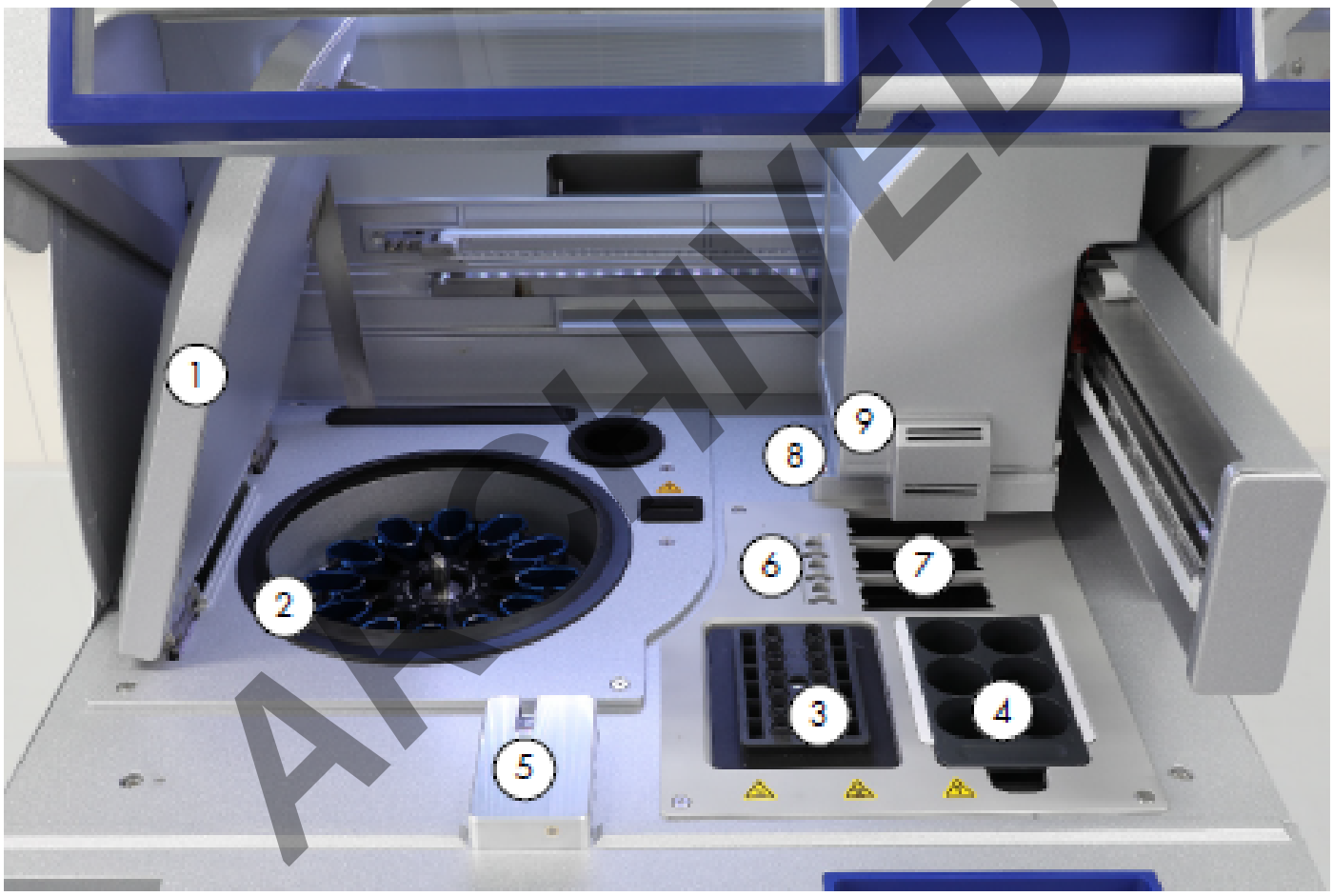
	Individual Volumes (µl)			
Number of samples	**G2**	**ProtK**	**DTT**	**Total Volume (µl)**
2	270	18	72	360
3	398	27	106	530
4	506	34	135	675
5	623	42	166	830
6	750	50	200	1000
7	863	58	230	1150
8	975	65	260	1300
9	1106	74	295	1475
10	1219	81	325	1625
(11 or) 12	1470	98	392	1960

APPENDIX B: BUFFER G2 MINIMUM VOLUMES

Number of samples	G2 (µl)
2	4900
3	6100
4	7300
5	8500
6	9700
7	10,900
8	12,100
9	13,300
10	14,500
(11 or) 12	16,900

APPENDIX C – INTERNAL VIEW OF QIACUBE CONNECT

1. Centrifuge lid	4. Reagent bottle rack	7. Tip racks
2. Centrifuge	5. Tip sensor and hood lock	8. Disposal slots for tips
3. Shaker	6. Microcentrifuge tube slots	9. Robotic arm



APPENDIX D: QIACUBE CONNECT LOADING SCHEME – If there are only 11 samples, use a blank water tube in the 12th spot to balance the centrifuge.