

DNA Extraction of Bone Samples		
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## Large Volume Demineralization Extraction Procedure with Qiagen M48 Low Elution

**\*NOTE:** This protocol allows for both nDNA QC'd reagents and Mito QC'd reagents. Any questions regarding reagent use can be directed to either the nuclear or mitochondrial DNA technical leaders.

### 1 Extraction Sample Set-up

- 1.1 Set up work area; obtain samples, conical tubes for controls, and reagents (0.5M EDTA and 20mg/mL Pro K).
- 1.2 Obtain two empty 1.5mL microcentrifuge tubes for the extraction negatives and label one as Extraction Negative 1 and the other as Extraction Negative 2.
- 1.3 **Have a witness confirm the order of your sample set. Have your witness verify the tube top label and sample label. This will be your "Bone Incubation" witness.**
- 1.4 Add 9mL 0.5M EDTA and 200  $\mu$ L Pro K to each tube.
- 1.5 Vortex thoroughly and parafilm all samples.
- 1.6 Place samples in shaker and incubate at 56°C at a speed of 124 RPM overnight. Shaker should default at these settings.
  - 1.6.1 To program the shaker use the "Select" button to highlight the fields on the right of the control panel. Once field is highlighted the up and down arrows can be used to set field to the appropriate number. Once samples are in the shaker, close the cover and select the "Start" button. Samples should begin shaking at set RPM's. Before opening the cover to remove samples, press the "Stop" button and allow samples to come to a stop. If shaker starts to beep after opening or closing cover hit the "Select" button once. (This beep is signaling that temperature has dropped from the setting that was selected.)

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## 2 Clean-up

- 2.1 Remove tubes from shaker and set temperature to 60°C, speed at 124 RPM.
- 2.2 Add 1.0mL of 1.0M KOH to each tube. Dispose of all KOH tips in the amber hazardous waste bottle labeled “potassium hydroxide”.

**\*\*NOTE: Eye protection must be worn when handling 1.0M KOH. Avoid contact of reagent with metal part of pipette when aliquoting from reagent container.**

- 2.3 Vortex thoroughly and place on shaker once it has reached 60°C for 5min.
- 2.4 Vortex all samples and place in large centrifuge at 2500 RPM for 3-5min.
- 2.5 Label 10K Amicon tubes (tops and sides) the same way the extraction sample set is labeled.
- 2.6 **Have a witness confirm the order of your samples ensuring that they are correctly set up for transfer to the correctly labeled Amicon tube. Have your witness verify the tube top label on the input tubes and the tube top label and side tube label on the Amicons. This will be your “Bone Clean-up” witness.**
- 2.7 Transfer the supernatant portion of the samples to Amicons. Throw away incubation tubes in the hazardous waste trash.
- 2.8 Spin Amicons in large centrifuge at 4000-4500 RPM for an initial 45-60min. **The Eppendorf centrifuge will only reach 4000 RPM.**
- 2.9 Continue spinning until samples are at or below the 500µL mark on the Amicon tube.
- 2.10 Once under 500µL, remove the top of the Amicon tube, pull out the filter portion and drain out the liquid in the bottom of the Amicon into a sink with running water.
- 2.11 Replace the filter in the tube. Add 5mL sterile or UltraPure water to each Amicon.
- 2.12 Spin again at 4000-4500 RPM for 10-15 until sample is at or below the 500µL mark on the Amicon tube.
- 2.13 Repeat steps [2.10-2.12](#) one more time for a total of 2 sterile or UltraPure water washes.
- 2.14 Label stratalinked M48 tubes the same way the incubation and Amicon tubes were labeled. **Print out input sample labels for M48 tubes.**

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- 2.15 **Have a witness confirm the order sample set to ensure that the Amicon sample will be transferred to the correctly labeled M48 tube. Have the witness verify the tube top label and side label of the Amicon tubes, and the M48 input tube tops and sample label. This will be your "M48 tube set-up" witness.**
- 2.16 Using a 200 $\mu$ L pipette and sterile or UltraPure water, bring the volume of the sample in the Amicon tube up to 500 $\mu$ L.
- 2.17 Using the pipette tip, move it across the bottom of the Amicon filter to re-suspend sample with sterile or UltraPure water. Tilt the Amicon so sample collects to one side and draw up the sample, placing it into the labeled M48 tube. Throw away Amicon tubes when finished in the biohazard trash.
- 2.18 **Samples should be processed on the M48 within 48 hrs of extraction clean-up. If M48 processing cannot be done immediately after extraction, keep samples in a freezer until procedure can be done.**

### 3 M48 Large Volume-Low Elution Procedure

**\*\*NOTE: G2 and Pro K are NOT added to the samples and the samples are not incubated as with other M48 Procedures. No new controls are introduced for this procedure.**

- 3.1 Open file on the M48 computer. Save this sheet by going to File→Save As and save the sheet to the "SampleName" folder on the desktop with "File Name:" in MMDDYY.HHMM format and "Save As Type:" set to CSV (Comma delimited)(\* .csv). Use the original extraction date and time.
- 3.2 Click "Save". A window stating "The selected file type does not support workbooks that contain multiple sheets" will open. Click "OK".
- 3.3 A second window asking "Do you want to keep the workbook in this format?" opens. Click "Yes".
- 3.4 Open instrument program on computer and set program to "**Large volume v1.1**".
- 3.5 Click on the "select" button and select "1.5 ml" for the size of the elution tubes
- 3.6 Select the number of samples 6, 12, 18, 24, 30, 36, 42, or 48.
- 3.7 Set sample volume to 500 $\mu$ L
- 3.8 Set elution volume to 50 $\mu$ L

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- 3.9 The next prompt asks to ensure the drop catcher is clean. In order to check this, click on “manual operation” and select “Drop Catcher Cleaning”. The arm of the robot will move to the front of the machine, and the drop catcher (a small plastic tray) will be right in front of you. Remove and clean with 70% ethanol. When the catcher is clean, replace the tray, close the door, and click “OK” in the window.
- 3.10 Make sure that the chute to the sharps container bin is clear for the tips to be discarded. Click “Next”.
- 3.11 The software will calculate the number of tips necessary for the run. Place tips in the tip rack(s) if necessary. **When filling racks, make sure that the pipette tips are correctly seated in the rack and flush with the robotic platform.** Tips are located in three racks. These racks may be filled one at a time, BUT you must fill a **whole rack** at a time. After a rack is filled, reset the tip rack by clicking on “Yes tip rack ...”. If no new tips are being added to the robot click “No”.

Tips needed for a run:

Samples	6	12	18	24	30	36	42	48
# Tips	30	42	54	66	78	90	102	114

- 3.12 After you are finished, click “Next”
- 3.13 Obtain stock bottles of reagents and **record lot numbers**. Fill the reagent reservoirs as stated below. All reagents are stored in their respective plastic reservoirs in the metal rack, labeled with the lot number of the reagent that they contain, and covered with Parafilm, **EXCEPT** the magnetic resin. The resin is disposed of after every extraction. Vortex the magnetic resin solution well, both in the stock bottle and in the reservoir, before adding it to the metal rack (see step [3.16](#) for preparation of MagAttract Resin). If you notice crystallization in any of the solutions, discard the solution, rinse the container out, and start again with fresh reagent.

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- 3.14 Remove the Parafilm and lids from the reagents, and fill the reservoirs to the appropriate level using solutions from the working solution bottles using the same lot as labeled on the reservoir. If not enough of the same lot of a solution remains, discard the remaining solution from the reservoir, rinse and re-label the reservoir with the new lot number. When filling the reservoirs **add approximately 10% extra to the volumes recommended below to account for the use of the large bore pipette tips:**

# of samples	Large reservoir Sterile or UltraPure Water (mL)	Large reservoir Ethanol (mL)	Large reservoir Buffer MW1 (mL)	Large reservoir Buffer MTL (mL)	Small reservoir Sterile or UltraPure Water (mL)	Elution buffer (TE <sup>-4</sup> ) (mL)	Small reservoir Poly A RNA - Magnetic Resin (mL)
6	10.0	11.8	7.2	5.9	3.5	1.6	1.5
12	18.4	22.6	12.9	10.3	5.9	1.9	1.7
18	26.9	33.4	18.6	14.7	8.4	2.2	1.9
24	35.3	44.2	24.3	19.0	10.8	2.5	2.1
30	43.7	55.0	30.0	23.4	13.3	2.8	2.3
36	52.2	65.8	35.7	27.8	15.7	3.1	2.5
42	60.6	76.6	41.4	32.1	18.2	3.4	2.7
48	69.0	87.4	47.0	36.5	20.6	3.7	2.9

**Note: Bottles of MW1 require the addition of ethanol prior to use. See bottle for confirmation of ethanol addition and instructions for preparation if needed.**

- 3.15 Follow software instructions to prepare reagent rack. Software will indicate the reagent, reagent position in the rack and amount of reagent to use.

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- 3.16 Refer to the table below for amounts of 1000ng/uL Poly A RNA stock solution to add for resin preparation:

Samples	Volume of 1000ng/uL stock PolyA RNA solution added to resin (uL)	Volume of Untreated MagAttract Resin (uL)	Total Volume of RNA Treated MagAttract Resin (uL)
6 samples	<b>4.4</b>	<b>1497.8</b>	1502.2
12 samples	<b>5.0</b>	<b>1697.5</b>	1702.5
18 samples	<b>5.6</b>	<b>1897.2</b>	1902.8
24 samples	<b>6.2</b>	<b>2096.9</b>	2103.1
30 samples	<b>6.8</b>	<b>2296.6</b>	2303.4
36 samples	<b>7.4</b>	<b>2496.3</b>	2503.7
42 samples	<b>7.9</b>	<b>2696.0</b>	2703.9
48 samples	<b>8.5</b>	<b>2895.7</b>	2904.2

- 3.17 The pretreated resin may be prepared in a 15mL conical tube and then added to the appropriate reservoir for addition to the platform in the amount dictated by the protocol.
- 3.18 Vortex the magnetic resin in the container before pipetting into M48 reagent container and vortex the M48 reagent container once the PolyA dilution has been added.
- 3.19 Place reservoirs into the metal rack in the following locations. The plastic reservoirs only fit into the rack one way. Check the directions of the notches which should point **into** the robot:

Size Reservoir	Rack Position	Software Tag	Reagent
Large reservoir	L4	Rea_4	Sterile or UltraPure Water
Large reservoir	L3	Rea_3	Ethanol (100%)
Large reservoir	L2	Rea_2	Wash Buffer 1 (Buffer MW1)
Large reservoir	L1	Rea_1	Lysis and Binding Buffer (Buffer MTL)
Small reservoir	S6	ReaS6	(empty)
Small reservoir	S5	ReaS5	(empty)
Small reservoir	S4	ReaS4	(empty)
Small reservoir	S3	ReaS3	Sterile or UltraPure Water
Small reservoir	S2	ReaS2	Elution Buffer (TE <sup>-4</sup> )
Small reservoir	S1	ReaS1	Magnetic Particle Resin

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- 3.20 Flip up the “container interlocks” and place the metal reservoir holder onto the left side of the robotic platform in the proper position. **DO NOT force the holder into place and be careful not to hit the robotic arm.** After correctly seating the metal holder, flip down the “container interlocks” and press “next”.
- 3.21 Click “Next” when you are prompted to write a memo.
- 3.22 Place the sample preparation trays on the robot. One tray for every 6 samples. Click “Next”.
- 3.23 Place empty, unlabeled 1.5mL elution tubes in the 65 degree (back) hot block, located on the right side of the robotic platform. Make sure tubes are in places for any blank samples. Click “Next”.
- 3.24 Print output labels for 1.5 mL screw top tubes for final sample collection in the robot.
- 3.25 Place the **labeled**, empty 1.5 mL sample collection tubes in the 8 degree (front) cold block for collection of final samples. Make sure tubes are in place for any blank samples.
- 3.26 **Prepare the PolyA dilution and add 2.5µL of PolyA dilution to each M48 sample tube based on the following table:**

Samples	Volume of 1000ng/µL stock PolyA RNA solution added to UltraPure water (µL)	Volume of UltraPure water (µL)
6	5	15
12	10	30
24	15	45
48	30	90

- 3.27 Vortex samples and centrifuge if needed. Do not obtain a pellet in M48 tube from over centrifuging. If pellet occurs, vortex slightly to re-suspend before placing sample on M48 instrument.
- 3.28 Fill “Blanks” with 500µL sterile or UltraPure H<sub>2</sub>O.
- 3.29 Make sure all of the lot numbers are recorded.
- 3.30 **Have a witness confirm the order and labels of both the sample tubes and the labeled 1.5 mL final sample collection tubes. Have your witness verify the input tube top and sample label, and the output tube top and sample label.** This witness should also verify that all plastic ware is in the correct position and correctly seated in the platform. This will be your “Robot Setup” witness.

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3.31 Click “Yes” when asked to input sample names.

### 4 Importing Sample Names

- 4.1 At the sample input page, click “Import”.
- 4.2 The Open window will appear. “Look in:” should automatically be set to a default of “SampleName”. If not, the correct pathway to the folder is My Computer\C:\Program Files\GenoM-48\Export\SampleName. (The SampleName folder on the desktop is a shortcut to this file.)
- 4.3 Select your sample name file and click “Open”. Verify that your sample names have imported correctly. Do not be concerned if a long sample name is not completely displayed in the small window available for each sample.
- 4.4 Manually type in the word “Blank” for all empty white fields.
- 4.5 Click “Next”.

### 5 Verifying Robot Set-Up and Starting the Purification

5.1 In addition to confirming the *position* of all plastic ware and samples, check the following conditions before proceeding:

All plasticware (tips, sample plates, tubes) is seated properly in the robotic platform	<input type="checkbox"/>
Metal reservoir rack is seated properly, UNDER the interlocks	<input type="checkbox"/>
Interlocks are down	<input type="checkbox"/>
Sample tubes, elution tubes and sample collection tubes have been added to the platform in multiples of 6 as follows:	
Empty 1.5 mL tubes are filling empty positions for both sets of elution tubes in the cold and hot blocks	<input type="checkbox"/>
2.0 mL sample tubes filled with 500uL of sterile or UltraPure H2O are in empty positions of the sample rack	<input type="checkbox"/>

- 5.2 After confirming the position and set-up of the plastic ware click “Confirm”.
- 5.3 Click “OK” after closing the door.

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- 5.4 Click “Go” to start the extraction. Check that the bag attached to the waste chute is open and clear.
- 5.5 The screen will display the start time, remaining time, and the completion time.
- 5.6 Monitor the extraction until the transfer of DNA sample from the sample tubes to the first row of sample plate wells to ensure proper mixing of magnetic resin and DNA sample.
- 5.7 At the end of the extraction, a results page will be displayed indicating the pass/fail status of each set of six samples.

### 6 Saving Extraction Report Page

- 6.1 At the end of the extraction, a results page will be displayed indicating the pass/fail status of each set of six samples. **DO NOT** click “Next” until you have exported the results. To export results, click on the “Export” button. The Save As window will appear. “Save In:” should be set to the “Report” folder on the desktop. This is a shortcut to the following larger pathway: My Computer\C:\Program Files\GenoM-48\Export\Report.
- 6.2 In “File Name:”, name the report in the format, MMDDYY.HHMM. Set “Save As Type:” to Result Files (\*.csv). Use the original extraction data and time. For instance, an extraction performed at 4:30pm on 5/14/06 would be saved as 051406.1630.csv.
- 6.3 Click “Save”.
- 6.4 Drag a copy of the result file into the appropriate LIMS SHARE folder.
- 6.5 Proceed with clean-up and sterilization.

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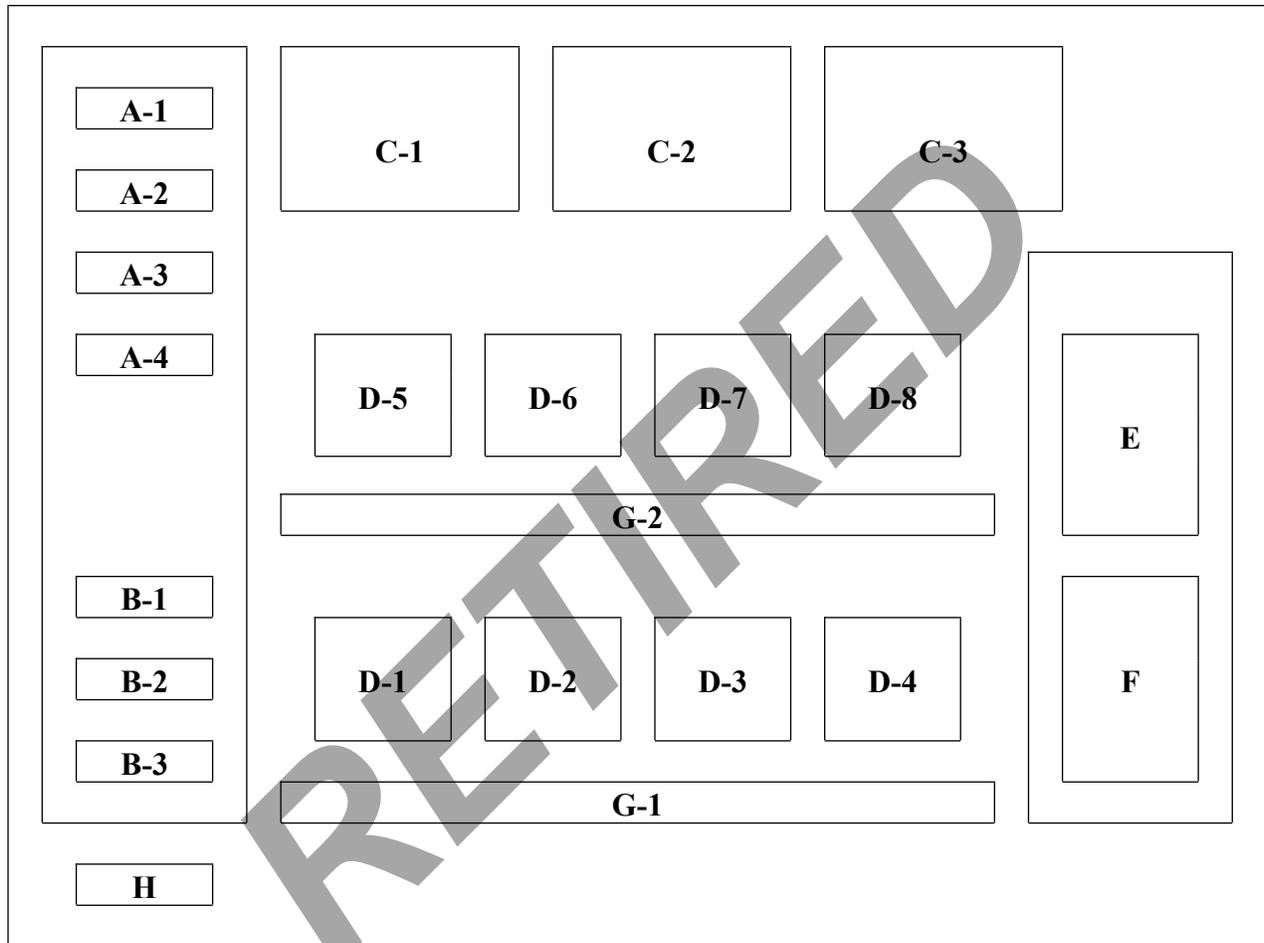
### 7 Post-Extraction Clean Up and UV Sterilization

- 7.1 Remove samples (from the 8 degree (front) cold block) from the robotic platform and cap with newly labeled screw caps.
- 7.2 Wipe down the robotic platform and waste chute with Ethanol. **DO NOT USE SPRAY BOTTLES.** Discard used pipette tips, sample tubes, and sample preparation plate(s).
- 7.3 Replace the lid on the magnetic resin reservoir and vortex remaining resin thoroughly. Discard the Magnetic resin immediately with a 1000uL pipette. Rinse the reagent container with de-ionized water followed by ethanol and store to dry.
- 7.4 Cover all other reagents and seal with Parafilm for storage. **MAKE SURE RESERVOIRS ARE LABELED WITH THE LOT NUMBER OF THE REAGENT THEY CONTAIN** and that the lot numbers have been recorded.
- 7.5 Replace tips on the instrument that were used during run. There are three racks, and all racks should be full. Ensure that the pipette tips are correctly seated in the rack and flush with the robotic platform.
- 7.6 Click "Next".
- 7.7 When prompted, "Do you want to perform a UV sterilization of the worktable?", click "Yes".
- 7.8 Select 1 Hour for the time of "UV sterilization" then click "yes" to close the software upon completion.
- 7.9 Store the extracts at 2 to 8°C or frozen.
- 7.10 In the LIMS system, navigate to the Data Entry page, assign the samples to a storage unit (cryobox), and import instrument.
- 7.11 Submit samples at neat and/or 1/100 dilutions, as needed for real-time PCR analysis to determine human DNA concentration (refer to the STR manual).
- 7.12 **COMPLETE THE M48 USAGE LOG WITH THE PURPOSE, PROGRAM, PLATE, AND ANY COMMENTS ARISING FROM THE RUN.**

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**8 BioRobot M48 Platform Diagram**



**Figure 1.** Diagram of Robotic Platform of the QIAGEN BioRobot M48.

- |         |                                   |
|---------|-----------------------------------|
| A (1-4) | Large Reagent Reservoir Positions |
| B (1-3) | Small Reagent Reservoir Positions |
| C (1-3) | Tube Racks                        |
| D (1-8) | Sample Plate Holders              |
| E       | Hot Elution Block (65°C)          |
| F       | Cold Final Elution Block (8°C)    |
| G (1-2) | Sample Tube Racks                 |
| H       | Waste Disposal Chute              |

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**9 Troubleshooting**

Error	Cause/Remedy
Resin/sample is being drawn up into pipette tips unequally	<b>Report problem to QA.</b> Resin buffer has evaporated. O-rings are leaking and need service.
Crystallization around 1 <sup>st</sup> row of wells in sample plate	Forgot to fill empty sample tubes with 500uL of sterile or UltraPure H <sub>2</sub> O
BioRobot M48 cannot be switched on	BioRobot M48 is not receiving power. Check that the power cord is connected to the workstation and to the wall
Computer cannot be switched on	Computer is not receiving power. Check that the power cord is connected to the computer and to the wall power outlet.
BioRobot M48 shows no movement when a protocol is started	BioRobot M48 is not switched on. Check that the BioRobot M48 is switched on.
BioRobot M48 shows abnormal movement when a protocol is started	The pipettor head may have lost its home position. In the QIAsoft M software, select “ <u>M</u> anual Operation/ Home”.
Aspirated liquid drips from disposable tips.	Dripping is acceptable when ethanol is being handled. For other liquids: air is leaking from the syringe pump. <b>Report problem to QA.</b> O-rings require replacement or greasing. If the problem persists, contact QIAGEN Technical Services