This is a non-controlled version.
****Note: controlled versions of this document are subject to change without notice.***

Plate-Based rRNA Depletion v3

I. Purpose

To remove cytoplasmic rRNAs (nuclear-encoded 5S, 5.8S, 18S and 28S rRNA), and mitochondrial rRNA species (12S and 16S rRNA) from total RNA using the RNase H-based kit from New England Biolabs (NEB).

II. Scope

All procedures are applicable to the BCGSC Library Core and the Library TechD groups.

III. Policy

This procedure will be controlled under the policies of the Genome Sciences Centre, as outlined in the Genome Sciences Centre High Throughput Production Quality Manual (QM.0001). Do not copy or alter this document. To obtain a copy see a Quality Systems associate.

IV. Responsibility

It is the responsibility of all personnel performing this procedure to follow the current protocol. It is the responsibility of the Group Leader to ensure personnel are trained in all aspects of this protocol. It is the responsibility of Quality Systems to audit this procedure for compliance and maintain control of this procedure.

V. References

Document Title	Document Number
NEBNext® rRNA Depletion Kit (Human/Mouse/Rat)	AM.0099

VI. Related Documents

Document Title	Document Number
Operation and Maintenance of the Agilent 2100 Bioanalyzer for RNA Samples	LIBPR.0018
Operation and Maintenance of the LabChipGX for RNA samples using the HT RNA Assay	LIBPR.0052
Quantifying RNA Samples Using the Qubit 4 Fluorometer	LIBPR.0186
Operation and Maintenance of the LabChipGX Touch HT for RNA samples using the High Throughput RNA Assay	LIBPR.0184
Total RNA Normalization on the Hamilton Nimbus	LIBPR.0121

This is a non-controlled version.
****Note: controlled versions of this document are subject to change without notice.***

Document Title	Document Number
96-well Plate-based Strand-specific cDNA Synthesis using Maxima H Minus on Hamilton NIMBUS	LIBPR.0132
Custom Magnetic Bead-Based Purification	LIBPR_WORKINST.0035
DNase I Treatment of RNA/Total Nucleic Acid	LIBPR.0143
Quantifying DNA samples using the Qubit 4 Fluorometer	LIBPR.0153
Automated DNA Quantification using the dsDNA Quant-iT High Sensitivity Assay Kit and VICTOR3V/VICTOR X3	LIBPR.0108

VII.Safety

All Laboratory Safety procedures will be complied with during this procedure. The required personal protective equipment includes a laboratory coat and gloves. See the safety data sheets (SDS) for additional information.

VIII. Materials and Equipment

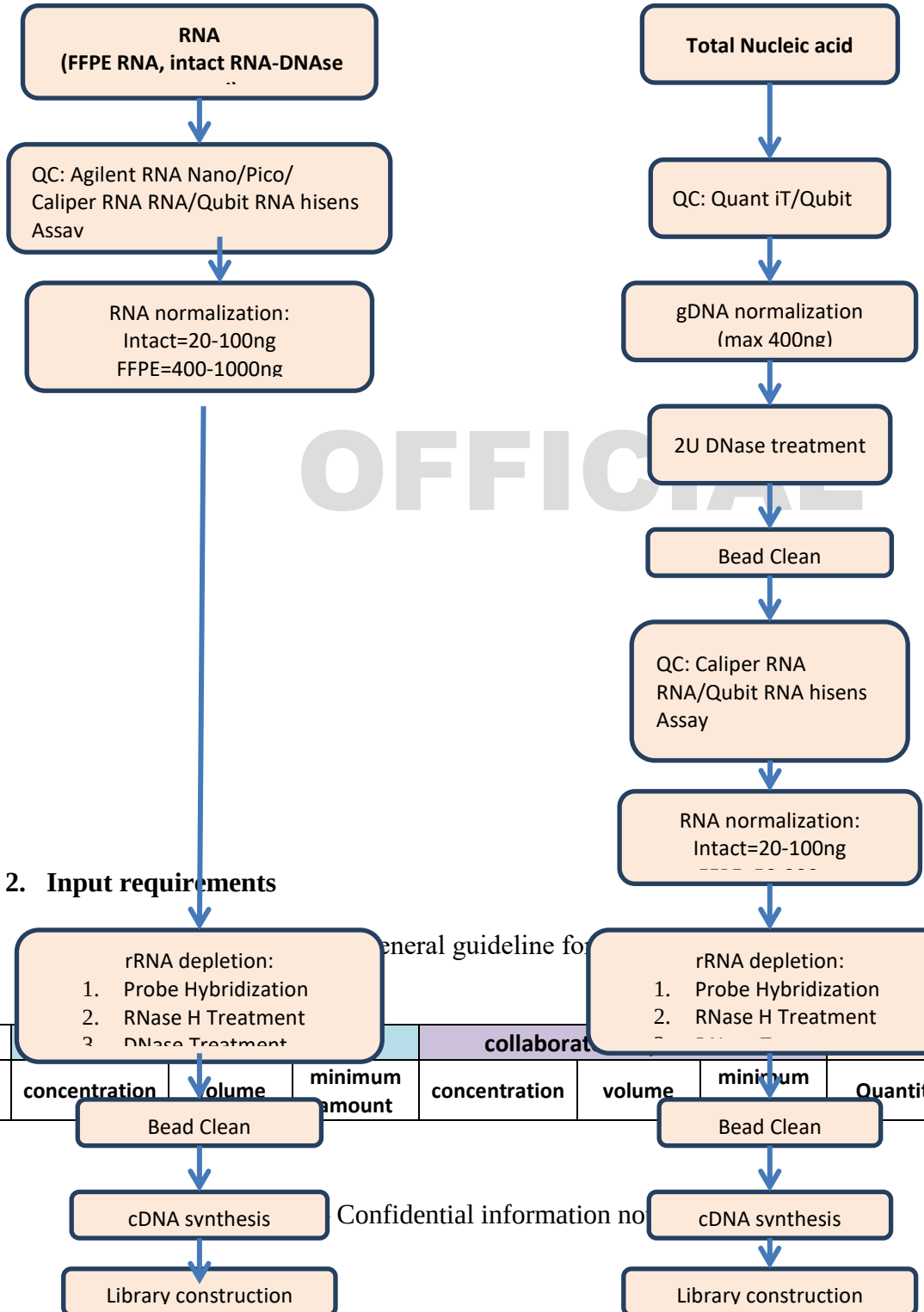
Name	Supplier	Number
Fisherbrand Textured Nitrile gloves - large	Fisher	270-058-53
RNAse Zap	Ambion	9780
Ice bucket – Green	Fisher	11-676-36
Wet ice	In house	N/A
RNAse free 1.5mL eppendorf tube	Ambion	12400
Gilson P2 pipetman	Mandel	GF-44801
Gilson P10 pipetman	Mandel	GF-44802
Gilson P20 pipetman	Mandel	GF23600
Gilson P200 pipetman	Mandel	GF-23601
Gilson P1000 pipetman	Mandel	GF-23602
Mandel P200 DF200 tips	Mandel	GF-F171503
Mandel P1000 DF1000 tips	Mandel	GF-F171703
VX-100 Vortex Mixer	Rose Scientific	S-0100
200µL Rainin tips	Rainin	RT-L200F
20µL Rainin tips	Rainin	RT-L10F
200µL Pipet-Lite	Rainin	L12-200
20µL Pipet-Lite	Rainin	L12-20
1250µL pipette tip, 96tips/rack, 480 tips/cs filter sterile	Mandel Scientific	TM-4445
Pipette-VIAFLO 8 Channel	Mandel Scientific	TM-4124
Large Kimwipes	Fisher	06-666-117
Black ink permanent marker pen	VWR	52877-310
Bench Coat (Bench Protection Paper)	Fisher	12-007-186
Small Autoclave waste bags 10”X 15”	Fisher	01-826-4
DEPC water	Ambion	9922

Name	Supplier	Number
Mini-centrifuge	Eppendorf	5417R
Thermo Scientific 0.2mL Ultra Rigid Skirted 96-well PCR Plate	Thermoscientific	AB1000-150s custom
Deep-well, 96-well, 1.2mL, U bottom, low pro, 50/cs	Fisher Scientific	AB1127
NEBNext® rRNA Depletion Kit (Human/Mouse/Rat)	NEB	E6310X
RNA MagClean DX	ALINE Biosciences	C-1005
Sterile Filtered Conductive 50µL Tips in Frames	Hamilton	235979
Sterile Filtered Conductive 300µL Tips in Frames	Hamilton	235938
Tape Pads	Qiagen	19570
Foil Tape	VWR	60941-126
AB1127 1.2mL plate	Thermo Scientific	AB1127
MagMAX 2mL deep well plate	Applied Biosystems	4388476
96 Well Reservoir, Low Profile	Thomas Scientific	1149J14
PCRMax Alpha Cycler 4	Froggabio/Cole Parmer	AC496
2100 Electrophoresis Bioanalyzer Instrument	Agilent	G2939AA
LabChip GXII	Perkin Elmer/Caliper	124582
NIMBUS Liquid Handling Workstation	Hamilton	
NIMBUS P50 tips Clear Sterile Tips, 5760 tips/case	Hamilton	235831
NIMBUS P300 tips Clear Sterile Tips, 5760 tips/case	Hamilton	235832
EZPierce 20µm Thermal foil	ThermoFisher	AB1720
ALPS 50 V Microplate Heat Sealer	Thermo Scientific	AB-1443

IX. GENERAL GUIDELINES

1. Workflows

The following is a flow chart depicting the various steps in the rRNA depletion (RBD) protocol that will be described in detail below:



2. Input requirements

eneral guideline fo orators'

				collaborat			assessment	
Starting Material	concentration	olume	minimum amount	concentration	volume	minimum	Quantity/Quality	Quality Value

Low input Intact RNA	3.3 ng/ul	6 ul	20 ng	≥4 ng/ul	10 ul	40 ng	Spot check Agilent Bioanalyzer **RNA Pico** chip	RIN > 5
							Caliper Hisens RNA Assay (greater than 24 samples)	RQS > 4.5
Intact RNA	8.3 ng/ul	6 ul	50 ng	≥ 12.5 ng/ul	10 ul	125 ng	Agilent Bioanalyzer: RNA Nano chip	RIN > 5
							Caliper RNA	RQS > 4.5
FFPE RNA* (FormaPure)	8.3 ng/ul	6 ul	50 ng	≥ 12.5 ng/ul	10 ul	125 ng	Agilent Bioanalyzer RNA Nano chip (less than 24 samples)	**>70% of RNA fragments should be >200nt**
							Caliper RNA (greater than 24 samples)	
FFPE RNA* (other)	66.7 ng/ul	6 ul	400 ng	≥100 ng/ul	10 ul	1000 ng	Agilent Bioanalyzer RNA Nano chip (less than 24 samples)	**>70% of RNA fragments should be >200nt**
							Caliper RNA (greater than 24 samples)	

***Consult with your supervisor if the % total is <70% for FFPE RNA fragment lengths >200nt. Collaborator must assume the risk of failed libraries before we proceed.**

3. Controls

There will be two positive controls for the ribodepletion library construction process:

a. UHR (Universal Human Reference) RNA or FG031 control with RNA amount matching the average starting amount of samples on the same plate. This will be the control for the entire library construction process.

b. 500ng UHR RNA or FG031 control will mainly be used to assess shearing and the ribodepletion process. DEPC water will serve as the negative control. Both positive controls and the negative control will go through the entire ribodepletion library construction process.

4. General Technical Precautions

4.1. The beads (**RNA MagClean DX**) to be used in this protocol are different from those used for other protocols as they are certified to be RNase-free.

4.2. Ensure proper personal protective equipment is used when handling sample plates, reagents and equipment.

4.3. Wipe down the assigned workstation, pipetman, tip boxes and small equipment with RNAse Zap (Ambion) followed by DEPC-treated water. Ensure you have a clean working surface before you start.

4.4. Double check the QA release and/or expiry date of each reagent and enzyme.

4.5. Reactions in plates should never be vortexed and plate covers are never to be re-used.

4.6. Retrieve and thaw all reagents at room temperature. Once thawed, pulse-vortex, quick spin, and keep reagents on ice. Enzymes should be left in the freezer until ready to use.

4.7. Ensure the waste bag for the Nimbus is empty.

4.8. Brews are prepared and dispensed manually. Note that what is dispensed into the final brew plate is the actual volume of the specified volume for each step below (without any dead volume).

4.9. Beads are manually dispensed into wells in a 1.2mL plate (AB1127). 20µL dead volume should be factored in. Ensure that you include 50µL/well dead volume for 70% ethanol and 25mL dead volume for DEPC H_2O on top of what is required for the actual washes and elution, respectively. The Nimbus adds an original sample or a sample from a given reaction to the brew and bead cleanups are performed on Nimbus. Follow the prompts and lay out from the Nimbus programs to execute a particular step.

4.10. The Nimbus mixes at 80% of total volume 10 times.

4.11. Note that where it is specified that you proceed immediately to the next step, plates can be briefly placed on ice (not more than 30 minutes) in the case of emergency. Make an active attempt to proceed as immediately as possible.

X. PROCEDURE

This is a non-controlled version.
****Note: controlled versions of this document are subject to change without notice.***

Note: If you are unsure of which Nimbus protocol version to use, please consult your supervisor.

1. Upstream Preparation

1.1. In the case that LIMS is down, brew calculators can be located on the worksheet listed below. Enter the number of samples to be processed and print the Ribodepletion Worksheet located in:

R:\Library Core\Work Sheets and Calculators\Ribodepletion

1.2. Retrieve the plate containing Total RNA/Total nucleic acid. If stored at -80°C, thaw it on ice followed by a quick spin at 4°C, 700g for 1 minute. Place the plate on ice.

2. Input QC and conditional DNase treatment:

Sample type	Input QC	DNase Treatment	Amount for RBD	Volume of RNA for RBD (µL)
Intact RNA >100ng submitted	Agilent RNA Nano / Caliper 80% samples >50ng	No DNase treatment prior to RBD	50-100ng	6
Intact RNA (low input) <100ng submitted	Spot check on Agilent RNA Pico 80% samples <50ng	No DNase treatment prior to RBD	20-50ng	6
FFPE RNA	Agilent RNA Nano / Caliper	No DNase treatment prior to RBD	400-1000ng and >70% of RNA fragments should be >200nt	6
FFPE / Intact Total Nucleic Acid	Qubit / Quant-iT	200-400ng DNA in 35µL for DNase treatment (2U)	50-100ng intact 20-50ng intact (low input) 50-200ng FFPE and >70% of RNA fragments should be >200nt	6
Positive Control (UHR)	n/a	n/a	500ng	6
Positive Control (UHR)	n/a	n/a	RNA amount matching the average starting amount of samples on the same plate	6

Note: If the volume of samples to obtain RNA amount required for RBD exceeds 8µL, concentrate the samples using the bead-based purification described in LIBPR_WORKINST.0035, starting with twice the minimum amount of RNA to conservatively account for the loss of RNA during purification. Top up the samples to a maximum volume of 65µL with DEPC water if needed. Choose the appropriate input

volume, 1.8:1 ratio of beads to samples and elution volume of 8µL when prompted by Nimbus.

Refer to LIBPR.0121 for sample normalization using Nimbus. If volumes required for normalization are below 2µL, an appropriate intermediate dilution may be needed as advised by your supervisor.

Refer to LIBPR.0143 for DNase treatment. Quantify DNase treated samples using Agilent RNA Nano / Caliper / Qubit RNA assay or spot check on Agilent RNA pico for low input samples.

3. Hybridization: Annealing of rRNA probes to target rRNAs

Note: All reagents for steps 3, 4, and 5 including the DNase I in Step 5 are from the NEBNext rRNA depletion kit.

3.1. The set up for 1 reaction is as follows:

Solution	1 rxn (µL)
RNA	6
Depletion solution (probes)	0.5
Hybridization Buffer	1
Total volume	**7.5**

Hyb Mix (1.5µL)

3.2. Make sure to mix each reagent well and quick spin before adding to the brew.

3.3. Prepare the brew and check off reagents as they are added. Mix the brew by repeated pulse-vortexing followed by a quick spin.

LIMS Calculator: RBD_Hybridization_0.5x

3.4. Log into Nimbus Program as follows to add the RNA to 1.5µL of the Hyb mix:

Open file: **Production** > *toggle to workflow* > **Ribodepletion v3 > Ribodepletion Scheduler v3.wfl > Hybridization**

3.5. After Nimbus program completion, heat seal the plates. Since the volume is small and the incubation includes high temperature, heat seal the plate using Adhesive foil, EZPierce 20 um Thermal foil (cat# AB1720, ThermoFisher). The equipment used for

this is ALPS 50V Microplate Heat Sealer (cat# AB-1443, Thermo Scientific). Inspect the reaction plates for any variations in volume. Please see Appendix B for instructions.

3.6. In the PCRmax Tetrad thermocycler, incubate the plate as follows:

TETRAD: RNAHYB

- 95˚C 2 minutes
- 95˚C -0.1˚C at 1 sec down to 22˚C (730 cycles)
- 22˚C 5 minutes

Attend to this immediately after the incubation is done (within 30 minutes).

3.7. After thermocycler program is finished, remove the plate and spin it at 4°C, 700g for 1 minute.

Note: *This is **NOT** a safe stopping point; proceed to the next step immediately*.

4. RNase H digestion: Degradation of RNA in rRNA/ DNA probe hybrid

4.1. The reaction set up for 1 reaction is as follows:

Solution	1 rxn (µL)
RNA in hyb reaction	7.5
DEPC water	0.5
RNase H Reaction Buffer	1
RNase H	1
Total volume	**10**

RNase H mix (2.5µL)

4.2. Make sure to mix each reagent well and quick spin before adding to the brew.

4.3. Prepare the brew and check off reagents as they are added. Mix the brew by repeated pulse-vortexing followed by a quick spin.

LIMS Calculator: RBD_ RNaseH_0.5x

4.4. Log into Nimbus Program as follows to add the RNA to 2.5µL of the RNase H mix:

This is a non-controlled version.
****Note: controlled versions of this document are subject to change without notice.***

Open file: **Production** > *toggle to workflow* > **Ribodepletion v3 > Ribodepletion Scheduler v3.wfl > RNaseH**

4.5. After Nimbus program completion, seal the plates and quick spin at 4°C for 1 minute. Inspect the reaction plates for any variations in volume.

4.6. In the Tetrad thermocycler, incubate the plate at 37˚C for 30 minutes (Heated Lid 47°C).

TETRAD: RBD>RBD_37

4.7. After incubation, remove the plate and spin it at 4°C, 700g for 1 minute.

Note: *This is **NOT** a safe stopping point; proceed to the next step immediately.*

5. Post-RBD DNase I treatment: Removal of rRNA DNA probes and residual sample gDNA contamination

5.1. The reaction set up for 1 reaction is as follows (all reagents for this reaction should be from the Ribodepletion kit E6310X):

Solution	1 rxn (µL)	
RNA from RNase H reaction	10	
DEPC water	11.25	DNase mix (15µL)
DNase Buffer (from NEBNext Kit)	2.5	
DNase I (from NEBNext Kit)	1.25	
Total volume	**25**	

5.2. Make sure to mix each reagent well and quick spin before adding to the brew.

5.3. Prepare the brew and check off reagents as they are added. Mix the brew by repeated pulse-vortexing followed by a quick spin.

LIMS Calculator: RBD_ DNase_0.5x

5.4. Log into Nimbus Program as follows to add the RNA to 15µL of the DNase mix:

Open file: **Production** > *toggle to workflow* > **Ribodepletion v3 > Ribodepletion Scheduler v3.wfl > DNase I**

5.5. After Nimbus program completion, seal the plates and quick spin at 4°C for 1 minute. Inspect the reaction plates for any variations in volume.

5.6. In the Tetrad thermocycler, incubate the plate at 37°C for 30 minutes (Heated Lid 47°C).

TETRAD: RBD>RBD_37

5.7. See step 6.1 for bead cleanup preparation during incubation. After incubation, remove the plate and spin it at 4°C, 700g for 1 minute.

Note: *This is **NOT** a safe stopping point; proceed to the next step immediately.*

6. Post–RBD purification using RNA MagClean DX beads

6.1. The input volume for this step is 25µL per well.

6.2. Prepare beads, ethanol and DEPC-treated water as described below.

Reagent	Labware	Volume/well
70% Ethanol	2mL MagMAX deep well plate	350µL
RNA MagClean DX	AB1127 plate	95µL*
DEPC treated water	EK 2036 Low Profile Reservoir	25mL

*Includes 50µL dead volume

6.3. The Nimbus will perform the purification of the rRNA depletion reaction using beads as follows:

Reaction	*Bead Vol manually dispensed (µL)	Bead Vol added by Nimbus (µL)	Bead Binding Time (mins)	Magnet Clearing Time (mins)	2X 70% EtOH* Wash Vol (µL)	Magnet Airdry Time (mins)	DEPC Elution Vol (µL)	Elution time (mins)	Magnet Elution time (mins)	Transfer Vol (µL)
RBD Reaction	**95**	**45**	**15**	**7**	**150**	**5**	**18**	**3**	**2**	**18**

*Must be at Room Temp for a minimum of 30 minutes before usage; failure to do so would result in a decrease in yield

6.4. Log into Nimbus Program as follows:

Open file: **Production** > *toggle to workflow* > **Ribodepletion v3** >

Ribodepletion Scheduler v3.wfl > Bead Clean RBD

*This is a safe stopping point***.** If needed, the plate containing the bead cleaned RNA can be stored at -80°C. Otherwise, proceed to cDNA synthesis as described in 96-well Plate-based Strand-specific cDNA Synthesis using Maxima H Minus on Hamilton NIMBUS– LIBPR.0132**.**

Appendix A: LIMS SOP

1. Start of Plate Library Construction

2. Bioanalyzer Run / Caliper Run – if working with intact RNA: QC samples on Agilent after 1st DNase treatment. Create Bioanalyzer Run – QC Category: Total RNA QC

   RNA Qubit Assay: if working with FFPE RNA or FFPE total nucleic acid that has been DNase treated in set less than 24 samples. LIMS: **RNA Qubit QC**

   OR

   Caliper Run – if working with FFPE RNA or FFPE total nucleic acid that has been DNase treated in set greater than 24 samples. QC Category: Total RNA QC

3. DNase Treatment: pipeline is RBD_3.0: Ribodepletion 3.0. Pass barcode to supervisor. Your supervisor may generate a new barcode for you if your samples need to be normalized before the probe hybridization step.

4. Ribodepletion: pipeline is RBD_3.0: Ribodepletion 3.0. Use the barcode your supervisor passes to you.

Appendix B: ALPS 50V Microplate Heat Sealer

NOTE: The seals should be stored in the foil seal packaging to maintain proper orientation. Failure to orient the foil seal with the adhesive side down in the plate sealer will result in the seal adhering to the instrument rather than the plate.

1. Turn on the ALPS 50V heat sealer and allow the instrument to warm up. The Heat on/off LED will flash during this time and stay on once the desired temperature is reached. The sealer should be pre-set for 165°C, 3 second seal time.

2. Place the foil seal on top of the input plate (shiny side up).

3. Place the plate on the plate carrier so that well A1 is in the back left corner. Avoid touching the heating surface while loading the sample plate to prevent injury.

4. Grasp the handle and lower to thermally compress the foil seal onto the input plate. Do NOT apply more pressure to the handle than necessary. When the correct pressure is achieved, an audible tone will sound and the timer will count down to zero.

5. Once the timer reaches zero, another audible tone will sound. Raise the handle to release the heater plate. Use a roller seal to ensure that all wells are properly sealed.

6. Rotate the plate so that well A1 is in the front right corner (H12 will be in the back left corner) and repeat the sealing steps 4 and 5.

7. Put a thermal pad on top of the output plate, then close and tighten the lid.