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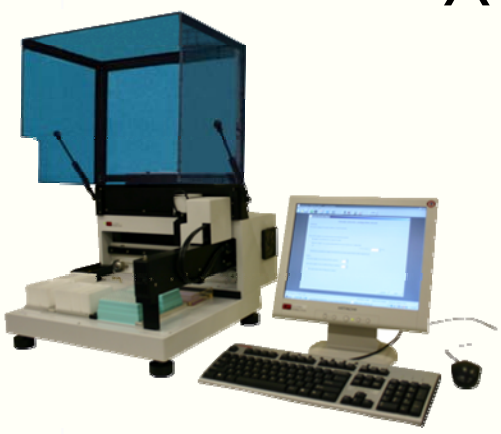
ROBOTICS
A Division of Corbett Life Science

Total RNA Extraction Protocol with MACHEREY-NAGEL NucleoSpin® 96 RNA kit

Cat#740 698.X (8 Well Strip System)
Cat#740 709.X (96 Well Plate)

X-tractor Gene™



MACHEREY-NAGEL

Contents

Appendices

Protocol Description

For use on the Corbett Robotics X-tractor Gene
Please download the run files for the X-tractor, to execute this protocol from the following link

http://www.corbettlifescience.com/control.cfm?page=Macherey-Nagel

Introduction
The total RNA extraction Protocol described here is designed for walk-away automated preparation of RNA from a variety of samples (for example tissues, cultured cells, yeast etc). Furthermore, the kit is suitable for clean-up of Trizol pre- purified samples. Extracted RNA is of high quality and suitable for a wide variety of downstream applications.

Purification kits are available in 96-well plates (**NucleoSpin® 96 RNA**) or in a flexible 8-well strip format (**NucleoSpin® 8 RNA**).

With the **NucleoSpin® 8/96 RNA** method, cells or tissue are lysed by incubation in a solution containing large amounts of chaotropic salt. This lysis buffer immediately inactivates RNases – which are present in virtually all biological materials – and creates in combination with RA4 appropriate binding conditions which favor adsorption of RNA to the glass fibre membrane. Contaminating DNA, which is also bound to the glass fibre membrane, is removed by DNase I which is directly applied onto the silica membrane during the preparation (RNase-free DNase I is supplied with the kit). Salts, metabolites, and macromolecular cellular components are removed by simple washing steps with three different buffers. Pure RNA is finally eluted under low ionic strength conditions with RNase-free water.

Sample size
For trouble-free operation, samples must be as consistent as possible. After lysis of cells or tissue a lysate free of particulates and debris is ideal for further processing. In addition, processing too much sample will cause the glass fibre membrane to block and the extraction to fail. The protocol is suitable for up to 30 mg of tissue or up to 2 x10^6 cells).

The **NucleoSpin® 8/96 RNA** kit provides reagents and consumables for purification of typically up to 40 µg (maximum binding capacity of each column is 100 µg) of pure total RNA from up to 30 mg tissue samples with an $A_{260/280}$ ratio between 1.90 and 2.10 and a typical concentration of 50-250 ng/µL.

Typical results:

Sample	Sample amount	Typical yields
Mouse liver	30 mg	30 µg
Mouse spleen	20 mg	40 µg
Mouse kidney	20 mg	20 µg
HeLa cells	2 x 10^6 cells	20 µg

Processing time
Total time required to complete the RNA extraction procedure depends on the number of samples processed. Following lysis incubation typically, a single column of 8 samples requires 45 minutes to complete. Each additional column of 8 samples adds a further 5 minutes to the total processing time. Thus a full 96-well plate requires about 95 minutes to complete.

IMPORTANT

Wear gloves and a laboratory coat throughout procedure.

Take care to avoid cross-contamination of samples and reagents.

Make sure reagent tubs, tubes and plates are clearly labelled and clean.

Protocol Validation

Verification Testing
Total RNA extraction protocol was functionally tested on the Corbett Robotics X-tractor Gene™ Automated Extraction System using **NucleoSpin® 96 RNA** kit provided reagents and consumables. Typical results for the extraction of total RNA from HeLa cells , mouse liver or pork liver tissue are shown below. Actual results will vary depending upon sample age, quality, type, and species of subject.

Samples
Pork or mouse liver samples of 15 mg or 1x 10^6 HeLa cells were used for each extraction. For investigation of reproducibility and consistency of extraction lysates were combined, mixed and split following the lysis incubation in order to get a homogenous lysate.

Cross Contamination Test
To maximise the detection of any potential contamination event lysates of HeLa cells and Lysis Buffer without sample material were arranged in alternating wells (in a "checkerboard" pattern – see *fig. 1*). Following RNA isolation RT-PCR analysis was done on wells without samples (NTCs) targeting *28s RNA* gene in a 40 cycle on step RT-PCR reaction in a LightCycler™ PCR using SYBR green™ detection. No amplification of NTCs was observed indicating no cross-contamination (see fig. 2 and fig. 3).

	1	2	3	4	5	6	7	8	9	10	11	12
A		x		x		x		x		x		x
B	x		x		x		x		x		x	
C		x		x		x		x		x		x
D	x	**x**	x		x		x		x		x	
E		x		x		x		x		x		x
F	x		x		x	**x**	x		x		x	
G		x		x		x		x		x		x
H	x		x		x		x		x		x	

Sample**(+)**

Buffer**(-)**

Fig.1 Illustration of the 'Checkerboard Pattern' utilised for the cross contamination analysis test.
The recovered samples at the indicated positions (x) off wells filled with buffer or samples were analyzed by RT-PCR. For results see Fig. 2

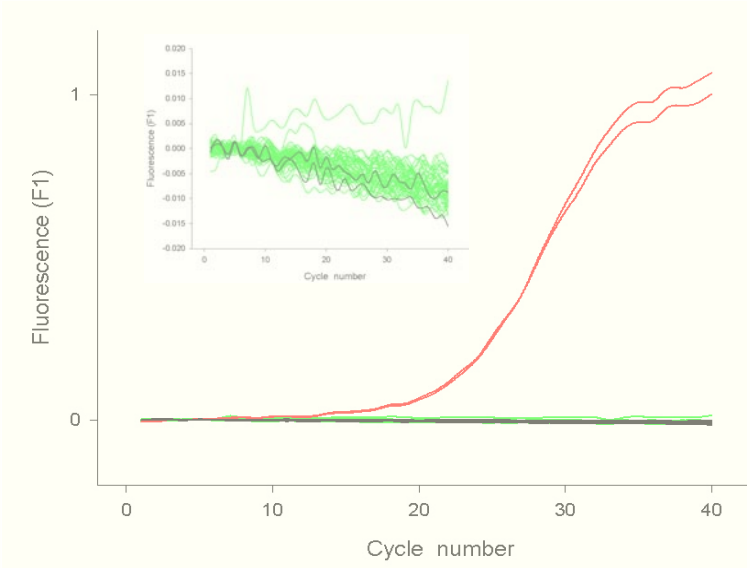


Fig. 2. Cross-contamination analysis. *Samples received from the 48 no sample extractions were analysed by real time RT PCR targeting 28sRNA gene. No cross contamination was detected.*

Quality of RNA isolated from cell or tissue
Aliquots of the purified RNA were analysed using Agilent Bioanalyzer 2100 using RNA 6000 Nano chip assay to estimate quality of isolated total RNA.

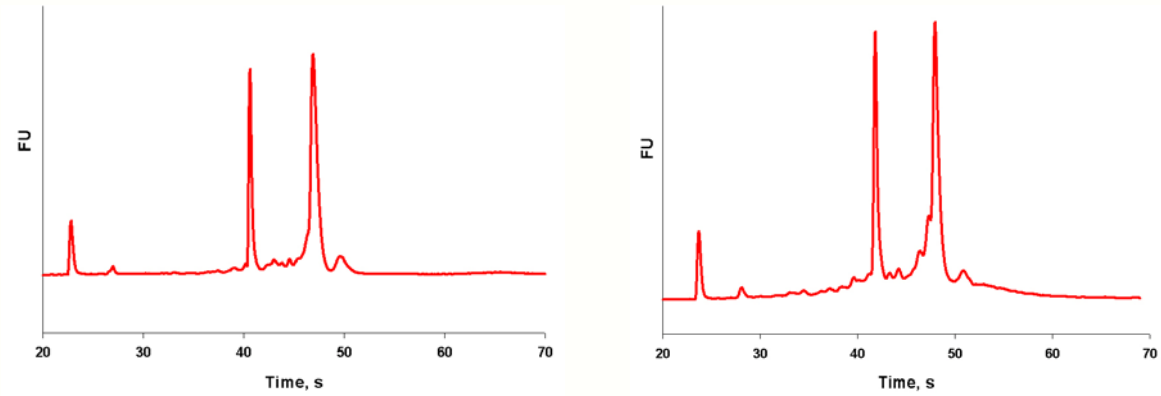


Fig 3: Typical results for analysis of isolated RNA samples using Agilent 2100 Bioanalyzer. *1 µl of either RNA isolated from HeLa cells or mouse liver tissue were analysed using the RNA 6000 Nano Assay. Electropherograms show high quality RNA without degradation. RNA Integrity Number (RIN) and values for ratio of ribosomal bands (28s/18s) are given below:*

RNA isolation from HeLa cells:

RIN: 9.8
28s/18s: 2.3

RNA isolation from mouse liver tissue:

RIN: 8.6
28s/18s: 2.1

Spectrophotometer Analysis
RNA samples isolated from either HeLa cells or pork liver (24 samples each were analysed for yield and purity (Fig. 3, Fig. 4) by UV spectrophotometry. Results are summarized below.

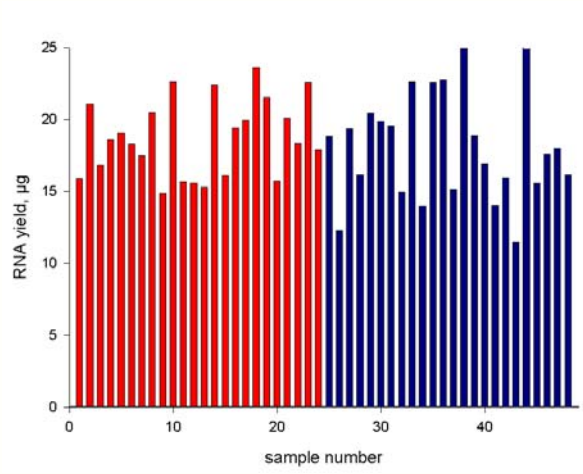


Fig. 4 Example RNA yield *sample 1-24 RNA isolation from 1 10⁶ HeLa cells, sample 25-48 RNA isolation from 15 mg pork liver.*

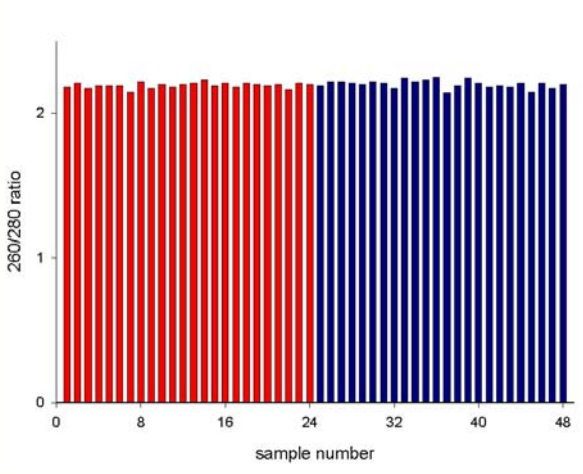
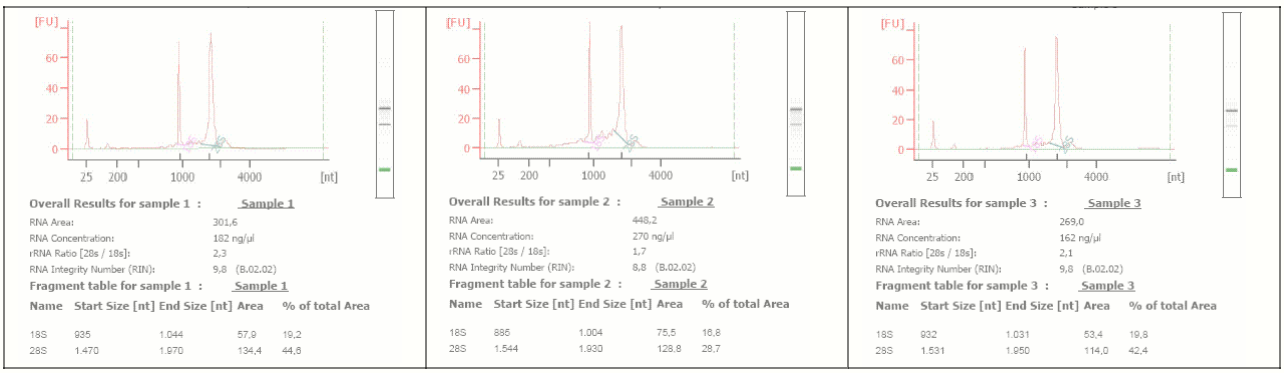


Fig 5. Example 260/280 ratios *sample 25-48 RNA isolation from 1 10⁶ HeLa cells, sample 25-48 RNA isolation from 15 mg pork liver.*

	Samples 1-24 (cells)			Samples 25-48 (tissue)		
	Yield	**Concentration**	**260/280 Ratio**	**Yield**	**Concentration**	**260/280 Ratio**
Maximum	23.63 µg	236 ng/µL	2.23	25.93 µg	259 ng/µL	2.25
Minimum	14.84 µg	148 ng/µL	2.15	11.48 µg	114 ng/µL	2.14
Average	18.71 µg	187 ng/µL	2.19	18.06 µg	180 ng/µL	2.20
Standard Deviation	2.67 µg	26.7 ng/µL	0.019	3.81 µg	38.1 ng/µl	0.028

Reproducibility of RNA isolation

HeLa cells were lysed in buffer RA1. Aliquots of 300 µl cell lysate (corresponding to 1 x 10⁶ cells) were used for isolation of RNA. Purified RNA from 12 randomly selected samples was analysed using the Agilent Bioanalyzer 2100. Results are shown in Fig 6



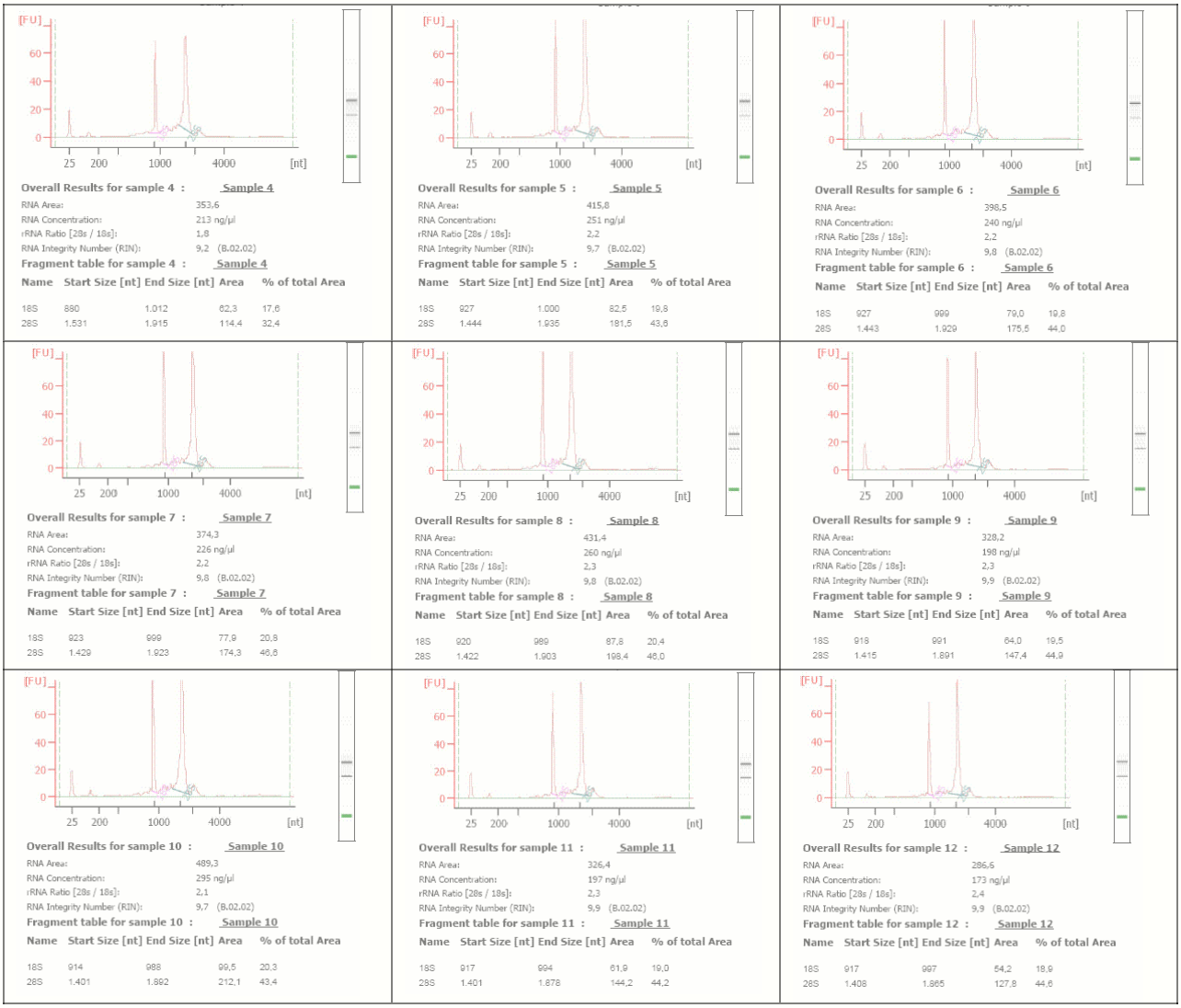


Fig 6: Results for analysis of isolated RNA samples using Agilent 2100 Bioanalyzer. *1 µL of RNA isolated from HeLa cells were analysed using the RNA 6000 Nano Assay. Electropherograms show high quality RNA . without degradation. RNA Integrity Number (RIN) and values for ratio of ribosomal bands (28s/18s) are summarized in the table below.*

	Samples 1-24 (cells)		
	Concentration	**rRNA ratio (28s/18s)**	**RIN**
Maximum	295 ng/µL	2.4	9.9
Minimum	162 ng/µL	1.7	8.8
Average	222 ng/µL	2.2	9.7
Standard Deviation	42 ng/µL	0.2	0.3

RT PCR Inhibitor Test and efficiency of DNA removal step
Reproducibility of real-time RT PCR C_T values and end-point product yield analysis showed no evidence of RT-PCR inhibitors in any of the extracted RNA samples. Absence of RT-PCR products present in samples amplified without reverse transcriptase action indicate no detectable DNA contamination in the isolated RNA samples.

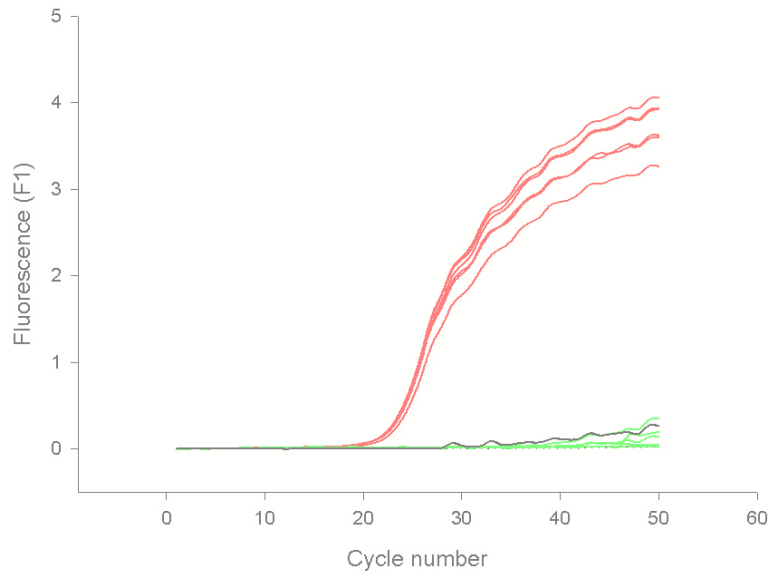


Fig. 7 Real-time RT PCR analysis of RNA extracted from HeLa cells. *6 randomly selected samples were analysed on a LightCycler™ real-time PCR instrument system in a RT-PCR SYBR green™ assay. Each sample was amplified with AMV (Avian Reverse Transcriptase, +RT) or without AMV (-RT). Results show strong and reproducible amplification only in the presence of reverse transcriptase indicating effective removal of DNA and absence of PCR inhibitors.*

Reproducibility of RNA yield from 6 randomly selected out 24 samples (isolated from HeLa cells) was measured as threshold cycle (C_T) values from real-time RT-PCR analysis. Sample RNA aliquots were amplified in a LightCycler™ instrument system using SYBR green™ chemistry. Results (Fig. 3) are summarised as follows:

Maximum C_T	22.93
Minimum C_T	22.68
Average C_T	22.80
C_T Std. Deviation	0.11

Reagents and Consumables Required

Item		Requirement for a Full 96-Well or 48 Well Extraction	Part No. or Part of (MN)	Supplier
Consumables				
NucleoSpin® RNA Binding Plate[1] (for 96 well plate extractions)		1 Plate	740 709.2[2]	**Local MN Supplier**
NucleoSpin® Starter Set A[3] (Accessory for 8-strip system)		1 Column Holder	740 682	
NucleoSpin® RNA Binding Strips (for 8-strip extractions)		6 strips	740 698[2]	
MN Square-well Block[1]		1 Plate	740 709.2	
MN Tube Strip Rack[1]		1 Rack	740 763	
200 µL Filtered Fine Bore Tips in Robotic Rack sterile		2 Racks	2097	**Corbett Office or Distributor**
Elution Plate with 0.65 mL Cluster Tubes[4]		1 Plate	2147	
Elution Plate Strip Caps (1 bag contains 12 strips of 8)[4]		1 Bag	1636	
Reagent Tubs	70 mL Tub	2 Tubs	2137 (Disposable) (2365 Reusable)	
	170 mL Tub	2 Tubs	2136 (Disposable) (2364 Reusable)	
	270 mL Tub	1 Tub	2314 Disposable (2363 Reusable)	
Reagent Tub Lids	70 mL Tub Lid	2 Lids	2505 (For use with disposable tubs) (2416 Reusable Tubs)	
	170 mL Tub Lid	2 Lids	2504 (For use with disposable tubs) (2415 Reusable Tubs)	
	270 mL Tub Lid	1 Lid	2503 (for use with disposable tubs) (2414 Reusable)	
Self adhesive PCR/Elisa plate Plastic Sealing Film (for sealing unused wells of capture plate)		1 Sheet	2411	

[1] Part of NucleoSpin® 96 RNA kit.
[2] Kit is available for different number of preparations. See ordering information of kit protocol.
[3] Required only when using 8 well strips.
[4] not required when using MN Tube Strip Rack (MN P/N 740 637)

Kits and Reagents		Part No:	Supplier
NucleoSpin® 96 RNA kit (including buffers, MN Square-well Block, RNase free water, RNase free DNase I, DNase I reaction buffer) Sufficient for 2 x 96, 4 x 96 or 24 x 96 preps	1 kit	740 709.2 740 709.4 740 709.24	MN
NucleoSpin® 8 RNA kit (including buffers, MN Tube Strips for collection of eluted RNA, MN Square-well Block, RNase free water, RNase free DNase I, DNase I reaction buffer) Sufficient for 12 x 8 preps or 60 x 8 preps NB: MN Accessory Starter Set A required to use 8-strip System (P/N 740 682)	1 kit	740 698 740 698.5	
Additional reagents to be ordered separately if required			
Lysis buffer RA1	50 mL 500 mL	740 961 740 961.500	
DNase I set (sufficient for 50 reactions)	1 set	740 963	
NucleoSpin® Filters (for filtration of cell and tissue lysates)	50	740 606	
NucleoSpin® RNA Filter Plate (for filtration of cell and tissue lysates)	4	740 711	

Accessories Required

Part No.	Corbett Robotics Description
1675	High Skirt Transfer Carriage
1697 1696	96-well Separator Plate 8-Strip Separator Plate NB: MN Accessory Starter Set A required to use 8-strip system (P/N 740 682)
2443	Elution Riser Block (16.25 mm)[1] Optional not required when using MN tube strip rack supplied with kit. For Corbett Robotics elution tubes only.
2139	Reagent Tub SBS Base plate

[1] Not required when using MN Tube Strip racks (MN P/N 740 637)

Reagent Handling and Storage

HAZARD INFORMATION

Buffers RA1, RA2 contain guanidine thiocyanate, alcohols and detergents. Always wear a laboratory coat, disposable gloves, and eye protection when handling solutions containing these chemicals.

Lyophilized DNase I: avoid skin contact.

Do not add bleach or acidic solutions directly to solutions containing guanidine or extraction waste.
Guanidine forms reactive compounds and toxic gases when mixed with bleach or acids.
For any items contaminated with these buffers, clean with suitable laboratory detergent and water to remove all traces of guanidine before cleaning with bleach or acidic solutions
For details refer to the MSDS (material safety data sheet) information available at the following web site:

www.mn-net.com

Reagent storage

Upon receipt of reagents, unpack and store the individual reagents as follows:

Reagent	Store Temp	Storage State
Buffer RA1	18-24° C	Not critical
Buffer RA2	18–24° C	Not critical
Buffer RA3	18–24° C	Not critical
Buffer RA4	18–24° C	Not critical
Buffer RNase free water	18–24° C	Not critical
DNase reaction buffer	18–24° C	Not critical
DNase I	4° C	Not critical

Reagent Preparation

Prior To Each Run
Before starting a run, bring all reagents to room temperature. Where necessary, gently mix and re-dissolve any precipitates by warming to 37°C until dissolved. Swirl gently to avoid foaming.
Identifying Required Reagent Volumes For Your Run
The robotics software will calculate for you the exact required volume of each reagent once you have selected the number of columns you will be extracting from.
The X-tractor Gene™ software will display the required volume of each reagent (inclusive of each reagent tub's allocated dead volume) in a hover box when you place the computer's mouse cursor over the reagents designated position.
DNase I working solution
Add to each vial of DNase I 400 µL nuclease free water and incubate for 1 minute at room temperature. Gently swirl the vial to dissolve DNase I completely. Dilute reconstituted DNase I with 2.8 ml DNase reaction buffer. When using less than 96 samples store DNase I after addition of water
Binding Solution / Wash buffer RA4
Add 96-100% ethanol as indicated. Close bottle tightly in order to prevent ethanol evaporation.
Wash Solutions
Buffer RA2: ready to use.
Buffer RA3: add 96-100% ethanol as indicated. Close bottle tightly in order to prevent ethanol evaporation.
Please refer to the software (see note above) for the calculated required volume.

Sample Storage

Sample storage
RNA is not protected against digestion until the sample material is flash frozen or disrupted in the presence of RNase inhibiting or denaturing agents. Therefore, it is important that samples are flash frozen in liquid N_2 immediately and stored at –70° C or processed as soon as possible. Samples can be stored in lysis buffer RA1 after disruption at –70° C for up to one year, at +4° C for up to 24 hours or up to several hours at room temperature. Frozen samples are stable up to 6 months. Frozen samples in buffer RA1 should be thawed slowly before starting with the isolation of total RNA.

Frozen samples should not be thawed more than once. Inappropriate storage of sample may lead to degraded RNA. Repeated freeze-thaw cycles may also lead to poor quality of purified RNA.

Sample Preparation

Sample Homogenization
Add up to 30 mg sample (see *Sample Preparation* for advice) to each lysis block well for the columns you wish to extract from.

- Add 300 µL of Lysis Buffer RA1 to up to 30 mg of sample (either manually or using separate X-tractor Gene™ protocol *MN RNA – Homogenization preload. CAS4* run file (for automated setup, see Appendix A).
- Cells: Cells are lysed by addition of buffer RA1 directly. For cells grown in a 96well plate lysis buffer RA1 volume has to be reduced to 130 µl. Following lysis the added volume of binding solution / wash buffer RA4 is reduced to 130 µl accordingly. For cells grown in larger wells or dishes the lysis use 300 µl lysis buffer RA1.
  Tissue: Following addition of buffer RA1 tissues have to be homogenized by suitable mechanical disruption (e.g. using rotor-stator homogenizers, or by action of stainless steel beads (Retsch Modell MM300 Mixer Mill or SPEX CertiPrep Model Geno/Grinder 2000).
- To remove debris and to reduce viscosity of the lysate following the homogenization NucleoSpin® RNA Filter plate or NucleoSpin® can be used. Debris may also be removed by centrifugation.

General Considerations
Sample preparation on the X-tractor Gene™ should be conducted in the same manner as for spin columns. The same issues you address with your samples for processing on spin columns are applicable to samples processed on the X-tractor Gene™.

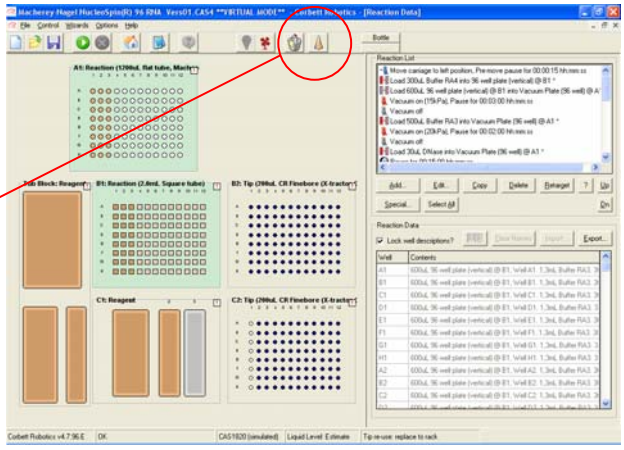
Preparing Difficult Samples
Avoid transferring material into the lysis plate that could cause pipette blockage (e.g. debris or particulate matter). To maximise nucleic acid recovery from samples debris are best removed by filtration or centrifugation after the homogenization step. Reduce starting amount of sample material. If necessary include pre-purification using Trizol reagent.

Blocked Wells
Glass fibre membranes can be blocked by samples with particulate matter or a high viscosity (highly concentrated DNA and DNA/protein lysates are usually very viscous). DNA is still present after homogenization in the crude lysate. Overloading of the membrane will also cause blockage, therefore apply no more than 2×10^6 nucleated mammalian cells. Once a membrane is blocked, buffer flow may halt and the well will require manual removal of most of the subsequent buffers loaded. Alternatively, the membrane can be pierced with a 27-gauge needle. Overloaded or blocked membranes lead to reduced yields and low quality RNA.

Setting Up to Execute a Run

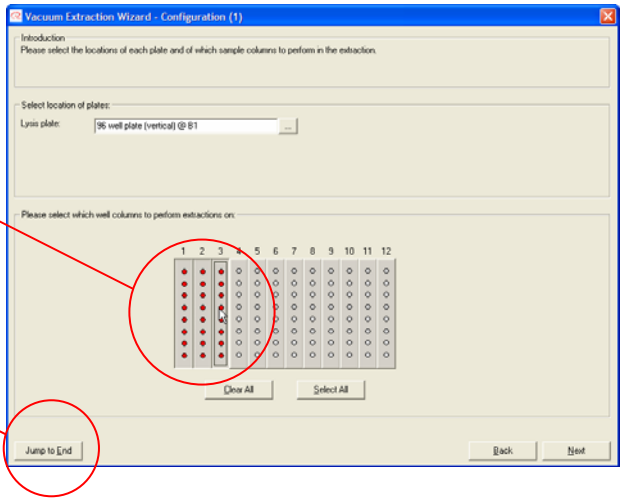
1. Turn on X-tractor Gene™.
2. Launch software.
3. Open *Macherey-Nagel NucleoSpin(R) 96 RNA V01.CAS4* run file (for 96-Well Plate)
   Open *Macherey-Nagel NucleoSpin(R) 8 RNA V01.CAS4* run file (for 8-Well Strip System)
4. Select the number of columns to extract from:

Open the Wizard by clicking on the wizard hat icon on the toolbar



Select the number of columns to extract from

Click Jump To End *button*



5. Once you have selected the required columns, exit the Wizard by clicking on the *Jump to End* button in the bottom left hand corner of the *Configuration* window.
6. This will take you to the *Review Protocol* screen. Confirm the protocol. If required, this screen can be printed to assist in robot setup or for your records. Click *Next*, and then *OK* to exit the Wizard.

   The X-tractor Gene™ will now calculate the required volumes of the described reagent for each tub and the number of tips required.

Loading Workspace Prior to a Run

Note: See Appendix B for Sample Introduction Method

1. Load the lysis block with filtered or centrifuged lysate onto the B1 position of the X-tractor Gene™.
2. If you have less than 96 samples to extract you will need to cover the unused columns of the capture plate with a piece of self adhesive sealing film. Covering the unused portion is essential for proper vacuum operation. DO NOT attempt to re-use the unused portion of the plate as repeated handling of the capture plate can result in cross contamination of subsequent extractions.
3. Observing sterile procedure set up the instrument deck with clean accessories, the required consumables and all reagents.
4. Ensure the separator plate is thoroughly clean and dry (see *Cleanup*).

> ***WARNING***
>
> **Do not dispense the required volumes of reagents into the reagent tubs until just prior to the start of the run.**
>
> **Keep reagents covered with the provided lids until you are ready to start the run. Leaving reagents in tubs for extended periods will result in evaporation (especially of alcohol solutions) and salt precipitate formation resulting in loss of binding conditions. For this reason reagents left over from a previous run should be disposed of and new or clean tubs loaded onto the deck with fresh regents.**

5. Prepare DNase I Solution as described above.
6. Finally load DNase, Wash buffers, and Elution Buffer or water as indicated into their assigned reagent tubs and cover the tubs with the supplied reagent tub lids.

> ***IMPORTANT***
>
> ***Samples are always processed in batches of 8 (whole columns).***
>
> ***If you do not use all the wells in a column, make sure the unused wells contain a substitute liquid (such as water or buffer) equal in volume to the sample volume.***
>
> ***Failure to do this can cause undue foaming of a short-filled well during a mix cycle.***

Run Protocol

Pre-Run Checklist

Click the *Start* icon and a pre-run checklist will appear. The checklist may include some of the following:
Please ensure:

- That the columns you wish to extract from are correctly selected and the unused columns of capture plate is sealed with self-adhesive microtiter plate plastic sealing film or PCR foil.
- That the elution plate is in position and its lid removed.
- Sufficient pipette tips are loaded and lids removed.
- Clean reagent tubs are loaded with the required volume of reagent, are in the correct positions, and their covers are on.
- All reagents and samples are at room temperature at the start of the run.

For first time users, the software option of user pause at the end of vacuum steps is enabled ,this requires the user to confirm that all the samples have flowed through the capture plate before continuing. This option can be turned off once proper sample preparation has been confirmed.

Start Run

Once you have confirmed that the X-tractor Gene™ workspace is correctly set up as described in the software and observed the pre-run checklist, click *Run* and the protocol will execute as described below.

Load Binding Solution

- Add 300 µL of Binding/Wash solution RA4.

Load Filter Plate

- Load 600 µL of lysate into the capture plate.
- Pre-mix 15 times.
- Apply a vacuum of 15 kPa for 3 minutes and check for slow or blocked wells (user pause on).

Wash Step 1

- Load 500 µL of Wash Buffer RA3 into the capture plate.
- Apply a vacuum of 20 kPa for 2 minutes (user pause on).

DNase I step

- Load 30 µL of DNase I solution into the capture plate.
- Incubate for 15 minutes.
- Do not use vacuum at the end of this step.

Wash Step 2

- Load 500 µL of Wash buffer RA2 into the capture plate.
- Incubate for 1 minute.
- Apply a vacuum of 20 kPa for 1 minute.

Wash Step 3

- Load 800 µL of Wash buffer RA3 into the capture plate.
- Apply a vacuum of 20 kPa for 1 minute.

Wash Step 4

- Load 500 µL of Binding /Wash solution RA4 into the capture plate.
- Apply a vacuum of 20 kPa for 1 minute.

Dry Sample

- Apply a vacuum of 60 kPa for 15 minutes.

Product Removal

- Load 130 µL of RNase free water into the capture plate.
- Incubate for 2 minutes.
- Apply a vacuum of 50 kPa for 1 minute.

Finish

- Recover elution plate with samples.
- Remove and discard used consumables, clean separator plate, sink, tubs and carriage in preparation for the next run.
- (Please refer to Appendix C for nucleic acid storage advice).

Post-Run Cleanup

Disposable Plasticware and Liquid Waste
Dispose of plasticware and liquid waste in accordance with laboratory guidelines for the sample type and reagent hazard.

> ***WARNING***
>
> **Do not add bleach or acidic solutions directly to solutions containing guanidine or extraction waste. Guanidine forms reactive compounds and toxic gases when mixed with bleach or acids. For any items contaminated with these buffers, clean with general laboratory detergent and water to remove all traces of guanidine before cleaning with bleach or acidic solutions.**

You can download reagent MSDS (material safety data sheet) information from the following web sites:

- www.corbettrobotics.com
- www.mn-net.com

Transfer carriages, waste sink and tip chute
Thoroughly rinse under cold tap water and allow to dry.

If further cleaning is desired then soak in 1% Sodium hypochlorite (final concentration of bleach) for >30 minutes. Rinse thoroughly with large amounts of water and allow to dry.

Do not apply hot water, autoclave or heat sterilize these components.

Separator Plates and Non-disposable Reagent Tubs
The separator plate and non disposable reagent tubs must be washed to ensure they are RNA/DNA and RNase/DNase-free. Ensure they are dry before re-using.

Do not autoclave or heat sterilize the separator plate and non disposable reagent tubs (do not exceed 100°C).

When washing the separator plate, scrub lightly with a brush, this will help dislodge air bubbles that can become trapped in the holes and prevent the plate from been cleaned thoroughly. Agitating the plate up and down will also help ensure the holes are properly washed.

1. First rinse with tap water to remove any guanidine salts.
2. Then to clean either soak in 1 % sodium hypochlorite (final concentration of bleach) for >30 minutes, then rinse thoroughly with large amounts of milliQ or Molecular Biology grade RNase-free water.

   Or

   Soak for 1 minute in 0.1 M NaOH, 1 mM EDTA followed by a 1 minute soak in 0.4 M HCl then rinse thoroughly with large amounts of milliQ or Molecular Biology grade RNase-free water.

   Alternatively, the plate may be cleaned with RNase Zap® (Ambion Inc, Austin, TX; P/N 9780).

Troubleshooting

Problem	Cause	Solution
Slow or blocked capture plate wells	Excessive sample	Reduce the amount of starting material. Do not use more than 30 mg of tissue or $2x10^6$ cells. To preserve the sample you are currently working with, remove it from the well and re-run on a different vacuum plate. Once you have recovered the sample, pierce the bottom of the glass fibre membrane through the nozzle with a 27-gauge needle to allow subsequently loaded buffers to pass through.
	Incomplete lysis of sample material	Increase homogenization time.
	Particulates and precipitates blocking membrane	Avoid debris carry over when aspirating sample into Lysis block. Use NucleoSpin® RNA Filter plate to remove debris or pellet particulates (2,500 x g, 10 minutes) and load sample supernatant into a new empty lysis block.
	Vacuum too low	Increase the vacuum or prolong the vacuum time. Ensure the vacuum applied complies with the extraction protocol.

Troubleshooting *cont.*

Problem	Cause	Solution
RNA degradation, poor RNA quality or low yield	RNA is degraded/ no RNA obtained	Create an RNase free environment on the worktable. Clean trough reservoirs with appropriate solutions. Wear gloves during all steps of the procedure. Change gloves frequently. Use of sterile, disposable polypropylene tubes is recommended. Do not fill back unused buffer from the trough reservoir into the bottle.
	Poor quality of sample material	Sample material not fresh or stored under inappropriate storage conditions.
	Samples have undergone multiple freeze-thaw cycles	Samples that have been frozen and thawed repeatedly will eventually experience RNA degradation. Use fresh samples where possible.
	Particulates and precipitates and clots blocking membrane	If using too much sample or if tissue lysate has not been properly homogenized or cleared using the NucleoSpin® RNA Filter Plate, clogging of the NucleoSpin® RNA Binding Strips/Plate may appear. To prevent this reduce sample amount and raise time for vacuum binding steps. If clogging happens during the run take the remaining lysate off the NucleoSpin® RNA Binding Strips/Plate, discard it, and proceed with the desalting step (buffer RA3).
	Incomplete elution	Increase volume of dispensed RNase free water. Make sure that all of the water gets in contact with the glass fibre membrane. No water drops should stick to the wall of the individual columns. Increase elution vacuum time in preference to increasing vacuum pressure.
	Reagents not applied or restored properly	Reagents not properly restored. Add the indicated volume of RNase-free water to the DNase I vial and 96 – 100% ethanol to buffer concentrates RA3 and RA4 and mix.

Troubleshooting *cont.*

Problem	Cause	Solution
Poor RNA yield	Excessive evaporation of reagents	The required volumes of reagents should not be dispensed into the reagent tubs until just prior to the start of the run. Leaving reagents in open tubs for extended periods will result in evaporation of water and alcohols. This will result in salt precipitates and loss of poor binding conditions
	Steps not followed correctly or wrong reagents used	This protocol requires that the correct volumes of reagents are used in a specific order. When done correctly the RNA will bind and remain bound to the membrane during the purification.
Low RNA purity (A260/280 ratio too low)	Inappropriate diluent for UV measurements	Ionic strength and pH influence A_{260} absorbance as well as A_{260}/A_{280} ratio. For absorbance measurement use 5 mM Tris buffer pH 8.5 as diluent.
Inhibition with downstream applications	Incorrect type or quality reagent or plasticware	Before continuing, it is essential to ensure you use original and fresh MACHEREY-NAGEL reagents and the recommended Corbett consumables (see section *Reagents and Consumables Required*). Low quality chemicals may cause inhibition effects, as can inhibitors leached from incorrect plasticware.
	Salt carryover during elution	Check Wash Buffer for salt precipitates. If there are any precipitates, carefully warm until they dissolve.
	Ethanol carry over during elution	Increase drying time for ethanol removal step. Dry plate at 50°C for 10 minutes.
	Reduced sensitivity	Determine the maximum volume of eluate suitable for your amplification reaction. Reduce or increase the amount of your eluate added accordingly.
	Elution cluster rack tubes autoclaved before elution	Do not autoclave elution cluster rack tubes. This may leach chemicals from the tubes, which may inhibit enzymatic reactions. Repeat the purification with a new set of elution cluster rack tubes. The Corbett Robotics or MACHEREY-NAGEL cluster rack tubes are RNase/DNase and RNA/DNA free.

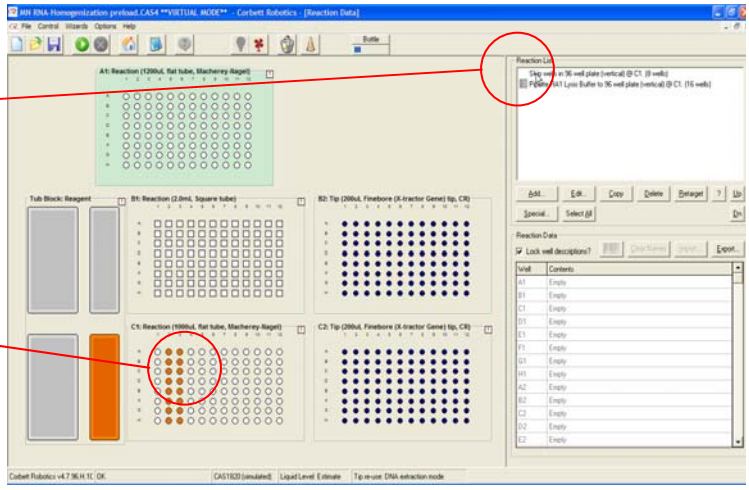
Appendix A

RA1 Lysis Buffer Homogenization Preload Protocol

1. Open the Robotics4 software and open the *MN RNA – Homogenization preload. CAS4* run file.
2. Click on the reaction plate at workspace position C1. This will open a *reaction list* on the right hand side of the screen.
3. To skip columns, highlight the *Skip wells in 96 well plate* option and then select *Edit*. In the *Reaction Configuration* window,

***3**. Double click on "Skip wells to open configuration window"*

***2**. Click on the C1 position to open the "Reaction List"*



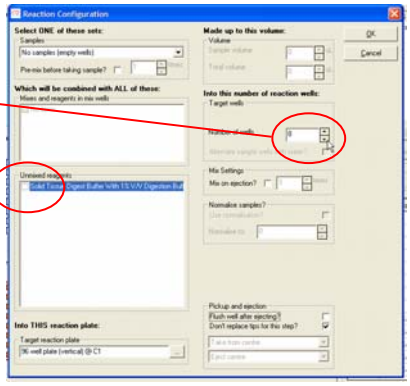
<u>To skip wells</u>

4. Select the number of wells (8 wells equals 1 column) you want to skip in the *skip well* option.
5. Ensure that in the *Unmixed reagents* pane, that *RA1 Lysis Buffer* is **<u>Not</u>** selected.
6. Click Ok.

<u>To Skip Wells</u>

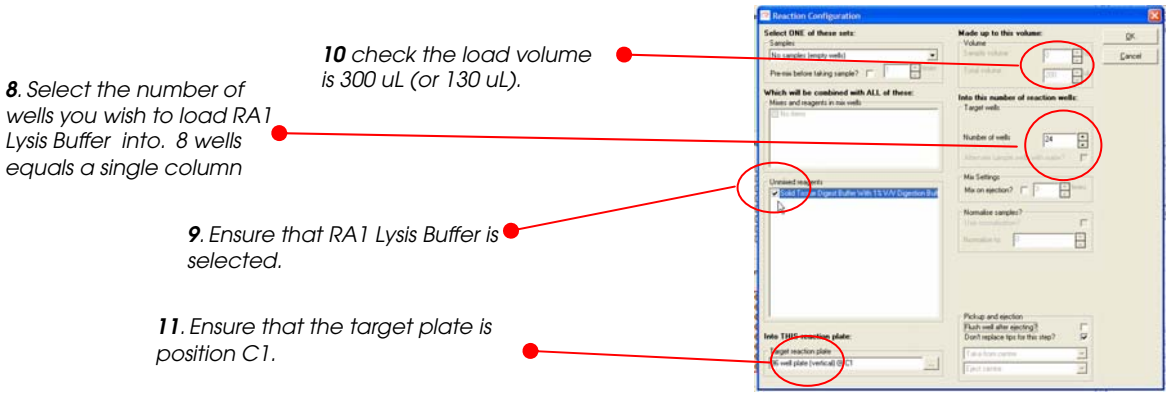
***4**. Select the number of wells you wish to skip. 8 wells equals a single column.*

***5**. Ensure that no reagents are selected.*



To Load Required wells with Digest Buffer

7. To select the number of columns you want to load with *RA1 Lysis Buffer,* highlight the *Pipette RA1 Lysis Buffer* option then double click (as per step 3).
8. In the *reaction configuration* window, enter the number of *target wells* you wish to load.
9. Ensure that in the *Unmixed reagents* pane, that *RA1 Lysis Buffer* is selected.
10. Ensure that the total volume in the *volume* pane displays 300 µL (or 130 µL for direct lysis in 96 well culture plates, remember to reduce buffer RA4 to 130 µL also).
11. Ensure that the target reaction plate is the 96 well plate in position C1.
12. Click *Ok.*



Load wells with Digest Buffer

13. Ensure the reagent tub described within the software has been loaded with the required amount of *RA1 Lysis Buffer* .
14. Click the *Start* icon and a pre-run checklist will appear. Select *Check all* once all messages have been acknowledged and addressed.
15. Click *Ok.*

Once the *RA1 Lysis Buffer* has been aliquoted into the wells of the plate, the tissue sample can be added (if not added previously).

Appendix B

Sample Lysis Volume

Note: A larger volume of lysis buffer RA1 (400 µL) allows a more consistent automated removal of 300 µL homogenized sample following disruption and homogenization of tissue samples in 96well plates. Please note that additional Lysis Buffer RA1(Cat # 740961 or Cat # 740961.500) is required . Furthermore, use of NucleoSpin® RNA Filterplate allows to remove debris from tissue homogenization efficiently.
For small or precious samples the homogenization volume (300 µL) can be maintained as per the original MN protocol. For cells grown in 96well plates the lysis buffer volume can be reduced to 130 µL. To adjust binding conditions the volume of binding / wash solution RA4 has to be reduced to 130 µL accordingly.

Choosing Sample Introduction Method

Selecting the correct method for introduction of homogenized sample into the extraction run.
There are two options for introduction of the digested sample into the X-tractor Gene™.

1. Manually load the required 300 µL of homogenized material directly into the lysis plate and then place the lysis plate into the B1 position.
   This **is** the **default** run file option

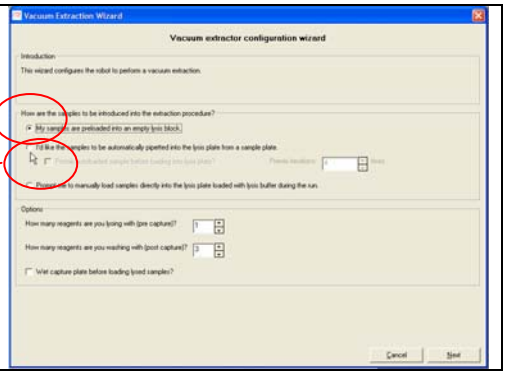
   For this option you will need to change the run file from its default settings, therefore:
   a. Open the *Macherey-Nagel NucleoSpin(R) 96 RNA V01.CAS4* run file.
   b. Open the Wizard and scroll back two screens to the first page. Ensure the "My samples are preloaded into an empty lysis block" option is selected.
   c. Place the square well 96-well lysis block with 300 µL of digested material per well in the B1 position.
   d. Scroll to the end of the wizard and confirm the protocol in the post wizard setup screen.

2. Have the X-tractor Gene™ transfer 300 µL. of homogenized supernatant from the pelleted "homogenization plate" to the "lysis plate". The Binding Buffer is then added to the homogenized sample.
   This is **not** the **default** run file option.

   To set up:
   a. Open the *Macherey-Nagel NucleoSpin(R) 96 RNA V01.CAS4* run file.
   b. Open the Wizard and scroll back two screens to the first page.
   Ensure the "I'd like the samples to be automatically pipetted into the lysis plate from a sample plate" option is selected, and the associated "premix sample" option is unchecked.
   c. Scroll to the end of the wizard and confirm the protocol in the post wizard setup screen.
   d. Place the round well 96-well block of digested material in the C1 position. Use a round well 96-well plate and within the plate configuration window, select the supernatant alternative of the plate.
   e. Manually height calibrate the plate to an appropriate height so that the pellet will not be disturbed during sample aspiration.
   f. Under Options>Run Settings, configure the sample pipetting speed to 60 µL/sec (**Default**)

***2**. Option 2 Manually load samples into lysis block*

1.** Option 1 **Default
The X-tractor Gene™ automatically transfers the required volume of digested supernatant off the digest pellet from the digest plate at position C1 into the lysis plate at position B1.



Appendix C

Nucleic Acid Storage

A working stock of DNA can be stored at 2 – 4 ˚C for several weeks. For long term storage DNA should be stored at -20 ˚C.

RNA should be stored at -80 ˚C at all times (and held at 2- 4 ˚C during use).

Note that the solution in which the nucleic acid is eluted in will affect it's stability during storage. Pure water lacks buffering capacity and an acidic pH may lead to acid hydrolysis. Tris or Tris-EDTA buffer contains sufficient buffering capacity to prevent acid hydrolysis.

Repeated freeze thaw cycles should be avoided as this can shear the DNA.

Disclaimers

Protocol Use
It is the user's responsibility to validate performance of this protocol for any particular application, since performance characteristics of this protocol and its product have not been validated for any specific application.

This protocol is for *in vitro* research use only.

It is not intended to identify any specific organism or for clinical use.

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