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 eRNA extraction from water samples filtered through 47 mm diameter filters (NucleoMag DNA/RNA Water Kit - MACHEREY NAGEL).

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ABSTRACT

The objective of this protocol is the **environmental RNA (eRNA) extraction from water samples filtered through 47 mm diameter filters**.

RNA extraction is performed using a **MagnaPure 32 Nucleic Acid Purification System** (Dutscher) and with the **NucleoMagDNA/RNA Water Kit** (Macherey Nagel), with a rDNase digestion step.

The procedure is based on **reversible adsorption of nucleic acids to paramagnetic beads** under appropriate buffer conditions.

The benefits of using the **MagnaPure 32 Nucleic Acid Purification System** (Dutscher) are **increased productivity and repeatability, as well as eliminating human error and the pain of repetitive work**.

This protocol is used **prior to molecular biology analysis** (e.g. qPCR, metabarcoding, ddPCR) to specifically target **both macro- and micro-organisms eRNA extracted from water samples**.

IMAGE ATTRIBUTION

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We use this protocol and it's working

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GUIDELINES

The main steps of the protocol are:

- Material and rDNase preparation
- Plate preparation
- Sample lysis
- eRNA extraction with the MagnetaPure 32 System (Dutscher)
- eRNA elution

MATERIALS

▪ **Materials:**

- 1000 µL pipette
- 100 µL pipette
- 10 µL pipette
- Scissors
- Vortex + benchtop centrifuge for 5 mL tubes
- Horizontal vortex with 5 mL tube holder (15 mL tube holders)
- Centrifuge for 2 mL tubes (relative centrifugal force needed: 11,000 x g)
- MagnetaPure 32 Nucleic Acid Purification System (Dutscher)
- Specific DNA/RNA-workstation (sterile area equipped with air filtration and UV systems)

▪ **Consumables:**

All tubes and tips must be sterile

- 1000 µL tips with filter
- 100 µL tips with filter
- 10 µL tips with filter
- 50 mL tubes: 4 to prepare aliquots, 3 for scissors decontamination 1 for rDNase dilution.
- 5 mL tubes: 1 per 8 samples to prepare NucleoMag B-Beads and MWA2 mix
- 2 mL tubes: 1 per sample to transfer lysate + 2 to prepare aliquots
- 0.5 mL tubes: 3 per sample to transfer eluted RNA
- 96-well plate with 2 mL deep-wells, U-Bottom (Macherey Nagel - 746032.DEEP): 1 per 16 samples
- Magnetic rod coverfor MagnetaPure 32 (Macherey Nagel – 747032.TC): 1 per 8 samples
- Plastic film to protect the 96-well plate
- Gloves

For any manipulation in a rare DNA/RNA room, provide complete equipment (disposable coat, cap, mask, shoe covers & gloves).

▪ **Reagents:**

- rDNase Set (Macherey Nagel - 740963)
- Reaction Buffer for rDNase (Macherey Nagel - 740834.60)

- NucleoMag DNA/RNA Water kit (Macherey Nagel)

Note: shelf life of reagents for 24 months from production

- To clean the scissors:

- o Solution to remove RNA (e.g. *RNase-off, RNA away*)
- o Ethanol 96% for molecular biology
- o Molecular biology grade water

▪ **Samples to be extracted:**

- Filters in 5 mL tubes and stored at -80°C until RNA extraction

BEFORE START INSTRUCTIONS

- **Filtration and preservation of the water samples through filters**

As eRNA degrades very rapidly, the water samples must be filtered and preserved very quickly after sampling (less than one hour if possible). If filtration is carried out in the field, the filters should be immediately frozen in liquid nitrogen and then stored at -80°C until the eRNA is extracted. If filtration is carried out in the laboratory, the filters can be stored at -80°C immediately after filtration and until the eRNA is extracted.

Note: As eRNA is very sensitive to RNase, which is active at temperatures above -70°C, it is very important to freeze the filters in liquid nitrogen or at -80°C very quickly after filtration.

This protocol is suitable for the RNA extraction from different types of 47 mm diameter filters:

Filter Type
Polyethersulfone (PES)
Polycarbonate (PC)
Cellulose nitrate (NC)
Cellulose mixed esters (CM)
Cellulose acetate (CA)

The filters should be preserved in 5 mL tubes for this RNA extraction protocol.

- **The following precautions must be applied:**

- Wear gloves throughout the extraction process
- Clean the bench with a RNA-removing solution (e.g. RNase-off).
- Use tips with filters to avoid contaminations
- All steps have to be performed under a specific DNA/RNA-work station (sterile area equipped with air filtration and UV systems)

For any manipulation in a rare DNA/RNA room, provide complete equipment (disposable coat, cap, mask, shoe covers & gloves).

- **Pre and post extraction equipment decontamination:**

- Clean a specific DNA/RNA-work station and apply UV

- MagnetaPure 32 System (Dutscher): Visual check for residues to be removed and UV decontamination – *see instrument manual*

- **Scissors decontamination** (*to be done before starting the protocol, and between each filter cutting*)

- *Prepare :*

1. one 50 mL tube with RNA-removing solution (e.g. DNA/RNase-off, DNA/RNA away)
2. one 50 mL tube with molecular biology grade water
3. one 50 mL tube with ethanol

- *Successively dip the scissors into each tube, opening and closing the scissors in each tube.*

Note: The cutting of filters is not a requirement, but it does have an impact on the extraction efficiency.

Material and rDNase preparation

1 Material preparation

- *To limit contamination of the kit buffers, it is recommended to aliquote them:*

- *Into 50 mL tubes for MWA1, MWA2, MWA3 and MWA4*
- *Into 2 mL tubes for NucleoMag B-Beads solution and RNase-free H₂O*

- *Tubes annotation*

- *one 2 mL tube per sample for lysate collection*
- *one 5 mL tube per 8 samples for the NucleoMag B-Beads and MWA2 mix preparation*
- *three 0.5 mL tubes per sample for RNA collection*

- *Scissors decontamination (to be done before starting the protocol, and between each filter cutting)*

- *Prepare :*

1. one 50 mL tube with RNA-removing solution (e.g. RNase-off)
2. one 50 mL tube with molecular biology grade water
3. one 50 mL tube with ethanol

- *Successively dip the scissors into each tube, opening and closing the scissors in each tube.*

Note: The cutting of filters is not a requirement, but it does have an impact on the extraction efficiency.

2 rDNase preparation

5m

1. Reconstitution of lyophilized rDNase:

- Add 4 mL of **reaction buffer for rDNase** to the rDNase vial
- Incubate for 00:02:00 - 00:03:00 at Room temperature
- Gently shake the vials to completely dissolve the rDNase

Note: Be careful not to mix the rDNase vigorously because the rDNase is sensitive to mechanical agitation

2. rDNAse Dilution:

- Transfer the **reconstituted rDNase** to a 50 mL tube
- Add 28 mL of **reaction buffer for rDNase**
- Gently shake the tube

Note: The resulting rDNase solution will be sufficient for 96 samples. If fewer than 96 samples are to be processed, it is advisable to aliquot the rDNase as it should not be frozen and thawed more than three times. Aliquots can be stored at -20 °C for at least 6 months.

Plate preparation 1/2

3

30m

In this step, the buffers provided by the kit are distributed in a 96-well plate.

For RNA extraction from filtered water samples, the 12 columns of the plate are divided into 2 sections of 6 columns each, allowing up to 16 samples to be extracted per plate.

- Annotate the 96-well plate as recommended below:

Solution	1	2	3	4	5	6	7	8	9	10	11	12	
Column name	D	3	3	L	4	E	D	3	3	L	4	E	
Samples between 1 to 8												Samples between 9 to 12	

Table 1: Recommended plaque annotation

Note: It is useful to mark the dividing line between columns 6 and 7 with a marker pen to provide a visual cue for filling the plate.

- Add the appropriate buffers into the appropriate wells of the plate (Table 2):

1st column / 7th column: Will be filled during plate preparation 2/2

2nd column / 8th column: 850 µL of **MWA3** (1st wash)

3rd column / 9th column: 850 µL of **MWA3** (2nd wash)

4th column / 10th column: Will be filled during plate preparation 2/2

5th column / 11th column: 850 µL of **MWA4** (3rd wash and bead drying)

6th column / 12th column: 50 µL or 65 µL of **RNase-free H₂O** (RNA elution)

Note: The choice of elution volume is based on the expected eRNA amount. The smaller the amount, the smaller the elution volume in order to obtain more concentrated RNA.

Solution	1	2	3	4	5	6	7	8	9	10	11	12				
Volume (µL)	<i>Plate preparation 2/2</i>		MWA3	MWA3	<i>Plate preparation 2/2</i>		MWA4	RNase-free H ₂ O	<i>Plate preparation 2/2</i>		MWA3	MWA3	<i>Plate preparation 2/2</i>		MWA4	RNase-free H ₂ O
Column name			850	850			850	50 or 65			850	850			850	50 or 65
	3	3			4	E			3	3			4	E		

Samples between 1 to 8 **Samples between 9 to 12**

Table 2 : Plate preparation 1/2

- Film and reserve the plate at Room temperature

4

- Preparation of the NucleoMag B-Beads and MWA2 mix:

- Prepare one 5 mL tube / maximum 8 samples
- Add **MWA2** only, the NucleoMag B-beads will be added during plate preparation 2/2 (*allow a margin of one sample for the mix preparation. For example: plan a mix for 9 samples if 8 samples are to be extracted*)

Sample Number	Volume NucleoMag B-Beads (μL)	Volume MWA2 (μL)
1	25	475
2	50	950
3	75	1425
4	100	1900
5	125	2375
6	150	2850
7	175	3325
8	200	3800
9	225	4275

Table 4: Volume required to prepare NucleoMag B-beads and MWA2 mix (*no margin*)

Sample Lysis

- 5 During this step, a mechanical and chemical lysis of the sample is performed.

10s



- Collect the 5mL tubes containing the filters from the freezer and place them On ice
- Add immediately 750 μL of **MWA1** buffer into each tube
- Cut the filter into small pieces directly into each tube using decontaminated scissors
Note: Scissors must be decontaminated between each sample (see section 1 "material preparation")
- Place the tubes on the vertical vortex 00:00:05 at median speed
- Place the tubes on the horizontal vortex 00:05:00 at maximum speed
- Place the 5 mL tubes into the benchtop centrifuge
- Pipette the lysate from the tube (approximately 650 μL) and transfer it into a 2 mL tube
- Centrifuge at 11000 x g, 00:00:30
- Replace the tubes into the rack and reserve them at Room temperature until their distribution into the 96-well plate

Plate preparation 2/2

- 6 Preparation of the NucleoMag B-Beads and MWA2 mix

15m

- NucleoMag B-Beads and MWA2 mix sediment quickly, vortex between each samples to ensure homogeneity*
- Vigorously vortex **NucleoMag B-Beads** tube

- For each tube containing MWA2 buffer (previously prepared), add the appropriate volume of **NucleoMag B-Beads** (see Table 4 above)
- Vortex

- NucleoMag B-Beads - MWA2 mix and lysate distribution
- Remove the film from the plate
- Add the appropriate solution into each well of the plate

4th column /10th column:

500 µL of **NucleoMag B-Beads and MWA2 mix**

450 µL of **Lysate** (supernatant from the 2 mL tubes)

- rDNase mix distribution
- Slowly agitate the **rDNase mix** tube (previously prepared- see step 2)
- Add the appropriate solution into each well of the plate

1st column / 7th column:

300 µL of **rDNase mix**

Solution	1	2	3	4	5	6	7	8	9	10	11	12
rDNase reaction mixture				NucleoMag B-Beads and MWA2 mix			rDNase reaction mixture			NucleoMag B-Beads and MWA2 mix		
Volume (µL)	300			500			300			500		
Solution				Lysate						Lysate		
Volume (µL)				450						450		
Column name	D			L			D			L		
	Plate preparation 1/2				Plate preparation 1/2				Plate preparation 1/2			
	Samples between 1 to 8						Samples between 9 to 12					

Table 5: Plate preparation 2/2

eRNA extraction with the MagnetaPure 32 System (Dutscher)

7

• Extraction part 1

- Place the plate into the MagnetaPure 32 System and insert the magnetic rod coverfor – *see instrument manual*

1h 20m

- Select the appropriate program for RNA extraction part 1

Step	Well	Name	Mix time (min)	Magnet (sec)	Wait time (min)	Volume (μ L)	Mix speed	Temp (°C)
1	4	Binding	8	10	0	950	8	OFF
2	3	MWA3	2,3	5	0	850	8	OFF
3	2	MWA3	2,3	5	5	850	8	OFF
4	1	Digest DNA	2,3	0	15	300	8	37

Table 6: MagnetaPure 32 System program for NucleoMag DNA/RNA Water Kit - RNA extraction part 1

- Start the run (*The run lasts approximately 00:35:00*)

- At the end of DNA digestion
- Remove the plate and add the appropriate solution into each well of the plate

1stcolumn / 7thcolumn:  350 μ L of MWA2

	1	2	3	4	5	6	7	8	9	10	11	12
Solution	MWA2						MWA2					
Volume (μ L)	350						350					
Column name	D						D					

Samples between 1 to 8 Samples between 9 to 12

Table 7: Addition of MWA2 buffer after DNA digestion

▪ **Extraction part 2**

- Place the plate back into the MagnetaPure 32 System (it is not necessary to change the magnetic rod coverfor)

- Select the appropriate program for RNA extraction part 2

Step	Well	Name	Mix time (min)	Magnet (sec)	Wait time (min)	Volume (μ L)	Mix speed	Temp (°C)
1	1	Rebind	8	10	0	650	8	OFF
2	5	MWA4	2,3	5	15	850	8	OFF
3	6	Elution	5	20	0	50 or 65	8	OFF
4	5	Release	0,5	0	0	850	10	OFF

Table 8: MagnetaPure 32 System program for NucleoMag DNA/RNA Water Kit - RNA extraction part 2

- Start the run (*The run lasts approximately*  00:30:00)

Transfer of eRNA extracts

30m

- 8
- At the end of the run, remove the plate and place it  On ice into the DNA/RNA-workstation
 - Remove the magnetic rod cover and start UV for decontamination – *see instrument manual*
 - In the DNA/RNA-workstation, transfer each **eRNA extract** into a 0.5 mL tube previously annotated.

Note: It is recommended to aliquot the eRNA extracts directly to avoid freezing and thawing cycles that could degrade the eRNA.

Note: RNA concentration and quality can be measured at this step (e.g. Nanodrop)

- Store eRNA extracts at  -80 °C