COSMO BIO CO., LTD.
Inspiration for Life Science

Nucleic Acid Purification Kit

MagExtractor *-RNA-*

Instruction Manual

(Code No.NPK-201)

Distributor

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Caution

All the reagents included in this kit are for experimental use and are never to be used for diagnostical or clinical purposes. When using this kit, make sure to follow common laboratory safety procedures carefully.

Hoffman-La Roche Ltd. retains a patent on the PCR method and special permission may be required from them depending on the stated goals of the experiment undertaken.

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1. Introduction

This kit is a reagent for total RNA extraction utilizing the absorbency power of RNA on magnetic beads inside a protein denaturing solution containing an RNA bond accelerator. The RNA obtained from culture cells and tissue with this kit can in large part be used for an RT-PCR (reverse transcription PCR). Aside for use as a reagent for the MFX-2100 fully automatic nucleic acid extractor, this kit can also be used with the manual extraction method. When performing extraction using the MFX-2100, please make sure to read its operation manual carefully.

Capabilities and features

- It is capable of extracting and purifying total RNA from various samples such as culture cells. The extracted RNA is mainly rRNA and mRNA.
- Does not use harmful solvents such as phenol or chlorophorm.
- High revolution centrifuge separation is not required due to magnetic particle separation.
- RNA separation in a short time span is rendered possible. When using the MFX-2100, 24 samples can be completely extracted in approximately 4 hours.

2. Cautions prior to use

The MagExtractor *-RNA-* is applicable to the following samples:

Sample	Amount	Yield Point	Remarks
Cultured cells	up to 5×10^6 cells	up to 10μg/10^6 cells	RNA yield point varies depending on cell type and culture conditions
Tissue	up to 30mg	up to 15μg/30mg	RNA yield point varies depending on tissue type and storage conditions
Yeast	cells collected. from a 0.5ml culture solution of ~10O.D.(660nm)	up to 20μg/5 O.D.	Pre-treatment using Zymolyase required, RNA yield point varies depending on culture conditions

- With direct extractions from whole blood and blood serum samples, the yield point is very low. In the case of whole blood, operations such as ficoll centrifuge separation of leukocytes are necessary.
- As with yeast, RNA extraction from Lysozyme treated colon bacilli is possible. However, genome DNA verified by electrophoresis may be present in the mixture.

3. Items included in this kit

There are regents from which 100 samples of RNA can be extracted in this kit. Store them at the following temperatures:

100ml	Lysis & Binding Solution (with protein denaturant)	4°C
66ml	Washing Solution I (with protein denaturant)	4°C or room temperature
176ml	Washing Solution II (low concentration buffer)	4°C or room temperature
10ml	Elution Solution (RNase-free distilled water)	4°C or room temperature
7ml	Magnetic Beads	4°C or room temperature
1ml	2-Mercaptoethanol (2ME)	4°C

・Should some of the reagents get on the hands or clothing, make sure to wash them out thoroughly. In the likely event that some should get in the eyes, wash them out well and consult a physician.

・A protein denaturant is contained in the Lysis & Binding Solution and in Washing Solution I. Handle with care.

・For manual operation, make sure to use Washing Solutions I and II only after having returned them to room temperature. It is also possible to store them at room temperature. (For extended storage periods, it is recommended to store at 4°C.)

・Mix the Lysis & Binding Solution and the 2-Mercaptoethanol (2ME) at a ratio of 100:1(V/V). The mixture can be used for up to 3 months at 4°C.

4. Protocol

[1] Required items not included in this kit

(1) Reagents

For the extraction of RNA from yeast, the following reagents are necessary.

・Zymolyase (20,000U/g; Sei Kagaku Kogyo(SSK) Co., Ltd.)

・Zymolyase buffer (0.9M Sorbitol, 0.1M EDTA, 50mM DTT (pH7.5)

(2) Devices and equipment

When using the MFX-2100:

・Special tube (Code No.: MFX-301 or MFX-302)

・Chip with special filter attached (Code No.: MFX-402A)

・Reagent setting tubes (50ml, 15ml, 2ml) *1

For manual extraction:

・1.5ml micro-tube magnetic stand*2

・Vortex mixer

・Heat block (water bath also possible) (65°C)

・Simple tabletop centrifuge (with a capacity of about 3,000 to 5,000 r.p.m.)

*1. The following items are recommended for use as the reagent setting tube: 50ml tube: Blue Max 2170 (Becton Dickinson), 15ml tube: Blue Max, Jr. (Becton Dickinson), 2ml micro-tube: 2220, 2200 (Ina・Optika), 72.693, 72.694 (Assist)

*2. Our Mag*ical Trapper* (Code No.: MGS-101) magnetic stand may be used.

[2] Preparing reagents

・Mix the Lysis & Binding Solution with the 2ME at a ratio of 100:1(v/v). When preparing only the needed amount, mix 700μl of Lysis & Binding Solution with 7μl of 2ME for each time. However, when preparing the entire amount allotted, add 1ml of 2ME in the bottle of Lysis & Binding Solution (100ml). Store this solution (Lysis & Binding Solution containing 2ME) at 4°C (for up to 3 months).

・When extracting manually, use Washing Solution I and II only after having returned them to room temperature.

[3] Pre-treatment method for samples

Because the RNA inside the samples is unstable, dissolve it in advance in the Lysis & Binding Solution containing 2ME*1. Before dissolving yeast, an enzymatic cell wall decomposition treatment process is required. Below is an example of the pre-treatment method for samples.

(1) Cultured cells

・Collect the cultured cells in a 1.5ml micro-tube by centrifuge separation, add in 700μl of Lysis & Binding Solution (containing 2ME) and mix carefully by pipetting until the viscosity diminishes. (Performing the above in a short time will result in low RNA yield.)

・After having mixed another 30 seconds with the vortex mixer, incubate at room temperature for 10 to 15 minutes.

(2) Tissue

・Dispense 750 to 900μl of Lysis & Binding Solution (containing 2ME) in a micro-tube and place on ice.

・Cut away a piece of tissue and quickly freeze it with liquid nitrogen. Afterwards, crush the tissue with a hammer and place pieces of frozen tissue (10 to 30mg) in the above Lysis & Binding Solution. (Soft samples can be used directly.)

・Use a micro-tube homogenizer to homogenize the tissue on ice.

・Mix in the vortex mixer for approximately 30 to 60 seconds until the viscosity diminishes.

・Centrifuge at 3000 to 5000 r.p.m. for 10 seconds *2 and transfer 620 to 750μl of the supernatant to a fresh micro-tube *3. At this time, continue pipetting if the viscosity is still high, or, take it into a syringe with a 21G needle and treat until the viscosity diminishes. (Persistent viscosity may result in low yield.)

・Incubate at room temperature for 10 to 15 minutes.

(3) Yeast *4

・Immediately before use, prepare Zymolyase 20T (20,000U/g) in 30mg/ml of Zymolyase buffer and make the Zymolyase solution (store on ice). (Refer to page 2 for reagent composition.)

・Collect the yeast after having separated the culture solution by centrifuge.

・Suspend the precipitate in 50μl of Zymolyase solution.(in case of colony, pick up with a platinum loop, and suspend in 50μl of Zymolyase solution)

・Suspend in a vortex mixer and incubate at 37°C for 5 to 20 minutes.

・Add 700μl of Lysis & Binding Solution (containing 2ME) directly into the treatment solution and stir carefully by pipetting.

・After having mixed another 30 seconds with the vortex mixer, incubate at room temperature for 10 to 15 minutes.

*1. The RNA is stable inside the dissolving-absorbing solution. If storing in this condition, store at -20 to 4°C.

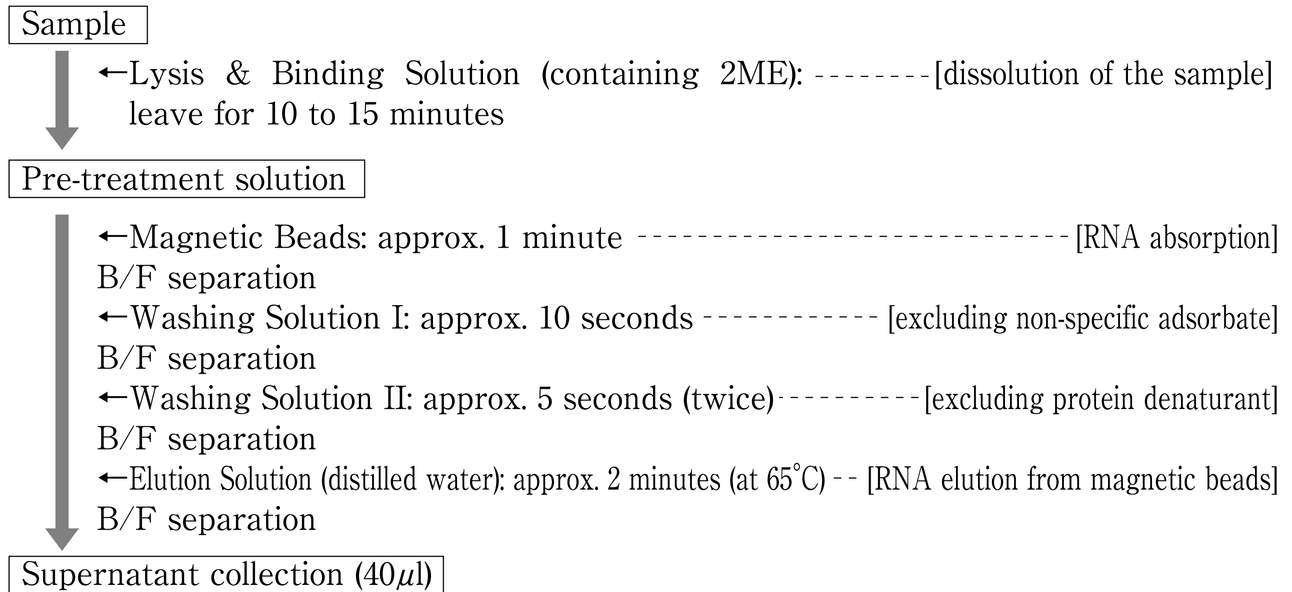
*2. Because the RNA may coprecipitate with foreign substances, avoid high revolution centrifuge.

*3. There may remain some insoluble matter. This does not represent a problem if the amount is not excessive. Having 620μl or more set in the device is permissible.

*4. Identical as when using colon bacilli, perform a Lysozyme treatment and the extraction can be performed using re-precipitated fungi. For colon bacilli, because the presence of genome DNA mixed in the RNA solution may be confirmed by electrophoresis, the DNase I treatment described on page 9 is recommended.

[4] Extraction flow

Extraction flow using the MagExtractor -*RNA*- is described below:



[5] RNA extraction using the MFX-2100

Before using the MFX-2100, make sure to read its operation manual thoroughly. For RNA extractions using the MFX-2100, a heating function is required.

(1) Selecting protocols

Two RNA extraction protocols for the MFX-2100 have been prepared. Depending on need, select either one.

① Normal protocol

- Elute RNA from 40μl *1 of eluate.
- The collected solution can be used directly for a DNase I treatment or a reverse transcription reaction.
- The collected solution can be measured for light absorbency.
- Extraction requires approximately 10 minutes for each sample.

② Ethanol *2 precipitate protocol

- Pour the reagent 40μl of eluate into the previously dispensed 500μl alcohol precipitate.
- If the RNA is not to be used immediately, it can be used to store the RNA for an extended period.
- RNA collection by centrifuge is necessary.
- Extraction requires approximately 10 minutes for each sample.

*1. The amount of eluate may vary in size.

*2. Set the reagent for the self-prepared alcohol precipitate onto the reagent stand. Dispense the alcohol precipitate reagent into the collection tube in advance and pour in 40μl of eluate.

Protocol	Entry No.	Shown on display
Normal	21	RNA: Normal
Ethanol precipitate	22	RNA: Ethanol

(2) Heating block and cooling block temperature setting

Set the heating and cooling blocks at the temperatures stated below:

	Heating block	Cooling block
Temperature setting	65°C	10°C

・For temperature setting, read the MFX-2100 operation manual thoroughly.

(3) Setting chips with special attached filter

Set chips with special attached filter (Code No.: MFX-402A) to the chip rack. Refer to the following table for quantities.

・Make sure to use chips with special attached filter (Code No.: MFX-402A).
・Setting more chips than prescribed does not pose any special problems.
・The chips have been gamma ray sterilized. Make sure to use gloves when setting.
・Line up the necessary quantity of chips onto the chip rack vertically from the bottom left. For more details on setting position, refer to the MFX-2100 operation manual.

For normal protocol:

Sample number	Chip count	Sample number	Chip count
1	5	13	31
2	7	14	33
3	9	15	35
4	11	16	37
5	13	17	39
6	15	18	41
7	17	19	43
8	19	20	45
9	21	21	47
10	23	22	49
11	25	23	51
12	27	24	53

For ethanol precipitate protocol:

Sample number	Chip count	Sample number	Chip count
1	6	13	33
2	8	14	35
3	10	15	37
4	12	16	39
5	14	17	41
6	16	18	43
7	18	19	45
8	20	20	47
9	22	21	49
10	24	22	51
11	26	23	53
12	28	24	55

(4) Setting the special tubes

Set the special tubes (Code No.: MFX-301) in A to E of the extraction rack, the heating and cooling blocks for the number of samples.

- To prevent the occurrence of troubles, do not set anything but the special tubes in A to E on the extraction rack and the heating block.
- A special 6-row tube (Code No.: MFX-302) can be set in A to E on the extraction rack.
- A screw cap type 1.5ml tube can be set onto the cooling block (suitable for RNA storage).
- Refer to the MFX-2100 operation manual for more details on setting method.

(5) Setting reagents

Transfer the reagent into the tubes of designated sizes and set them onto the reagent rack in place. The necessary amount of reagent varies depending on the number of samples. Set the reagent according to the table on page 7.

- Use a pipette if dispensing a small amount of reagent. For a large amount, use the calibrations imprinted on the side of the reagent tube as a guide and proceed.
- Stir the magnetic beads well in advance and set them onto the reagent rack.
- The reagent composition for the alcohol precipitate set when using the ethanol precipitate protocol can be determined as desired. (Ex.1: distilled water: 3M sodium acetate (pH 5.2): ethanol = 7:1:25, Ex.2: distilled water: 5M ammonium acetate: isopropanol = 0.9:1:2)
- The remaining reagent after extraction can be reused. If storing for an extended period, take note that the composition of the solution may change depending on the level of dryness.
- Do not set an amount of reagent that exceeds the designated amounts. Doing so may cause contamination of the nozzle and liquid dripping.

Set position	Reagent name	Tube
1	Washing Solution II	50ml tube
2*1	Ethanol	
7	Magnetic Beads	2ml micro-tube
9	Washing Solution I	15ml tube
10	Elution Solution	

*1. Set the reagent for self-prepared ethanol (isopropanol) precipitate only when using the ethanol precipitate protocol.

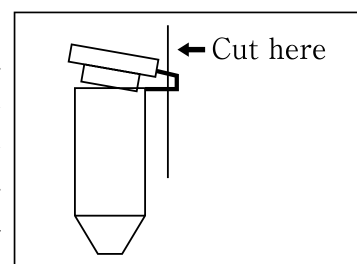
Dispersal amount for each reagent

Reagent		Washing Solution II	Alcohol Reagent for precipitate	Magnetic Beads	Washing Solution I	Elution Solution
Tube size		50ml	50ml	2ml	15ml	15ml
Set position		1	2	7	9	10
		Amount of reagent dispensed to reagent tubes (ml)				
Quantity of samples	1	5	5	0.5	1.5	0.5
	2					
	3	7.5			2	
	4				3	
	5				4	1
	6	15		0.8		
	7				5	
	8					
	9	20	10		6	1.5
	10				7	
	11			1.1		
	12	25			8	
	13					
	14				9	
	15	30				
	16			1.4	10	
	17	35	15		11	2
	18					
	19				12	
	20					
	21	40		1.7	13	
	22					
	23				14	
	24	42				

(6) Pre-treatment of samples and settings

Treat the samples as per the pre-treatment method described on page 3. Set the treated samples in their sample set positions on the extraction rack. For the sample set positions, the front of the holes on the extraction rack is numbered 1 to 24.

- A screw-cap type 1.5ml sample tube is recommended for sample preparation. Set in the set position with the cap off.
- When using a normal 1.5ml tube, cut the cap off carefully with scissors and set. Make sure to exercise caution when cutting.
- Set the pre-treatment solution amount at 620 to 750μl. For culture cells and yeast, set at 700 to 750μl. Set the tissue fragment solution at 620μl or above as a guideline to compensate for the loss caused by the precipitate. (Having the solution amount set at 600μl or less will cause it to foam when mixed resulting in low yield.)



(7) Initiate extraction and collect samples

Start the unit following the instructions below:

- Verify whether the samples, reagent tubes (reagent amount), tubes, and special chips are set as per the operation manual.
- Verify the set position of each rack (lock) and whether the stage is completely contained in the back. Close the front cover.
- Enter the protocol number and the number of samples in the liquid crystal display screen.
- Verify whether "press start key" is displayed on the screen and press "START".
- Extraction initiates and the liquid crystal display screen shows "in operation".
- When all extractions are completed, "press start key" will be redisplayed. Open the front cover and take out the sample.

[6] RNA extraction using the manual method

Prepare 700µ of a pre-treatment solution sample prepared according to the sample pre-treatment method described on page 3.

Pre-treated sample (700µl)

←50µl Magnetic Beads (add in after having tumble stirred until uniformly mixed)
←Stir 20 seconds [*1] (vortex mixer intensity: maximum)
Leave at room temperature for 40 to 60 seconds
B/F separation [*2] [*3]
←600µl Washing Solution I
Stir 10 seconds (vortex mixer intensity: maximum)
B/F separation
←800µl Washing Solution II
Stir 5 seconds (vortex mixer intensity: maximum)
B/F separation
←800µl Washing Solution II
Stir 5 seconds (vortex mixer intensity: maximum)
B/F separation
After spindown, re-extract supernatant
←40µl eluate
Stir 5 seconds (suspend particles)
Leave for 2 minutes at 65°C
Stir 5 seconds
Spindown

Supernatant

- Dilute part of the collected solution 10 to 20 times, measure the A_{260} and calculate the RNA concentration [*5].

*1. Mix with the vortex mixer set at maximum intensity.

*2. Set the tube onto the magnetic stand and bring the magnet beads towards the magnetic until it has lured out all the beads that adhered to the cover because of the persistent tumble mixing. Afterwards, strongly shake the magnetic stand to let any solution adhering the cover fall out. Extract the supernatant with a pipette.

*3. If there is no magnetic stand, centrifuge for about 5 seconds with a simple tabletop centrifuge at approximately 3000 r.p.m. The particles may become difficult to loosen because of the action of the centrifuge. Therefore, verify whether the particles have suspended during mixing.

*4. Proceed with the extraction very carefully.

*5. Calculate the RNA concentration (ng/µl) as $A_{260} \times 40 \times$ dilution rate.

[7] DNase Ⅰ treatment

When performing an RT-PCR, if the intron pinching pair of primers cannot be prepared because the mRNA composition is unknown or if Pseudo-genes, with a composition identical to mRNA, are present with the genomes, the effect of a minute amount of genome DNA mixed into the solution must be considered. It is then necessary to perform an enzymatic decomposition treatment on the mixed-in DNA using RNase-free DNase I especially if a small mRNA expression is amplified by the RT-PCR. An example of this is shown bellow:

(1) Items prepared

- 10 × DNase I buffer *1
- RNase-free DNase I (10U/μl)
- DEPC treated water
- TE saturated phenol
- Chloroform: isoamyl alcohol (24:1)
- 5M ammonium acetate solution (pH nonadjusted)
- glycogen solution (2mg/ml) (for molecular biology use)
- Isopropanol
- 70% ethanol solution

(2) Protocol

① DNase Ⅰ treatment

RNA solution	X (μl)
DEPC treated water	8.5-X
10 × DNase I buffer	1
RNase-free DNase I (10U/μl)	0.5
Total	10 (μl)

- Let react on ice for 10 to 30 minutes. *2

② Phenol-chloroform treatment / isopropanol suspension

10μl DNase I treated solution

←100μl DEPC treated water
←100μl TE saturated phenol
Mix and suspend well in a vortex mixer
Leave on ice for 5 minutes
Separate by centrifuge at 12,000 r.p.m. x 5 min. at 4°C
Transfer the supernatant to another micro-tube
←equal amount of chloroform: isoamyl alcohol (24:1)
Mix well and separate by centrifuge at 12,000 r.p.m. x 5 min. at 4°C.

100μl of supernatant

←5μl glycogen solution (2mg/ml)
←100μl 5M ammonium acetate solution
←200μl isopropanol
Stir in a vortex mixer
Leave at -20°C for 30 to 60 minutes
Separate by centrifuge at 12,000 r.p.m. × 10 to 15 min. at 4°C and extract the supernatant *3
←1ml of 70% ethanol
←Softly tumble stir
←Separate by centrifuge at 12,000 r.p.m. × 2 min. at 4°C and extract the supernatant
Spindown and completely extract supernatant

Precipitate

←DEPC treated water or reverse transcription reaction solution
Reverse transcription reaction *4

*1. 100mM Tris-HCI and 20mM $MgCl_2$ (pH 7.5)

*2. DNase I functions well on ice. Performing the reaction at a high temperature make the RNA difficult to break down.

*3. Glycogen precipitation can be verified. Exercise caution as to not allow it to be aspirated by the pipette.

*4. Prepare a Negative control that hadn't undergone a reverse transcription reaction simultaneously and verify whether the PCR amplification is derived from the RNA.

9. Trouble shooting

Refer to the following countermeasures should any troubles occur.

[1] Low RNA yield*1

Cause	Remedy
Decomposition process with Lysis & Binding Solution inadequate	When dissolving the sample with dissolving-absorbing solution, mix by pipetting until the solution's viscosity completely diminishes.
Time left in Lysis & Binding Solution short	After suspending the sample in dissolving-absorbing solution, incubate at room temperature for 15 minutes or over
Excess sample	If having treated an excessive sample amount, the particles will coagulate and the yield point will be reduced. Make another examination with a lower sample amount. Proceed very carefully if performing automatic extraction with the MFX-2100.
Excess centrifuge	If extracting insoluble matter such as a tissue sample, centrifuging for an extended period at 5000 r.p.m. or above may make the RNA coprecipitate with foreign substances.
Insufficient reagent set	When performing automatic extraction with the MFX-2100, if the amount of reagents set is insufficient, the extraction may not proceed normally. If the RNA amount is extremely low, verify whether there remain any reagents in the extraction tube.

*1. The RNA yield point may depend greatly on the type of cell and tissue used.

[2] Low $A_{260/280}$

Cause	Remedy
Surplus sample	Lower sample amount and examine.
RNA concentration	If the RNA concentration is low when measuring light absorption, the $A_{260/280}$ value may be low.
RNA diluted solution	Compared with diluting with a buffer, measuring after diluting RNA with distilled water may show a low $A_{260/280}$ value.

[3] The RT-PCR is not proceeding well

Cause	Remedy
Genome DNA is Contaminated	Verify whether any genome DNA *2 Genome DNA *2 may be introduced through the sample. Treat the solution according to the DNase I treatment protocol described on page 9. When performing an RT-PCR, it is recommended to also verify whether there are bands derived from genome DNA that underwent a PCR in the samples that haven't undergone a reverse transcription.
RNA decomposition	Refer to [4]

*2. For colon bacilli, genome DNA contamination may be verified by electrophoresis.

[4] RNA decomposition

Cause	Remedy
RNA heat treatment	The RNA may decompose if heated for an extended period in a reacting buffer. When heating for an extended period, use a buffer with an RNase inhibitor added. Do not exceed 70°C when heating.
DNase I treatment conditions	Depending on the DNase I treatment conditions, the RNA may decompose. It is recommended to proceed following the protocol for the DNase I treatment conditions on page 9. There is also a protocol that renders DNase I deactivated by the application of heat. However, by heat treating at 80°C or above in a DNase I buffer, RNA decomposition may be accelerated.
Surplus sample	In an RNA solution extracted using an excessive sample amount, there may be some RNase remaining in the solution. Lower the sample amount and re-examine.

[5] Magnetic beads coagulate and do not loosen

Cause	Remedy
Excess sample	If having treated a sample in surplus, the particles will coagulate and the yield point may be low. Re-examine with a lowered sample mount.
Dissolution treatment using Lysis & Binding Solution inadequate	When dissolving the sample with Lysis & Binding Solution, mix with a pipette until the solution's viscosity diminishes.
Time left in Lysis & Binding Solution short	After having suspended the solution sample in the Lysis & Binding Solution, incubate at room temperature for 15 minutes or over.

[6] The collected RNA solution is tinted

Cause	Remedy
Hemoglobin and chlorophyll remaining	If having used a sample with a large amount of hemoglobin and chlorophyll, the collected solution may be tinted lightly. Lower the sample amount and examine. For the collection of samples from animal organs, avoid the introduction of blood constituents. Tinting can be eliminated by: (DNase I treatment (0°C) → phenol, chloroform treatment → isopropanol precipitate
Magnetic beads mixed in	Having magnetic beads mixed into the collected solution may cause it to have a brownish tint. Magnetic beads do not inhibit enzymatic reactions, therefore, centrifuge lightly and extract.

6. References

1) Vogelstein, B., and Gillespie, D. (1979) Preparative and analytical purification of DNA from agarose. *Proc. Natl. Acad. Sci.* **76**: 615-619.
2) Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wertheim-van Dillen, P.M.E., and van der Noordaa, J. (1990) Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* **28**: 495-503.

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