

CONTENTS

Introduction	3
Frequently Asked Questions	4
Kit Contents	5
Materials and Equipment Needed	5
Standard Protocol	6
Short Protocol	9
Downstream Applications	10

Storage: Upon receipt, store the kit at 2-8°C.

Shelf Life

Storage options	Shelf life
Kits stored at 2-8°C	6 months
Enzyme Mix stored at -20°C All other components stored at 2-8°C	1 year

Limited Product Warranty

It is imperative that the users strictly adhere to this manual. Failure to do so will void TrimGen's guarantee of this product. TrimGen Corporation makes no other warranties of any kind, expressed or implied, including without limitation, warranties of merchantability or fitness for a particular purpose.

Notice to Purchaser

The product is provided as "Research Use Only, Not for use in diagnostic procedures". The purchaser must determine the suitability of the product for their particular use.

The purchase of WaxFree™ RNA products includes a limited, nonexclusive license to use the kit and systems. This license does not grant rights to use the kit and systems for reproduction of the WaxFree™ RNA kit and systems, to modify the WaxFree™ RNA kit and systems for resale, or to use the WaxFree™ RNA kit and systems to manufacture commercial products without written approval of TrimGen Corporation. No other license, expressed, implied or by estoppel is granted.

Product Safety and Liabilities

When working with the kit reagents, always wear a suitable lab coat, disposable gloves, and protective goggles. TrimGen Corporation shall not be liable for any direct, indirect, consequential or incidental damages arising out of the misuse, the results of use, or the inability to use this product.

Introduction

WaxFree™ RNA kit is designed to extract RNA from

1. Formalin fixed, paraffin-embedded (FFPE) tissue
2. Fine needle aspiration (FNA) samples
3. Fresh or frozen tissue
4. Cells

WaxFree™ RNA kit is a homogenous extraction method, which eliminates RNA loss inherent in traditional column, beads and phenol-chloroform extraction methods. The R-resin and the enzyme mix are optimized to maximally release RNA from tissues to increase the yield of RNA. The extracted RNA from one paraffin section (size 1 x 1cm, 10µm thickness) is sufficient to perform up to 15 RT-PCR reactions.

The kit uses Q-Solution, a non-toxic solution to efficiently remove paraffin and formalin residual from tissue.

The WaxFree™ RNA kit can be used with the **Standard Protocol** or **Short Protocol**. The applications for each protocol are listed in the table below:

Tissue Type	Standard Protocol	Short Protocol
FFPE Slides	✓	✓
FFPE sections in tube	✓ Recommended	✗
Tissue dissected from paraffin slide or block	✗	✓
Fine needle aspiration (FNA) sample	✗	✓
Fresh or frozen tissues	✗	✓
Culture cells	✗	✓

Frequently Asked Questions

Q: Can I use the kit to extract RNA from old paraffin sample?

A: Yes. We successfully extracted RNA from 10 year old paraffin sample and the RT-PCR results were excellent.

Q: Can I use the kit to extract DNA?

A: Yes. The final extract includes RNA and DNA from tissue.

Q: Can I amplify 300bp product from the RNA sample?

A: No. The RNA in the paraffin sample is already partially degraded during formalin fixation. The average size of RNA is about 100-120 bp.

Q: Can I design the PCR primers in same exon?

A: No. If the PCR primers are located in same exon, it could amplify the genomic DNA instead of RNA because most RNA extraction methods have DNA contamination. To avoid the artificial DNA amplification, you should design the forward primer in one exon and the reverse primer in next exon.

Kit Contents

The kit provides extraction reagents for 50 samples.

Component	Quantity
Q-Solution	50 ml
Wash Buffer	60 ml
Enzyme Mix	0.5 ml
R-Resin	7.5 ml
WR-Filter	50

V/2 90522

Materials and Equipment Needed

2ml sterile screw-cap microcentrifuge tubes

Laboratory incubator

Heat Block (1-2)

Vortex Mixer

Microcentrifuge

70% Ethanol (optional for Standard Protocol ONLY)

RNase-Free DNase I is not included in the kit. The enzyme can be purchased separately from TrimGen (Cat. No. DE-50), or from other vendors.

Standard Protocol

Pre-heat a heat block or incubator to 45°C. Set another heat block to 90°C.

1. Collect 1.5 or 2 ml tubes (screw cap) and label the tubes with sample ID.

2. Collect sample

Paraffin section on slide: One section with tissue size 1-2 cm² and 5-20 μm thickness. Scrape the tissue from slide and transfer to a tube.

Paraffin section prepared in tube: directly go to step 3.

Paraffin tissue block: Trim away surrounding paraffin. Cut and transfer 10-30mg of tissue to a tube.

Fine needle aspiration sample: transfer entire sample to a tube.

For solid tissue with high cell density such as brain or liver, reduce the amount of tissue for extraction.

3. Add 0.8 ml of **Q-Solution** to each tube.
4. Screw cap on and vortex for 30 seconds at high speed.
5. Incubate the tube at 45°C for 20 minutes.
6. Vortex the tube 30 seconds at high speed.
7. Centrifuge the tube at 10,000 x g (about 12,000-14,000 rpm in most tabletop centrifuge) for 10 minutes.
8. Discard the supernatant using a pipettor or by aspiration. Be careful not to disturb the tissue pellet.
9. Add 1 ml of **Wash Buffer** to the tube.
10. Screw cap on and vortex 10 seconds at high speed to re-suspend the pellet.

V/2 90522

- 11. Centrifuge the tube at 10,000 x g (about 12,000-14,000 rpm in most tabletop centrifuge) for 10 minutes.
- 12. Discard the supernatant using a pipettor or by aspiration. Be careful not to disturb the pellet.
- 13. Re-suspend **R-Resin** thoroughly by shaking the bottle several times. Transfer 120 µl (60 µl for small paraffin tissue or FAN sample) of the **R-Resin** to each tube.

The step 14 and 15 are designed for skin or muscle tissues only, for other type of tissues go to step 16.

- 14. Cap the tube and vortex 10 seconds at high speed.
- 15. Incubate the tube at 90°C for 10 minutes, then cool the tube to room temperature 20-25°C.

- 16. Add 7 µl (3.5 µl for small paraffin tissue or FAN sample) of **Enzyme Mix** to each tube. Cap the tube and mix the content by flicking the tube.

For multiple samples

Prepare pre-mixed **R-Resin** with **Enzyme Mix** using formula below:

R-Resin = Sample # x 120 (60 for small tissue) x 1.1

Enzyme Mix = Sample # x 7 (3.5 for small tissue) x 1.1

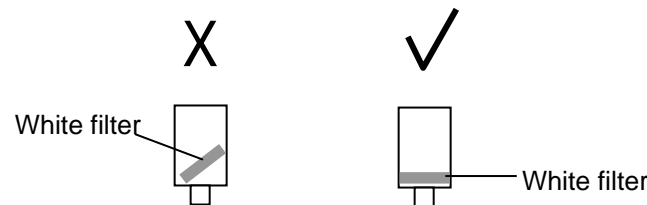
Transfer 127µl (63µl for small size tissue) of the mixture to each tube.

Return the Enzyme Mix to -20°C for storage.

- 17. Incubate the tube at 45°C for 1 hour.
- 18. Heat the tube at 90°C for 10 minutes.

V/2 90522

- 19. Place **WR-Filter** into a 1.5 ml tube and label the tube with sample ID. Make sure the white filter is at the bottom of the column (see below)



- 20. After incubation, transfer the entire extraction mix to **WR-filter**.
- 21. Centrifuge the tube at 1,000 x g (about 1500-3,000 rpm in most tabletop centrifuge) for 3 minutes.
- 22. **Discard the WR-Filter**. The solution in the tube is the final extract, which contains RNA ready for reverse transcription reaction or one-step RT-PCR.

Note:

Storage of RNA is not recommended even at – 80°C. The extracted RNA should be converted to cDNA after extraction. The cDNA can be stored at –20°C.

- 23. RNA Concentration Measurement: see page 11.

V/2 90522

Short Protocol

Pre-heat a heat block or incubator to 45°C. Set another heat block to 90°C.

1. Collect 1.5 or 2 ml tubes(screw cap) and label the tubes with the sample ID.

2. Collect sample

Paraffin section on slide: One section with tissue size 1-2 cm² and 5-20µm thicknesses. Scrape the tissue from slide and transfer to a tube.

Paraffin tissue block: Trim away surrounding paraffin. Cut and transfer 10-30 mg of tissue to a tube.

Fine needle aspiration sample: transfer entire sample to a tube.

Fresh or frozen tissues: Cut and transfer 10-30mg tissues to a tube.

Culture cells: Transfer 100 µl cell suspension (10³-10⁶ cells) to a 2 ml tube.

For solid tissue with high cell density such as brain or liver, reduce the amount of tissue for extraction.

Blank control:

If you are planning to measure the RNA concentration after the extraction, you need to set up a **Blank Control** tube. Add an extra tube to Step 2 and process it as a sample, but without adding any tissue. This tube is the **Blank Control** for OD_{260/280} calibration.

3. Re-suspend the **R-Resin** by thoroughly shaking the bottle several times. Then, transfer **120µl*** of the **R-Resin** to each tube (for small size tissue add 60µl, see table 1).

V/2 90522

4. Add **7 µl*** of **Enzyme Mix** to each tube. (for small size tissue add 3.5µl, see table 1).

***Table 1**

Tissues Size	R-Resin (µl)	Enzyme Mix (µl)
FFPE on slide 0.5-2 cm ²	120	7
FFPE on slide < 0.5 cm ²	60	3.5
FNA	60	3.5
Fresh tissue 10-30 mg	120	7
Fresh tissue < 10 mg	60	3.5
Cells > 10 ⁵ – 10 ⁸	120	7
Cell < 10 ⁵	60	3.5

V/2 90522

For multiple samples

Prepare pre-mixed **R-Resin** with **Enzyme Mix** using formula below:

$$\text{R-Resin} = \text{Sample \#} \times 120 \text{ (60 for small tissue)} \times 1.1$$

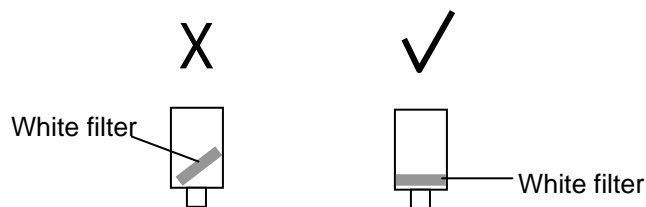
$$\text{Enzyme Mix} = \text{Sample \#} \times 7 \text{ (3.5 for small tissue)} \times 1.1$$

Transfer 127µl (63µl for small size tissue) of the mixture to each tube.

5. Screw cap on and mix the content by flicking the tube.
6. Incubate the tube at 45°C for 1 hour.
7. Heat the tube at 90°C for 10 minutes.

Return the Enzyme Mix to -20°C.

8. Place **WR-Filter** into 1.5 ml tubes and label the tubes with sample ID. Make sure the white filter is at the bottom of the column (see below):



V/2 90522

9. After incubation, transfer the extraction mix to **WR-filter**.
10. Place the **WR-Filter** in a new tube and centrifuge the tube at 1,000 x g (about 1,500-3,000 rpm in most tabletop centrifuge) for 3 minutes.
11. Discard the **WR-Filter**. The solution in the tube is the final extract, which contains RNA ready for reverse transcription reaction or one-step RT-PCR.

Note:

Storage of RNA is not recommended even at -80°C . The extracted RNA should be converted to cDNA after extraction. The cDNA can be stored at -20°C .

RNA Concentration Measurement: Measure by OD₂₆₀ method

Aliquot 5 μl of final extract to new tube. Dilute the final extract with 45 μl water.

Calibrate the UV spectrophotometer using the diluted **Blank Control** and adjust the OD₂₆₀ and OD₂₈₀ to zero to remove any background absorbance caused by the reagents. Then, measure the diluted samples at OD_{260/280} to calculate the RNA concentration.

The following equation can be used to determine the concentration of the extracted RNA.

A convenient calculation form is available online:

www.trimgen.com/wf/DNA-calculation.xls

$$\text{RNA Conc. (ng/}\mu\text{l)} = (62.9 \times \text{OD}_{260} - 36 \times \text{OD}_{280}) \times \text{dilution factor} / 0.5$$

RNA Quality

WaxFree™ is a homogeneous extraction method. The proteins from tissue remain in the final extracts and cause a reduction in the OD ratio. A typical OD_{260/280} ratio ranges from 0.8 to 1.3. The low OD ratio will not affect the reverse transcription or RT-PCR.

Measure by fluorescent method

The RNA concentration can be accurately measured by RiboGreen® method (Invitrogen, Cat. No. R-R-32700).

<http://probes.invitrogen.com/media/pis/mp32700.pdf>

V/2 90522

Downstream Applications

(Reagents are not included in this kit)

Reverse transcription

Use 10-25µl of the final extracts as template for total 50µl of reverse transcription reaction. The reverse transcribed cDNA can be stored at -20°C.

PCR amplification

The PCR enzyme and condition varies in different laboratories. Before starting routine operation, we recommend that the first time user perform a sample titration test to find a proper sample amount for your PCR amplification. As an example, use 5µl, 10µl, 15µl of RT products for 25µl PCR reactions to determine the best sample volume for PCR.

The forward and reverse PCR primers should be designed in different exons to prevent the artificial amplification of genomic DNA.

One-Step RT-PCR

Use 5 - 10 µl of the final extraction supernatant as a template for total 50 µl of One-step RT-PCR reaction.

Sample normalization for quantitative PCR

The OD 260 absorbance of final extraction can be used as a reference to normalize the difference between samples.

V/2 90522

Troubleshooting Guide

Problem	Suggestions
The removal of paraffin is incomplete	<p>Too much tissue sample WaxFree™ RNA standard protocol is optimized for a maximum of 3 FFPE sections (5-20 µm thick, up to a 3 cm² each).</p> <p>Trim off excess paraffin before extraction.</p> <p>For larger sections reduce the number of sections used for extraction</p> <p>Add more Q-solution for de-paraffinization.</p>
The final RNA extract has yellow or brown color, the RT-PCR does not work well	<p>The paraffin samples such as bone marrow, spleen, and liver contains high blood component. The hemoglobin in the sample is the cause of the color and also inhibits the RT or PCR reaction. It is necessary to remove these molecules by further purification using a spin column (TrimGen, Spin 50 Cat No. TC-50) or other commercial RNA purification kit.</p>
The OD _{260/280} ratio is below our QC criteria, can I use the extracted RNA for RT-PCR	<p>Yes. The RNA quality assessment is different from conventional extraction methods. A typical OD_{260/280} ratio for the WaxFree™ ranges from <u>0.6 to 1.5</u>. <u>The low OD ratio will not affect the RT-PCR reaction.</u> For special applications such as a microarray study, further purification may necessary.</p>
The OD ₂₆₀ is too high	<p>Need Blank Control tube Use the Blank control tube to calibrate the spectrophotometer, then measure</p>

V/2 90522

	<p>the OD.</p> <p>For larger size tissue sections, or solid tissues with high cell density, the final RNA extract needs to be diluted with water.</p>
<p>The RNA concentration calculated from the OD_{260/280} readings is low and the RT-PCR does not amplify properly</p>	<p>The RNA concentration is low Add more final extract to the RT-PCR reaction. However, the excess of final extract may inhibit the RT-PCR reaction. It is necessary to titrate the final extract for the RT-PCR reaction.</p> <p>Less RNA released For some tissues such as skin and muscle, the 1-hour enzyme digestion is not long enough to extract RNA efficiently from these tissues. Increasing the enzyme digestion time to 3 hours or overnight at 45°C will increase the yield of RNA.</p>
<p>The RNA concentration calculated from the OD_{260/280} readings is high and RT-PCR does not work.</p>	<p>RNA concentration is too high: Dilute the extraction supernatant with nuclease-free water then perform the RT-PCR.</p> <p>PCR amplicon size is too big The formalin fixation will damage RNA. The average size of RNA in the FFPE sample is about 100-150 bp. When the designed amplicon size is too big, the PCR may not work because the genome RNA in the FFPE tissue already broken. The RNA quality depends on the tissue type, storage time, and fixation conditions. Our customers have successfully amplified 150 bp PCR products from 10-year-old samples.</p>

V/2 90522

<p>Some of the R-resin are leaking to the collection tube.</p>	<p>Yes. You need to spin the collection tube at 10,000 x g to bring down the R-resin and then use the supernatant.</p>
<p>Can I use the final extract to perform the RT-PCR?</p>	<p>Check your centrifuge to reduce the spin speed to 1,000 x g.</p>

V/2 90522