Technical Manual

ReliaPrep™ FFPE Total RNA Miniprep System

INSTRUCTIONS FOR USE OF PRODUCTS Z1001 AND Z1002.

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ReliaPrep™ FFPE Total RNA Miniprep System

All technical literature is available on the Internet at: www.promega.com/protocols/ Please visit the web site to verify that you are using the most current version of this Technical Manual. Please contact Promega Technical Services if you have questions on use of this system. E-mail: techserv@promega.com

1. Description

Formalin fixation and paraffin embedding (FFPE) is a common method for archiving tissue specimens. The ability to extract RNA from these samples provides the potential for correlating disease state and tissue morphology with gene expression. Historically, extraction of RNA from FFPE tissues has been a challenge because the formalin fixation process cross-links proteins and nucleic acids and introduces modifications to RNA by addition of monomethyl groups to nucleotides (1,2). These modifications can inhibit subsequent analysis of RNA isolated from FFPE tissues.

The ReliaPrep™ FFPE Total RNA Miniprep System optimizes lysis conditions to reverse these RNA modifications without the need for overnight digestion. This system also uses a deparaffinization method that does not rely on xylene

or other organic solvents. By eliminating the washing steps involved in xylene deparaffinization, this method can help retain small RNA fragments.

Note: The cross-linking introduced by the formalin fixation and paraffin embedding process results in nucleic acids that are partially degraded. **The degree of fragmentation will vary depending on sample type, the age and storage conditions of the sample and the conditions used during formalin fixation.** When designing downstream amplification assays, best results will be achieved when targeting regions of 200 nucleotides or less (1,2).

2. Product Components and Storage Conditions

Product	Size	Cat.#
ReliaPrep™ FFPE Total RNA Miniprep System	10 reactions	Z1001

Contains sufficient reagents to perform total RNA isolations from 10 FFPE samples. Includes:

- 5ml Mineral Oil
- 1ml Lysis Buffer (LBA)
- 250µl Proteinase K Solution (PK)
- 250µl $MnCl_2$ (0.09M)
- 3.25ml BL Buffer
- 3ml Wash Solution
- 1 vial DNase I (lyophilized)
- 70µl DNase Buffer
- 1.25ml Nuclease-Free Water
- 1 package Collection Tubes (10 tubes/package)
- 1 package ReliaPrep™ FFPE Binding Columns (10 columns/package)
- 2 packages Elution Tubes (5 tubes/package)

Product	Size	Cat.#
ReliaPrep™ FFPE Total RNA Miniprep System	100 reactions	Z1002

Contains sufficient reagents to perform total RNA isolations from 100 FFPE samples. Includes:

- 50ml Mineral Oil
- 20ml Lysis Buffer (LBA)
- 1.1ml Proteinase K Solution (PK)
- 2 × 750µl $MnCl_2$ (0.09M)
- 32.5ml BL Buffer
- 30ml Wash Solution
- 4 vials DNase I (lyophilized)
- 700µl DNase Buffer
- 13ml Nuclease-Free Water
- 2 packages Collection Tubes (50 tubes/package)
- 2 packages ReliaPrep™ FFPE Binding Columns (50 columns/package)
- 2 packages Elution Tubes (5 tubes/package)

Storage Conditions: Store at room temperature.

3. General Considerations

3.A. Avoiding Introduction of Contaminating RNases

- Wear gloves at all times during the extraction procedure.
- Use sterile, disposable tubes and aerosol-resistant pipette tips.

3.B. Minimizing Genomic DNA Carryover

In tissues with high genomic DNA content, the eluted RNA may still contain low levels of genomic DNA. The presence of genomic DNA may interfere with some downstream RNA assays (for example, amplification assays that are designed within a single exon). For downstream applications where it is critical that the RNA be free of contaminating genomic DNA, reducing the amount of starting tissue can help reduce the possibility of genomic DNA carryover.

To test for the presence of contaminating genomic DNA in the purified RNA, a control qRT-PCR lacking the reverse transcriptase may be performed. If the DNase treatment of the RNA was effective, then no real-time amplification product should be observed.

When possible, we recommend designing downstream amplification assays in which the gene-specific primers span an intron to avoid amplification of genomic DNA. Alternatively, the user may wish to incorporate a post-elution DNase treatment step using RQ1 RNase-Free DNase (Cat.# M6101).

4. Preparation of Solutions

1X Wash Solution

10 reaction size: Add 12ml of 95–100% ethanol to the bottle containing 3ml of concentrated Wash Solution.

100 reaction size: Add 120ml of 95–100% ethanol to the bottle containing 30ml of concentrated Wash Solution.

Note: After adding ethanol, mark on the bottle that you have performed this step. This reagent is stable at 22–25°C when capped tightly.

DNase I

For all prep sizes: Add Nuclease-Free Water (supplied) in the amount indicated on the DNase I vial to each vial of lyophilized DNase I.

Note: The DNase I enzyme is sensitive to physical inactivation. Gently mix by swirling the vial of solution; **do not** vortex. We recommend dispensing the rehydrated DNase into working aliquots using sterile, RNase-free microcentrifuge tubes.

Note: Store reconstituted DNase I at –20°C.

5. Preparation of FFPE Sections

Materials to Be Supplied by the User

- ClickFit Microtube, 1.5ml (Cat.# V4741) or other 1.5 or 2ml microcentrifuge tube

Use of the ClickFit Microtube, 1.5ml, is recommended to prevent inadvertent opening of caps during heated incubation.

1. Using a sterile blade, trim excess paraffin off the tissue block.
2. Cut 5–50µm sections from FFPE blocks using a microtome.

   Note: If you are extracting RNA from tissue sections that have been applied to microscope slides, use a sterile blade to scrape the sections from the slide.
3. Place the sections in a 1.5 or 2ml microcentrifuge tube (not provided). The equivalent of ≤100µm of tissue slices may be processed per reaction.

   Note: In tissues with high gDNA content, reducing the amount of tissue in the extraction can help reduce the possibility of gDNA carryover.
4. Proceed immediately to Section 6, RNA Isolation.

6. RNA Isolation

Materials to Be Supplied by the User

- 95–100% ethanol
- 100% isopropanol
- 80°C heat block
- 56°C heat block

Note: We recommend deparaffinization of FFPE samples using mineral oil (provided). For other methods of deparaffinization, refer to Section 9, Alternative Methods of Deparaffinization.

6.A. Deparaffinization Using Mineral Oil

1. Add mineral oil to the sample:
   - For sections ≤ 50 microns, add 300µl of mineral oil
   - For sections ≥ 50 microns, add 500µl of mineral oil
2. Incubate at 80°C for 1 minute.
3. Vortex to mix.

6.B. Sample Lysis

1. Add 100µl of Lysis Buffer to the sample.
2. Spin at 10,000 × *g* for 15 seconds at room temperature. Two phases will be formed, a lower (aqueous) phase and an upper (oil) phase.
3. Add 10µl Proteinase K directly to the lower phase; mix the lower phase by pipetting.
4. Incubate at 56°C for 15 minutes.
5. Incubate at 80°C for 15 minutes.
6. Remove the tubes from the 80°C heat block, and place them on ice for 1 minute to cool, then place tubes at room temperature for 2 minutes.

6.C. DNase Treatment

While the samples are equilibrating to room temperature, prepare the DNase treatment mix.

1. For each isolation to be performed, combine the following ingredients:

$MnCl_2$, 0.09M	13µl
DNase Buffer	7µl
DNase I enzyme	10µl

Notes:

- Prepare only the amount of DNase treatment mix required.
- Thaw and keep the DNase I enzyme on ice during use.
- The DNase I enzyme is sensitive to physical inactivation. Mix by gentle pipetting. Do **not** vortex.
- Prepare the DNase treatment mix immediately before use. The components of the DNase treatment mix should be stored separately and mixed fresh for each set of RNA extractions.

2. Add 30µl of freshly prepared DNase treatment mix directly to the lower phase of the sample; mix by gently pipetting.
3. Incubate for 15 minutes at room temperature (20–25°C).

6.D. Nucleic Acid Binding

1. Add 325µl of BL Buffer to the sample.
2. Add 200µl of 100% isopropanol.
3. Vortex briefly to mix.
4. Spin at 10,000 × *g* for 15 seconds at room temperature. Two phases will be formed, a lower (aqueous) phase and an upper (oil) phase.
5. For each sample to be processed, place a Binding Column into one of the Collection Tubes provided.

   Note: Wear gloves when handling the columns and tubes.
6. Transfer the entire lower (aqueous) phase of the sample to the Binding Column/Collection Tube assembly. Cap the column. Discard the mineral oil (upper phase).

   Note: The mineral oil is inert and will not interfere with extraction if some of the oil phase is carried over to the Binding Column.
7. Spin the assembly at 10,000 × *g* for 30 seconds at room temperature.
8. Discard the flowthrough. Reinsert the Binding Column into the Collection Tube.
9. Proceed immediately to Section 7, Column Washing and Elution.

7. Column Washing and Elution

1. Add 500µl of 1X Wash Solution (with ethanol added) to the Binding Column. Cap the column.
2. Spin at 10,000 × *g* for 30 seconds at room temperature.
3. Discard the flowthrough, and reinsert the Binding Column into the same Collection Tube used in Section 6.D, Nucleic Acid Binding.
4. Add 500µl of 1X Wash Solution (with ethanol added) to the Binding Column. Cap the column.
5. Spin at 10,000 × *g* for 30 seconds at room temperature.
6. Discard the flowthrough, and reinsert the Binding Column into the Collection Tube used for the Nucleic Acid Binding (Section 6.D).
7. Close the cap. Spin the Binding Column/ Collection Tube assembly at 16,000 × *g* for 3 minutes to dry the column.

   Note: It is important to dry the column to prevent carryover of ethanol to the eluate.
8. Transfer the Binding Column to a clean Elution Tube (provided).
9. Add 30–50µl of Nuclease-Free Water to the column and cap the column.
10. Spin at 16,000 × *g* for 1 minute at room temperature. Remove and discard the Binding Column.
11. Cap the Elution Tube, and store the eluted RNA at –20°C or –70°C.

8. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Partially dissolved paraffin	Large amounts of paraffin may require additional mineral oil to dissolve completely. Prior to deparaffinization, use a sterile blade to trim away excess paraffin that does not contain tissue.
Low RNA yield	RNA yield may vary depending on tissue type; some tissues may not contain large quantities of intact total RNA. Consider increasing the amount of starting material that is processed.
	RNA yield may vary depending on sample type, the age and storage conditions of the sample, and the conditions used during formalin fixation. This method of nucleic acid extraction has not been tested with tissues fixed by methods other than formalin.
	Ethanol was not added to the Wash Solution prior to use. Confirm that ethanol was added to the Wash Solution; record addition on the bottle label.
RNA appears degraded	The formalin fixation and paraffin embedding process results in RNA that is partially degraded. When designing downstream amplification assays, best results will be achieved when targeting regions of 200 nucleotides or less.
	Isolated RNA is susceptible to degradation by ribonucleases. To avoid introducing contaminating RNases, wear gloves at all times during the extraction procedure and use sterile disposable, tubes and aerosol-resistant pipette tips.
Downstream amplification reactions appear inhibited	Ethanol carryover in the eluted RNA sample can inhibit downstream enzymatic reactions. Prior to the final RNA elution step, confirm that the Binding Column is dried by spinning at maximum speed for 3 minutes. Dry the column with the cap open.

8. Troubleshooting (continued)

Symptoms	Causes and Comments
Downstream amplification reactions appear inhibited (continued)	Some RNA assays may be inhibited by the presence of genomic DNA. For downstream applications where it is critical for the RNA to be free of contaminating genomic DNA, incorporate a post-elution DNase treatment step using RQ1 RNase-Free DNase (Cat.# M6101).
Genomic DNA carryover	The DNase I enzyme is sensitive to physical inactivation. Resuspend and store the lyophilized DNase according to directions. Do not freeze-thaw the DNase more than three times after it has been rehydrated.
	The DNase treatment mix must be prepared fresh for each set of RNA isolations. Do not store the DNase treatment mix. Do not vortex the DNase treatment mix.
	In tissues with high genomic DNA content, the eluted RNA may still contain low levels of genomic DNA. For downstream applications, where it is critical that the RNA be free of contaminating genomic DNA, reducing the amount of starting tissue in the extraction may help reduce the possibility of genomic DNA carryover. Alternatively, we recommend incorporating a post-elution DNase treatment step using RQ1 RNase-Free DNase (Cat.# M6101).
The lysate has not passed completely through the Binding Column after centrifugation	Large amounts of tissue can result in concentrated lysates. If the lysate has not passed completely through the Binding Column after centrifugation, repeat the centrifugation step, increasing the speed to 16,000 × *g*. Overloading the column can result in reduced yield; consider reducing the amount of starting material.

9. Alternative Methods of Deparaffinization

9.A. RNA Isolation with Deparaffinization Using Xylene

Materials to Be Supplied By the User

- 95–100% ethanol
- 100% isopropanol
- 100% xylene
- 37°C controlled-temperature heat block
- 56°C controlled-temperature heat block
- 80°C controlled-temperature heat block
- ClickFit Microtube, 1.5ml (optional; Cat.# V4741)

Xylene is a hazardous chemical.

Use of the ClickFit Microtube, 1.5ml, is recommended to prevent inadvertent opening of caps during heated incubation.

Deparaffinization

1. Add 1ml of 100% xylene to the sample.
2. Vortex to mix.
3. Spin for 2 minutes at maximum speed.
4. Remove the xylene without disturbing the pellet.
5. Add 1ml of 95–100% ethanol.
6. Vortex to mix.
7. Spin for 2 minutes at maximum speed.
8. Remove the supernatant without disturbing the pellet.
9. Centrifuge for 30 seconds at maximum speed to collect remaining drops of ethanol; remove as much residual ethanol as possible without disturbing the pellet using a fine pipette tip.
10. Dry pellet for 5–15 minutes at 37°C to evaporate residual ethanol.

Lysis

1. Resuspend the pellet in 100µl of Lysis Buffer.
2. Add 10µl of Proteinase K to the sample; vortex briefly to mix.
3. Incubate at 56°C for 15 minutes.
4. Incubate at 80°C for 15 minutes.
5. Remove the tubes from the 80°C heat block, and place them on ice for one minute to cool, then place tubes at room temperature.

9.A. RNA Isolation with Deparaffinization Using Xylene (continued)

DNase Treatment

While the samples are equilibrating to room temperature, prepare the DNase treatment mix.

1. For each isolation to be performed, combine the following ingredients:

$MnCl_2$, 0.09M	13µl
DNase Buffer	7µl
DNase I enzyme	10µl

Notes:

- Prepare only the amount of DNase treatment mix required.
- Thaw and keep the DNase I enzyme on ice during use.
- The DNase I enzyme is sensitive to physical inactivation. Mix by gentle pipetting. Do **not** vortex.
- Prepare the DNase treatment mix immediately before use. The components of the DNase treatment mix should be stored separately and mixed fresh for each set of RNA extractions.

2. Add 30µl of freshly prepared DNase treatment mix directly to the lower phase of the sample; mix by gently pipetting.
3. Incubate for 15 minutes at room temperature (20–25°C).

Nucleic Acid Binding

1. Add 325µl of BL Buffer to the lysed sample.
2. Add 200µl of 100% isopropanol.
3. Vortex briefly to mix.
4. For each sample to be processed, place a Binding Column into one of the Collection Tubes provided. Wear gloves when handling the columns and tubes.
5. Transfer the entire sample to the Binding Column/Collection Tube assembly. Cap the column.
6. Spin the assembly at 10,000 × *g* for 30 seconds at room temperature.
7. Discard the flowthrough, and reinsert the Binding Column into the same Collection Tube.
8. Proceed immediately to Section 7, Column Washing and Elution.

9.B. RNA Isolation without Deparaffinization

Materials to Be Supplied By the User

- 80°C heat block
- 56°C heat block
- 100% isopropanol
- ClickFit Microtube, 1.5ml (optional; Cat.# V4741)

Use of the ClickFit Microtube, 1.5ml, is recommended to prevent inadvertent opening of caps during heated incubation.

Sample Lysis

1. Add 100µl of Lysis Buffer to the sample.
2. Incubate at 80°C for 1 minute.
3. Vortex to mix, then spin at maximum speed for 1 minute.
4. Add 10µl of Proteinase K directly to the aqueous portion of the sample; mix by pipetting.
5. Incubate at 56°C for 15 minutes.
6. Incubate at 80°C for 15 minutes.
7. Remove the tubes from the 80°C heat block, and place them on ice for 1 minute to cool. Then place tubes at room temperature.

DNase Treatment

While the samples are equilibrating to room temperature, prepare the DNase treatment mix.

1. For each isolation to be performed, combine the following ingredients:

$MnCl_2$, 0.09M	13µl
DNase Buffer	7µl
DNase I enzyme	10µl

Notes:

- Prepare only the amount of DNase treatment mix required.
- Thaw and keep the DNase I enzyme on ice during use.
- The DNase I enzyme is sensitive to physical inactivation. Mix by gentle pipetting. Do **not** vortex.
- Prepare the DNase treatment mix immediately before use. The components of the DNase treatment mix should be stored separately and mixed fresh for each set of RNA extractions.

2. Add 30µl of freshly prepared DNase treatment mix directly to the lower phase of the sample; mix by gently pipetting.
3. Incubate for 15 minutes at room temperature (20–25°C).

Nucleic Acid Binding

1. Add 325µl of BL Buffer to the lysed sample.
2. Add 200µl of 100% isopropanol.
3. Vortex briefly to mix.
4. For each sample to be processed, place a Binding Column into one of the Collection Tubes provided. Wear gloves when handling the columns and tubes.
5. Transfer the entire aqueous portion of the sample to the Binding Column/Collection Tube assembly. Cap the column.
6. Spin the assembly at 10,000 × *g* for 30 seconds.
7. Discard the flowthrough, and re-insert the Binding Column into the same Collection Tube.
8. Proceed immediately to Section 7, Column Washing and Elution.

10. References

1. Lewis, F. *et al*. (2001) Unlocking the archive—gene expression in paraffin-embedded tissue. *J. Pathol.* **195**, 66–71.
2. Farragher, S.M. *et al*. (2008) RNA expression analysis from formalin fixed paraffin embedded tissues. *Histochem. Cell Biol*. **130**, 435–45.

11. Related Products

DNA Purification Systems

Product	Size	Cat.#
ReliaPrep™ FFPE gDNA Miniprep System*	100 reactions	A2352
ReliaPrep™ gDNA Tissue Miniprep System*	100 preps	A2051
ReliaPrep™ Large Volume HT gDNA Isolation System	96 × 10ml preps	A1751
ReliaPrep™ Blood gDNA Miniprep System*	100 preps	A5081
Maxwell® 16 FFPE Plus LEV DNA Purification Kit	48 preps	AS1135

*Additional Sizes Available.

Biochemical Reagents and Labware

Product	Size	Cat.#
Mineral Oil	12ml	DY1151
Nuclease-Free Water*	50ml (2 × 25ml)	P1193
RQ1 RNase-Free DNase	1,000u	M6101
Recombinant RNasin® Ribonuclease Inhibitor*	2,500u	N2511
ClickFit Microtube, 1.5ml	1,000/pack	V4741

*Additional Sizes Available.

PCR Reagents

Product	Size	Cat.#
GoTaq® qPCR Master Mix	200 reactions	A6001
GoTaq® Hot Start Polymerase	100u	M5001
GoTaq® Hot Start Green Master Mix	100 reactions	M5122

Additional Sizes Available.

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