MELT™ Total Nucleic Acid Isolation System

(Part Number AM1983)

Protocol

P/N 1983M Revision D **Revision Date: October 27, 2008**

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I. Introduction

A. Product Description

The MELT™ Total Nucleic Acid Isolation System includes a novel alternative to mechanical disruption of tissue as a front-end to RNA or DNA isolation. A critical first step in any nucleic acid isolation protocol is to free the RNA or DNA from the cellular architecture. For tissue samples, this is almost always accomplished by mechanical disruption. Physical disruption of tissue samples, however, is cumbersome, limited to low throughput, and potentially hazardous because of operator exposure to aerosols from the open tube. The MELT Total Nucleic Acid Isolation System is simpler, easier, and safer, and is amenable to high throughput tissue processing without the need for tedious homogenization. As a result, many samples can be processed more quickly while also minimizing cross-contamination. The MELT Total Nucleic Acid Isolation System was developed for RNA isolation, but with minor procedure modifications, DNA can also be recovered from MELT lysates (this is outlined in section *V.A. DNA Purification from MELT Lysates* starting on page 27).

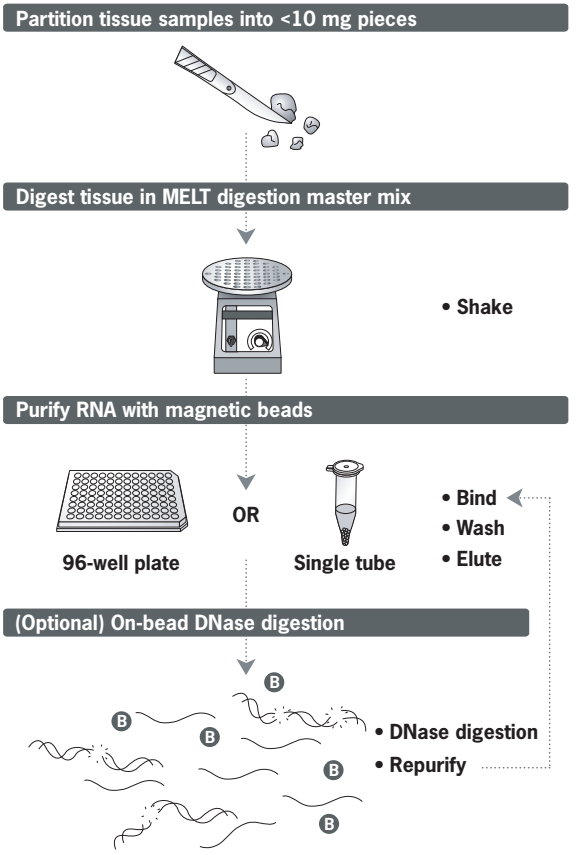
The MELT System uses a unique, hands-free, closed-tube enzymatic digestion to process up to 10 mg of fresh or frozen tissue per sample. Unlike chaotropes such as guanidine, MELT reagents irreversibly destroy RNases, and thus MELT lysates can be safely stored for up to one week at ambient temperatures without compromising RNA quality or yield. This creates opportunities for convenient storage and shipment of MELT lysates. The RNA isolation strategy uses the streamlined MagMAX™ magnetic bead-based technology that is equally amenable to manual and automated processing.

B. Overview of the Procedure

The MELT Total Nucleic Acid Isolation System is designed to purify total RNA from ≤10 mg samples of fresh or frozen tissue. The MELT System has been tested extensively with the tissues listed in Figure 2 on page 3 and is recommended for use with most animal tissues that do not contain comparatively high levels of RNases and that are not extremely hard or fibrous. It is not compatible with adipose tissue or tissues that have been stored in Ambion® RNA*later*® or RNA*later*®-ICE solutions.

Instead of using mechanical tissue disruption, the MELT System procedure (Figure 1 on page 2) employs a cocktail of proteases, an optimized buffer, and the Ambion Vortex Adapter-60 (P/N AM10014) to liquefy tissue samples in ~10 min in a closed-container system while maintaining RNA integrity and maximizing RNA yield (Figure 3 on page 3).

Figure 1. MELT™ Total Nucleic Acid Isolation System Procedure Overview



The resulting lysate is stable for at least 6 days at room temperature (Figure 6 on page 6), or it can be stored at –20°C or –80°C for up to one week.

Following the MELT digestion, Ambion MagMAX™ nucleic acid-binding magnetic beads are added to the lysate to capture RNA. The magnetic particles are drawn to a magnet, and cell debris and other contaminants are washed away. Reagents are supplied for an optional on-bead treatment with Ambion TURBO™ DNase to degrade any contaminating genomic DNA. After washing the RNA-bound beads to remove the TURBO DNase reagents and any small DNA fragments/nucleotides generated during the TURBO DNase digestion, the purified RNA is eluted in as little as 20 µL of Elution Buffer.

The entire procedure can be carried out in a single microcentrifuge tube, or the MELT digestion can be carried out in microcentrifuge tubes and the resulting lysates can be processed in a single 96-well plate. Total RNA isolated with the MELT Total Nucleic Acid Isolation System is suitable for common downstream applications such as qRT-PCR, Northern blot analysis, ribonuclease protection assay (RPA), and RNA amplification and microarray analysis (see Figures 4 and 5). Note that microRNA is not quantitatively recovered using the MELT System.

A video showing the MELT Total Nucleic Acid Isolation System in action can be viewed by following the link on the MELT System web catalog page:

www.ambion.com/catalog/CatNum.php?1983

Mouse Tissues	Average Total RNA Yield	Expected Digestion Time
Brain	4 µg	≤10 min
Liver	12 µg	≤10 min
Duodenum	8 µg	≤10 min
Jejunum	8 µg	≤10 min
Ileum	8 µg	≤10 min
Colon	6 µg	≤10 min
Ovary	7 µg	≤10 min
Thyroid	6 µg	≤10 min
Kidney	8 µg	12 min
Heart	4 µg	12 min
Thymus	12 µg	12 min

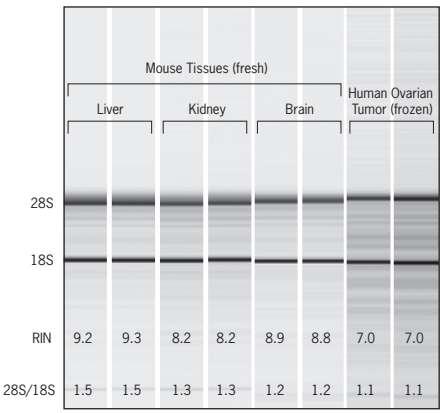


Figure 2. Average Total RNA Yield from 3–5 mg of Selected Mouse Tissues using the MELT™ System.

Average RNA yield from at least four experiments using 3–5 mg of the indicated tissues in the MELT Total Nucleic Acid Isolation System are shown. Yield was determined using A_{260} measurements with the NanoDrop® ND-1000A Spectrophotometer. Note that these are empirical data obtained using tissue samples that were handled rapidly to maximize RNA integrity and yield. RNA yield can vary from species to species, among developmental stages, and with tissue collection methods. For updated information: www.ambion.com/catalog/CatNum.php?1983

Figure 3. RNA Isolated from Fresh and Frozen Mouse and Human Tissues using the MELT™ System.

RNA was isolated from ~7 mg of the indicated tissues using the MELT Total Nucleic Acid Isolation System. Samples were subjected to on-bead DNase digestion. 1 µL of each RNA sample was analyzed on an RNA LabChip® using the Agilent® 2100 bioanalyzer. RIN values were determined using the Agilent 2100 expert software package (Agilent Technologies). The human ovary tumor sample was obtained through Ambion's network of certified tissue providers. Unlike the mouse tissues, we were unable to control the dissection and initial handling of this sample, which may have affected the quality of the purified RNA.

C. Product Applications

Compatible tissues

The MELT Total Nucleic Acid Isolation System is recommended for use with most mammalian tissues. It is not, however, recommended for the following sample types:

- Tissues that contain very high levels of ribonuclease, such as pancreas or spleen
- Extremely hard or fibrous tissues, such as bone or skin
- Adipose tissue

IMPORTANT

MELT Total Nucleic Acid Isolation System is not compatible with tissues that have been stored in RNAlater or RNAlater-ICE. For more information on choosing the ideal kit and reagents for your tissue and/or application, visit this resource page at: http://www.ambion.com/prod/isolation.

Gene expression analysis

The quality and purity of the RNA obtained with the MELT Total Nucleic Acid Isolation System makes it suitable for virtually any downstream application. The most popular of these applications are RT-PCR, and RNA amplification and labeling for microarray analyses.

Quantitative and endpoint RT-PCR

Real-time qRT-PCR is a powerful tool for measuring the abundance of mRNA transcripts. RNA isolated from fresh or flash-frozen tissues using the MELT System is appropriate for both qRT-PCR and endpoint RT-PCR.

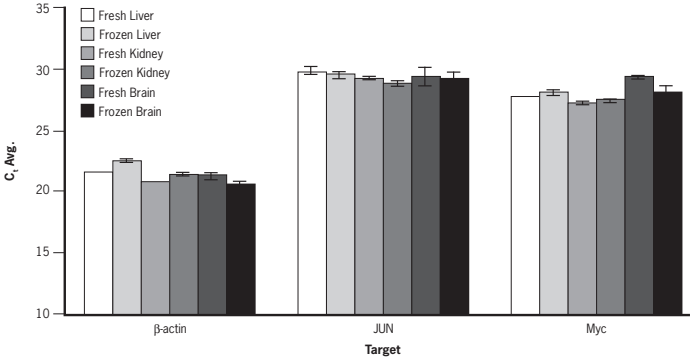


Figure 4. qRT-PCR of Total RNA Isolated with the MELT™ Total Nucleic Acid Isolation System

Total RNA (2 ng) was analyzed in real-time one-step qRT-PCR (10 µL) using MessageSensor™ RT conditions. Assays were performed on an Applied Biosystems 7900HT Real-Time PCR System (standard cycling conditions) with three different TaqMan® Gene Expression Assays. C_t values are represented as averaged triplicates.

The on-bead TURBO DNase digestion typically results in >10 C_T separation between experimental samples [RT-plus] and negative control [RT-minus] samples. The RNA can be further subjected to a more rigorous off-bead DNase digestion using the Ambion DNA-*free*™ Kit (P/N AM1906).

RNA amplification for array analysis

Gene expression profiling using gene arrays can simultaneously measure the expression levels of thousands of genes in a single experiment. RNA samples are typically prepared for array analysis by amplification and labeling in a T7 RNA Polymerase-driven process commonly called the Eberwine, IVT, aRNA, or cRNA method.

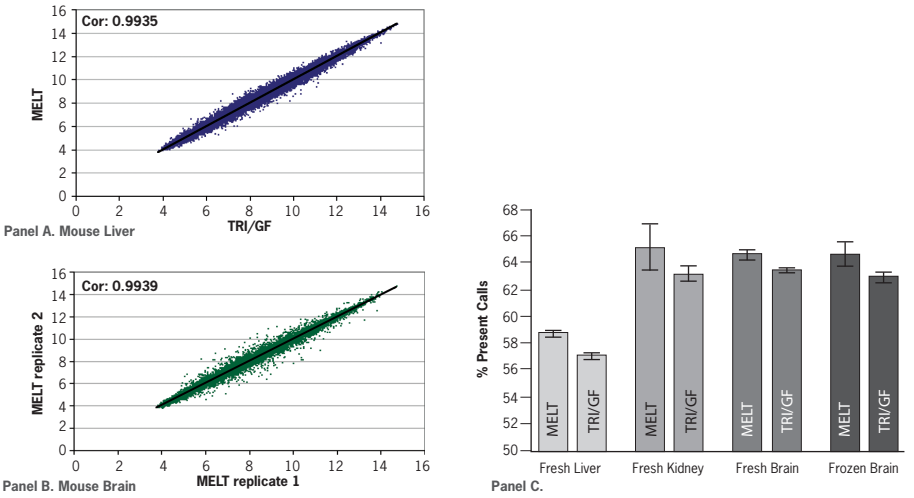


Figure 5. MELT™ Total Nucleic Acid Isolation System Performance on an Affymetrix® GeneChip® Array.

RNA was prepared from fresh and frozen mouse tissues using either the MELT Total Nucleic Acid Isolation System (MELT) or the method recommended in the Affymetrix® *Eukaryotic Sample Preparation Guide*. The Affymetrix recommended procedure consists of a front-end purification using TRI Reagent® followed by further purification with the Qiagen® glass-fiber filter-based RNeasy™ Micro Kit (TRI/GF). Total RNA (1 µg) was amplified using the Ambion MessageAmp™ II aRNA Amplification Kit. Fragmented aRNA was then hybridized to Mouse Genome 430A 2.0 arrays, and scanned with a GeneChip® Scanner 3000. Data were captured and analyzed on GeneChip Operating Software (Affymetrix). The results shown were normalized for array signal intensities. Biological replicates are represented for the two methods for fresh mouse liver and kidney tissues as well as fresh and frozen mouse brain tissue. Panel A shows the correlation between results from fresh mouse liver RNA obtained using the MELT and TRI/GF methods. Panel B shows the correlation within the MELT method with fresh mouse brain. Panel C represents the Percent Present calls obtained using either the MELT or TRI/GF RNA isolation procedures from the indicated fresh and frozen mouse tissues.

In Figure 5, microarrays hybridized with aRNA amplified from RNA isolated either with the MELT Total Nucleic Acid Isolation System or with the Affymetrix®-recommended RNA isolation procedure

(TRI Reagent® followed by glass-fiber filter purification) were compared. The two methods yielded comparable microarray results that were highly correlated by several key statistical measures.

RNA preservation

A major threat to RNA integrity in tissues is RNase activity. Eukaryotic RNases are a diverse group of catalysts; however, RNase A-type nucleases are the greatest threat to RNA integrity during processing of most tissues. MELT reagents digest cellular RNases to harmless peptide fragments that cannot reactivate. In contrast, the RNA in tissue lysates prepared using chaotropes such as guanidinium is rapidly degraded during room temperature storage.

In the absence of nucleases, cellular RNA can remain intact for long periods of time in tissue lysates. The MELT System produces tissue lysates that can be processed, stored for days, and readily transported at ambient temperatures for use in a range of downstream molecular analyses (Figure 6).

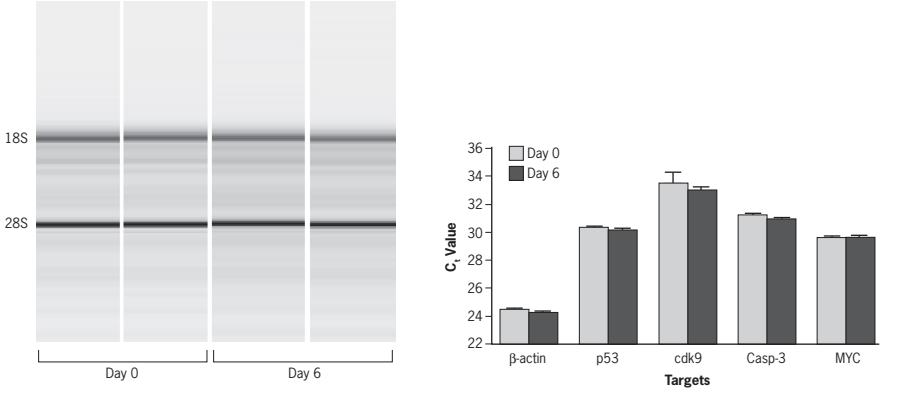


Figure 6. RNA Stability in MELT™ Lysates for up to 6 Days at Room Temp (22–25°C).

Total RNA was isolated from frozen mouse liver with the MELT Total Nucleic Acid Isolation System. RNA was purified from a fraction of the lysate immediately (Day 0), and the remaining lysate was left at room temperature (22–25°C) for 6 days (Day 6). On the left, Agilent® bioanalyzer data from duplicate RNA samples isolated on Day 0 and Day 6 are shown. The bands representing 28S and 18S rRNA show no significant deviation in RNA quality. On the right, data from qRT-PCR analysis with 5 TaqMan® Gene Expression Assays (Applied Biosystems) are shown. In this experiment, 2 ng of RNA isolated at Day 0 and Day 6 were compared; <0.5 C_t deviation between the two samples was seen indicating that RNA was preserved in the MELT lysate for 6 days at room temperature.

Other applications

High quality total RNA isolated from the MELT Total Nucleic Acid Isolation System is also suitable for Northern blot and ribonuclease protection assay (RPA) analysis. Ambion offers the NorthernMax® (P/N AM1940) and NorthernMax-Gly (P/N AM1946) Kits which contain a complete set of RNase-free reagents for conducting formaldehyde- or glyoxal-based Northern analysis, respectively. The RPA is an

extremely sensitive procedure for the detection and quantitation of individual RNA molecules in complex samples; Ambion provides the RPA III™ Kit (P/N AM1414, AM1415) to make RNA analysis simple and straightforward.

D. Kit Components and Storage Conditions

The MELT Total Nucleic Acid Isolation System contains reagents to isolate RNA from 50 samples.

Amount	Component	Storage
200 µL	MELT Cocktail	–20°C
5 mL	MELT Buffer	room temp*
500 µL	Binding Beads	4°C*
10 mL	Binding Solution Add 16 µL 14.3 M β-Mercaptoethanol per 1 mL Binding Solution just before use	4°C
25 mL	Wash Solution 1 Concentrate Add 8.25 mL 100% Ethanol before first use	room temp
60 mL	Wash Solution 2 Concentrate Add 48 mL 100% Ethanol before first use	room temp
1 ea	Processing Plate/Plate Seal	any temp†
100 µL	TURBO™ DNase (20 U/µL)	–20°C
5 mL	TURBO DNase Buffer	4°C or room temp
5 mL	Elution Buffer (for RNA elution only)	4°C or room temp
10 mL	Nuclease-free Water	any temp†
50 ea	Elution Tubes–0.5 mL RNase-free tubes	room temp

* Do not freeze these components.
† Store at –20°C, 4°C, or room temp.

E. Required Material Not Provided With the Kit

Reagents

- 14.3 M β-Mercaptoethanol (β-ME) for the Binding Solution
- 100% ethanol, ACS reagent grade or equivalent
- 80% ethanol, made with ACS reagent grade 100% ethanol or equivalent and nuclease-free water (not provided)
- 10% bleach solution for cleanup and decontamination after working with animal tissue samples
- RNase decontamination solution (e.g., RNase*Zap*®, P/N AM9780), to inactivate RNases on equipment that will come into contact with samples
- (Optional) 1X PBS (e.g., prepared from PBS 10X, pH 7.4, P/N AM9624), to keep fresh tissue samples moist during partitioning

- 1.5 mL non-stick RNase-free microcentrifuge tubes, (e.g., P/N AM12450)
- For the DNA isolation procedure (section V.A on page 27), the following reagents are also required:
  – 100% isopropanol, ACS or molecular biology grade
  – (Optional) RNase A, (e.g., P/N AM2270, AM2271)

Equipment

- The Ambion Vortex Adapter-60 (P/N AM10014) is needed to provide the specific shaking motion required for adequate sample digestion.
  The Vortex Adapter-60 includes a snap-on 6-inch platform adapter and two foam inserts, one to hold ≤60 microcentrifuge tubes, and the other to hold a single standard 96-well plate. It is compatible with the Vortex-Genie® 2 and 2T Mixers, and the VWR® Mini Vortexer.

IMPORTANT

After testing several vortex adapters for hands-free mixing, we concluded that the Vortex Adapter-60's flexible 6 inch platform provides a unique shaking motion that is critical for rapid and thorough tissue digestion using the MELT System. We strongly recommend using the Ambion Vortex Adapter-60 for tissue digestion with this kit.

- Scalpel and tissue forceps, or for frozen samples: dry ice, and (optional) liquid nitrogen and mortar and pestle
- Magnetic stand:
  – for 96-well plates, we recommend the Ambion Magnetic Stand-96 (P/N AM10027)
  – for RNA isolation in microcentrifuge tubes, we recommend the Ambion Six Place Magnetic Stand (P/N AM10055)
- Vacuum with fine pipettor tip attached, for rapid aspiration
- Nuclease-free tubes and tips
- Multichannel pipettor or high throughput processing equipment, for processing samples using the Processing Plate

For assessing RNA quantity and integrity

- Spectrophotometer, e.g. the NanoDrop® ND-1000A UV-Vis Spectrophotometer, or RiboGreen® RNA Quantitation Assay and Kit (Invitrogen Inc.) fluorescence-based RNA assay.
- Agilent® 2100 bioanalyzer and RNA LabChip® Kit.

F. Related Products Available from Applied Biosystems

Magnetic Stand-96 P/N AM10027	The Ambion Magnetic Stand-96 has powerful magnets positioned to capture beads to one side of the well. This capture pattern makes it very easy to remove supernatants manually without disturbing the beads, and therefore may be preferred by beginning users. In some applications, however, pellets formed with the Magnetic Stand-96 may be difficult to resuspend. If this occurs, we recommend the 96-well Magnetic-Ring Stand (P/N AM10050).
6 Tube Magnetic Stand P/N AM10055	Designed to hold up to six standard microcentrifuge tubes, the 6 Tube Magnetic Stand houses a strong magnet for fast, efficient separation of magnetic beads from solutions.

RNase*Zap*® P/N AM9780, AM9782, AM9784	RNase Decontamination Solution. RNase*Zap* is simply sprayed, poured, or wiped onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap*.
DNA-*free*™ P/N AM1906	DNase treatment and removal reagents. This product contains Ambion's ultra-high quality RNase-free DNase I and reaction buffer for degrading DNA. It is ideal for removing contaminating DNA from RNA preparations. A novel reagent for removing the DNase without the hassles or hazards of phenol extraction or alcohol precipitation is also included.
MessageSensor™ RT Kit P/N AM1745	The MessageSensor RT Kit for one-step qRT-PCR includes an optimized set of reagents for exceptionally sensitive reverse transcription. The kit is designed to be used for single-tube amplification of mRNA using either real-time or end-point amplification strategies.
MessageAmp™ aRNA Amplification Kits see our web or print catalog	Ambion offers a full line of MessageAmp Kits tailored for different array analysis applications. The MessageAmp II Kit offers maximum flexibility; samples can be amplified using either single- or double-round amplification, and the reagent cocktails are configured to accommodate modification. For arrays requiring biotin-labeled samples, Ambion offers the MessageAmp Premier and MessageAmp III RNA Amplification Kit. For preparation of fluorescently-labeled samples, we recommend the Amino Allyl MessageAmp II Kits which are available with and without Cy™3 and Cy5. Bacterial RNA can be amplified using the MessageAmp II Bacteria RNA Amplification Kit. We also offer the MessageAmp II-96 and Amino Allyl MessageAmp II-96 aRNA Amplification Kits for high throughput applications.

II. Set-up and Tissue Preparation

A. Planning and Reagent/Equipment Preparation

Planning the procedure

There are several options for the MELT procedure. We recommend that you decide which options you will incorporate into the procedure before starting.

Processing Plate method vs. single-tube method

We provide instructions for purifying RNA in either the 96-well Processing Plate provided with the kit, or using a separate microcentrifuge tube for each sample (single-tube method). For most experiments, we recommend using the Processing Plate method for RNA isolation. The advantages and disadvantages of each method are provided below.

Processing Plate Method:	Single-tube Method:
• Better for processing multiple samples	• Minimizes cross-sample contamination
• Streamlined and faster procedure	• Slightly more time-consuming
• Elute in 20 µL	• Elute in 40 µL

RNA Purification from half (50 µL) of the MELT lysate

If you plan to isolate RNA from only half of the lysate (50 µL) in order to reserve the other half for DNA isolation (procedure in section V.A starting on page 27) modify steps III.C.1 and III.C.2 on page 18 as follows:

- Step 1: Use only 50 µL Binding Solution and 50 µL of 100% ethanol per sample (use the same volume of Binding Beads).
- Step 2: Dispense 110 µL of the Binding Bead master mix per sample.

Deciding whether to include the on-bead TURBO DNase digestion

The on-bead TURBO DNase digestion (section III.D starting on page 20) is optional. We have found that in real-time PCR the on-bead TURBO DNase digestion will typically result in 1000-fold DNA removal of common housekeeping genes.

If your application does not require that DNA be removed, then you may want to skip the on-bead TURBO DNase digestion. Alternatively, if your application requires a more rigorous elimination of genomic DNA, consider skipping the on-bead TURBO DNase digestion and treating your samples using the Ambion® DNA-*free* Kit (P/N AM1906) after completing the MELT procedure.

Reagent preparation

Prepare Wash Solutions

1. Add 8.25 mL 100% ethanol (ACS reagent grade or equivalent) to the bottle labeled Wash Solution 1 Concentrate and mix well.
2. Add 48 mL ACS grade 100% ethanol (ACS reagent grade or equivalent) to the bottle labeled Wash Solution 2 Concentrate and mix well.
3. Mark the labels to indicate that the ethanol was added, cap the wash solution bottles tightly to prevent evaporation, and store the prepared wash solutions at room temperature.

The prepared solutions will be referred to as Wash Solution 1 and Wash Solution 2 in the procedure.

Equipment preparation

RNase decontamination

We recommend using RNase*Zap* Solution (P/N AM9780) to inactivate RNases on all equipment that will come into direct contact with samples. Allow treated equipment to dry before use.

6 Tube Magnetic Stand

For the MELT procedure, place the magnet in the tilted position inside the magnetic stand.

Vortex setup

Securely fasten the 6-inch platform adapter onto the vortex mixer. Before beginning the MELT procedure, become familiar with the slow and more vigorous mixing settings of the vortexer with the tube or plate holder in place.

- ***Determine the MELT digestion vortexer setting***
  The MELT digestion requires vigorous mixing using the tube holder. Attach the microcentrifuge tube holder to the vortex mixer via the platform adapter. Place 100 µL of MELT Buffer in a 1.5 mL microcentrifuge tube and seat it securely on the vortex mixer; use another tube containing 100 µL of water as a counterbalance. Set the vortexer to just below the maximum speed (6 to 7 for the Vortex-Genie 2 and 2T Mixers, and 7 to 8 for the VWR Mini Vortexer) and observe the motion of the assembly. Adjust the vortexer speed to agitate very rapidly without causing the tube contents to turn to foam, and without causing instability ("walking") of the vortexer on the lab bench. Note that some foam in the tube is expected; mixing too hard, however, will cause the entire 100 µL of MELT Buffer to become foam.
- ***Determine the TURBO DNase digestion vortexer setting***
  The on-bead TURBO DNase digestion requires gentle mixing. The setting most suitable for the on-bead TURBO DNase digestion step on the Vortex-Genie and the VWR Mini Vortexer is between 1 and 3. Load the microwell plate or microcentrifuge tube holder with a 96-well plate or a few microcentrifuge tubes containing 100 µL of water (depending on the reaction vessel you plan to use). Observe

the motion of the water with the mixer on setting #1 and gradually increase the speed to a maximum of setting #3 to identify a gentle but thorough mixing speed.

For frozen samples: dry ice

When working with frozen tissue samples larger than ~10 mg, have enough dry ice readily available in a closed container for breaking the tissue into ≤10 mg pieces.

Protective attire

It is good practice to wear appropriate lab safety attire, for example lab coat, eye covers, and double gloves when handling tissue samples.

B. Preparing and Handling Frozen Tissue for RNA Isolation

Keep samples at –80°C until you are ready to begin

It is imperative to maintain frozen tissue either at –80°C, for long term storage, or on dry ice for short periods of time, to allow for tissue handling and partitioning. RNA in tissue that has undergone a freeze/thaw cycle without protection by MELT reagents will be degraded. Since small masses of tissue (≤10 mg) can thaw very rapidly, it is particularly important to keep them at –80°C until they are ready for partitioning.

1–10 mg pieces (≤10 mg total)

For adequate enzymatic disruption, samples must be in fragments that are less than 10 mg. Note that a 10 mg tissue fragment is quite small—roughly the size of a 3 mm cube. Figure 7 below can be used to help estimate the weight of tissue fragments. Instructions and suggestions for partitioning tissue samples are provided below.

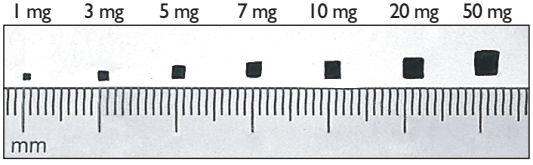


Figure 7. Estimating the Mass of Small Tissue Samples

Approximately 1–2 mm thick pieces of mouse liver tissue with the indicated weights were placed on a ruler and photographed. Most other soft tissues are of similar density.

When processing only a few samples, we recommend using 2–3 smaller pieces of tissue with a maximum combined weight of 10 mg, rather than a single ≤10 mg fragment in MELT reactions.

Recommendations for keeping samples frozen during partitioning

- Prepare a ***closed container*** (such as an ice chest) containing enough dry ice to create a cold vapor environment.
- To further safeguard against defrosting of small tissue fragments, prechill the container (such as a small tip box or a mortar and pestle decontaminated with RNase*Zap*) that will be used to hold the frozen tissue over dry ice during partitioning.
- Plan to use either a sharp scalpel with a handle and a nonflexible metal blade, or a mortar and pestle with liquid nitrogen, to fragment frozen tissue into 1–10 mg pieces. You will also need a pair of forceps manufactured with small 'grip' teeth near the point to grasp frozen tissue firmly. Decontaminate containers and tools with RNase*Zap*, and pre-chill in dry ice.

Instructions for partitioning tissue samples

There are two suggested strategies for breaking frozen tissue samples into ≤10 mg pieces: cutting the tissue with a scalpel, or freezing it in liquid nitrogen and breaking it by gently crushing in a mortar and pestle. We present detailed instructions for both of these methods below. The most important aspect of either procedure, however, is to work quickly and to keep frozen samples frozen ***completely***.

IMPORTANT

Tissue fragments should be smaller than ~3 mm cubes (10 mg) for processing. 1 mg tissue pieces are very effective in the MELT System, but there is a balance between partitioning samples into very small pieces and deterioration of RNA quality caused by over-handling the tissue.

Use a scalpel to chop tissue into ≤10 mg pieces

Working quickly in a prechilled container that is resting on dry ice in an ice chest, slice or carve the frozen tissue to 1–10 mg sized pieces (see Figure 7 as a guide). Partitioning frozen tissue into milligram pieces can be a challenge; we recommend the following technique to carve tissue effectively and rapidly. Grasp the frozen tissue firmly with chilled forceps. Position the scalpel tip on the surface of the pre-chilled container at a ~45 degree angle just in front of the tissue. Working at the edge of the tissue mass, press down firmly in a chopping motion without raising the tip of the scalpel, and chisel off a small fragment of tissue. Continue this chisel-like action until you have more tissue fragments than you need. Keep all the tissue fragments in the container resting on dry ice until you are ready to start the tissue digestion. (Keep the container closed when it is not in use.)

Break tissue by freezing in liquid nitrogen and gently crushing

Set up an ice chest with dry ice and a mortar and pestle that has been treated with RNase*Zap*. Fill the mortar with liquid nitrogen to a depth of 0.5–1 cm and drop in the tissue sample (tissue that was stored in foil can remain in the foil or can be removed from the foil). Allow the tissue to equilibrate to the temperature of the liquid nitrogen for several seconds, and add more liquid nitrogen if necessary so that the tissue is about half immersed. Next, gently press on the tissue with the pestle to partially crush it. The strategy should be to use the pestle to break the tissue into 1–10 mg pieces in the presence of liquid nitrogen, but ***not*** to

crush and grind it into a powder as is often done for RNA isolation. Keep the tissue fragments frozen in the container resting on dry ice until you are ready to start the tissue digestion.

Estimate the weight of tissue fragments

Weighing tissue is the most accurate way to quantify the amount of starting material, however, it is simply not practical for most experiments. Instead, use Figure 7 as a guideline to estimate appropriately-sized tissue fragments. Figure 2 on page 3 is also a helpful resource to provide an estimate of whether sufficient RNA can be recovered for downstream analyses with a single 10 mg sample. If more RNA is needed, isolate RNA using several MELT reactions with fragments from the same tissue sample.

C. Preparing and Handling Fresh Tissue for RNA Isolation

To prevent RNA degradation, quickly use a scalpel to partition tissue into ≤10 mg pieces at room temperature or in a container over wet ice. Small pieces of tissue will dry out quickly, especially in areas with strong air ventilation. For the best results, we recommend preparing only a few tissue fragments at a time or, if multiple samples are required, cutting tissue fragments into groups of three or four pieces for easier handling. Visually inspect small tissue fragments during partitioning and, if necessary, moisten them with 1–2 µL of 1X PBS to prevent dehydration.

One of the key advantages of the MELT Total Nucleic Acid Isolation System is that no hands-on attention is needed once the MELT digestion is started. Samples can be prepared and then immediately placed in the reaction tube for the digestion. The next sample can then be processed with minimal risk of sample cross-contamination.

D. Cleanup and Decontamination

We recommend wiping the benchtop and all utensils and containers used for tissue handling with a 10% bleach solution to clean and decontaminate them. Diluted bleach solutions will lose some potency over time, so use within ~5 days of preparation.

III. Tissue Digestion and RNA Isolation

A. Preparing for the Tissue Digestion

1. Add 16 µL of 14.3 M β-ME per 1 mL of Binding Solution needed

We recommend adding β-mercaptoethanol (β-ME) to the quantity of Binding Solution needed for an experiment on the day it will be used.

a. 100 µL of Binding Solution is needed per sample. Determine the total required volume of Binding Solution needed for the samples to be processed that day, and place it in an RNase-free tube appropriate for the volume.

b. Add 16 µL of 14.3 M β-ME per 1 mL of Binding Solution, and mix thoroughly. Keep at room temperature.
Although we recommend preparing only enough β-ME/Binding Solution for the experiment at hand, the mixture is considered stable for 1 week at 4°C. If you store the mixture at 4°C, bring it to room temperature before using it for the MELT procedure.

2. Plan the MELT digestion master mix, and place the required amount of MELT Buffer into an RNase-free tube at room temp

a. Calculate the reagent volumes needed to prepare 100 µL of MELT digestion master mix for each sample to be processed.

IMPORTANT

Keep reaction tubes and MELT Buffer at room temperature (22–25°C). To avoid reagent precipitates, do not place reaction tubes or master mix on ice.

Table 1. MELT Digestion Master Mix (One Sample)

Amount	Component
96 µL	MELT Buffer
4 µL	MELT Cocktail
100 µL	Volume/reaction

b. Dispense the total required volume of MELT Buffer into a non-stick RNase-free tube, and set it aside at room temperature. Do not add the MELT Cocktail until just before starting the digestion reaction in step B.1 below.

B. MELT Tissue Digestion

The MELT tissue digestion is conducted in a single, closed tube.

1. Complete the MELT digestion master mix and aliquot 100 µL into reaction tubes

a. Once the tissue has been partitioned into 1–5 mg fragments, complete the MELT digestion master mix by adding 4 µL of MELT Cocktail per sample, as calculated in step A.2, to the tube containing the MELT Buffer.

b. Mix by gently flicking the tube or by inverting the tube several times, briefly spin down, then aliquot 100 µL into labeled non-stick 1.5 mL RNase-free microcentrifuge tubes.

2. Add tissue samples to MELT digestion master mix and shake for 10 min


NOTE

If processing multiple unique samples that require equipment sterilization between each sample type, we recommend grouping MELT digestions into 3–4 reactions, then freezing the lysates at –20°C after step 3 for later batch RNA purification.

a. Place ≤10 mg tissue into MELT digestion master mix.
Place tissue fragments with maximum total mass of 10 mg into a tube containing MELT digestion master mix (see sections II.B and II.C starting on page 12 for instructions on separating fragments from larger tissue samples).

b. Start mixing immediately.
Close the tube, ***immediately*** place it in the installed Vortex Adapter-60 microcentrifuge tube insert, and turn on the vortex mixer. It is important to submerge the tissue in the MELT digestion mixture as quickly as possible.

c. Incubate with rapid shaking for 10 min at room temp.
As described in *Vortex setup* on page 11, adjust the speed on the vortexer to mix samples very rapidly (just below maximum speed), but not so fast that reactions turn to foam, or that the vortexer becomes unstable on the bench top. Note that some foam is expected to form in the tube; there may be as much as 0.5 cm of foam on top of the reaction mixture.


IMPORTANT

*To avoid reagent precipitation **do not** place reaction tubes on ice.*


NOTE

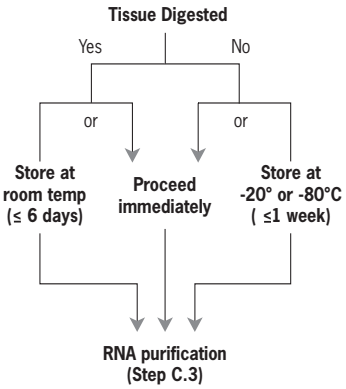
To proceed immediately to RNA purification after MELT digestion, prepare and dispense the Binding Bead master mix (steps C.1 and C.2) during step 2.

3. Inspect samples and, if necessary, continue shaking for 5 min more

After 10 min, inspect the solution for visible tissue fragments.
The MELT digestion is complete when the solution is a homogenous mixture with no visible tissue fragments (even translucent fragments). For samples that are completely digested, continue to step 4.

If tissue fragments are visible, return the reaction tubes to the vortex mixer, and continue shaking for an additional 5 min.
If tissue fragments are visible after 15 min, it may indicate that more than 10 mg of tissue was used, that the tissue fragments were too large, or that the MELT Total Nucleic Acid Isolation System is not efficient with that tissue type. Any cellular RNA that has not been exposed to the MELT digestion master mix by 10–15 min after starting the digestion reaction is susceptible to degradation by RNases, and therefore may not be intact. Quality RNA may still be isolated if the RNA is purified immediately.

4. Proceed to RNA Purification, or store the MELT lysate



If all of the tissue fragments are digested, and the lysate is visibly opaque, proceed immediately to RNA purification, or store the lysate at room temperature before proceeding.

- Proceed to step C.3 on page 18, to clarify the lysate and immediately purify the RNA. (Prepare and dispense the Binding Bead master mix, steps C.1 and C.2, during the MELT digestion.)
- Alternatively, store the MELT lysate at room temperature for up to 6 days (see Figure 6 on page 6) before proceeding with RNA purification.

NOTE

Storage of MELT lysates at room temperature is recommended only when all of the tissue fragments are digested, and the lysate is visibly opaque.

If tissue fragments are still visible, proceed immediately to RNA purification, or store the lysate at –20° or –80°C before proceeding.

- Proceed to step C.3 on page 18, to clarify the lysate and immediately purify the RNA. (Prepare and dispense the Binding Bead master mix, steps C.1 and C.2, during the MELT digestion.) This option is recommended for obtaining the highest possible quality RNA from your samples when the tissue is not completely digested.
- Alternatively, MELT lysates can be stored at –20° or –80°C for up to one week. Quick-freeze MELT lysates (on dry ice, or simply place tubes in the frost that has accumulated in the freezer). This option is recommended if visible tissue remains and you cannot proceed immediately to RNA purification.

C. RNA Purification

1. Prepare a Binding Bead master mix

During the MELT digestion (step B.2), prepare a Binding Bead master mix according to the number of samples, following the table below.

Table 2. Binding Bead master mix (per 100 µL MELT lysate sample)

Add in this order:	
Amount	Component
10 µL	Binding Beads*
100 µL	Binding Solution (containing β-ME, step A.1)
100 µL	80% Ethanol†
210 µL	Volume per reaction

* Mix the Binding Beads thoroughly by vortexing before dispensing.

† If you are adding each reagent individually to a Processing Plate, mix by pipetting up and down ~5 times after adding ethanol.

2. Dispense 210 µL of Binding Bead master mix per sample into an empty well or tube

For processing fewer than 96 samples using the Processing Plate, cover the entire Processing Plate with the Plate Seal, then cut away the Plate Seal to expose the number of wells in the Processing Plate that will be used during the purification. The Plate Seal will then protect unused wells during purification.

Vortex the Binding Bead master mix, and dispense 210 µL per sample into the wells of 96-well Processing Plate provided (Processing Plate method) or into empty RNase-free microcentrifuge tubes (single-tube method).

Set aside the dispensed Binding Bead master mix at room temperature until the MELT digestion is complete (step B.3).

3. Centrifuge MELT lysates for 3 min at >10,000 x g and transfer to the dispensed Binding Bead master mix

Frozen lysates: Defrost frozen lysates at room temperature for 15–20 min. Do not defrost at 4° C or on ice. Monitor progress with occasional vortexing or flicking, until the lysates are completely in liquid form. Lysates should appear clear, not cloudy. Proceed to step a.

a. Centrifuge the reaction tubes for 3 min at >10,000 x g to clarify the MELT lysates.

   This clarifying spin is necessary for optimum bead handling of highly viscous tissue lysates. Experienced users may find that it can be skipped if lysates are not viscous. Aspirate the lysate with a micropipettor to evaluate its viscosity. Examples of tissues that typically need a clarifying centrifugation include mouse lung, small intestine, and colon.

b. After centrifugation, carefully transfer the clarified MELT lysate (100 µL) into a Processing Plate well or RNase-free microcentrifuge tube containing Binding Bead master mix. While aspirating the lysate, avoid disturbing the debris at the bottom of the tube, even though it may not be easily visible.

4. Mix by gently pipetting up and down ~10 times and incubate for 2 min at room temp

a. Pipette the solution up and down ~10 times with a pipettor to mix. Small clumps of Binding Beads may be visible; this does not affect the efficiency of the RNA purification process.

If bubbles form during the mixing process, use a clean pipette tip to pierce the bubbles and release any trapped beads.

b. Allow the RNA to bind to the beads for a total of 2 min at room temperature, including mixing time. User a timer to monitor binding time.

5. Magnetically capture the Binding Beads for 1–3 min, and discard the supernatant

IMPORTANT

Follow the suggested binding and capture times closely to maximize RNA quality.

a. Transfer the Processing Plate or reaction tube to a magnetic stand. Allow the Binding Beads to be captured for 1–3 min (maximum 5 min) at room temperature. For optimum RNA yield, capture beads for 5 min on the magnetic stand.

b. With the plate or tube still positioned on the magnetic stand, carefully vacuum/aspirate/pipette as much of the solution as possible from the wells/tubes without disturbing the Binding Beads.

IMPORTANT

Avoid aspirating Binding Beads when removing supernatant from the captured beads. We find it helpful to reduce the aspiration speed, or to aspirate supernatants using a long flexible pipette tip (first remove filter) attached to the vacuum hose. Use the side of the well or tube opposite the captured beads as a guide to remove the supernatant from the well.

6. Wash with 300 µL Wash Solution 1

a. Remove the Processing Plate or reaction tube from the magnetic stand and add 300 µL Wash Solution 1.

b. Pipette up and down at least 5 times to disperse the beads.

c. Return the plate or tube to the magnetic stand and recapture the beads for 1–2 min.

d. With the plate or tube still positioned on the magnetic stand, carefully vacuum/aspirate/pipette as much of the solution as possible from the wells/tubes without disturbing the Binding Beads.

IMPORTANT

Remove all of Wash Solution 1.

7. Wash with 300 µL Wash Solution 2

Following the instructions in steps 6.a–d, wash the Binding Beads with 300 µL of Wash Solution 2.

8. Continue to the TURBO DNase digestion or skip it and proceed to the final wash and RNA elution

- To include the on-bead TURBO DNase digestion, proceed to step III.D.1 below.
- To omit the on-bead TURBO DNase digestion, go directly to section *III.E. Final Wash and RNA Elution From the Binding Beads* starting on page 21

D. (Optional) On-bead TURBO DNase Digestion and Post-digestion Cleanup

1. Prepare a TURBO DNase master mix

Use the table below to prepare a TURBO DNase master mix to treat all the RNA samples in your experiment.

For optimum DNA removal, equilibrate the non-enzymatic reagents for the on-bead TURBO DNase treatment to room temperature (22–25°C) before use. (Store the TURBO DNase at –20°C except just before use, when it can be stored on ice at the lab bench).

Table 3. On-bead TURBO DNase digestion master mix (one reaction)

Amount	Component
98 µL	TURBO DNase Buffer
2 µL	TURBO DNase 20/µL
100 µL	Volume per reaction

2. Air dry the Binding Beads for 5 min at room temp

a. After removing Wash Solution 2 in step C.7 on page 20, and with the Processing Plate or reaction tube still on the magnetic stand, open the lid and allow the Binding Bead pellets to air dry for 5 min at room temperature. This step removes residual ethanol that could prevent optimal TURBO DNase digestion.
Do not exceed 5 min or the beads may overdry, making it difficult to resuspend the RNA.

b. Remove the Processing Plate or reaction tube from the magnetic stand.

3. Add 100 µL TURBO DNase Digestion master mix and incubate for 10 min with gentle agitation

a. Add 100 µL of TURBO DNase Digestion master mix directly onto the air-dried beads. It is not necessary to mix by pipetting since mixing will occur in step b.

b. Position the Processing Plate or reaction tubes securely on the vortex adapter and gently agitate for 10 min at room temperature. The incubation time should be as close to 10 min as possible; use a timer.
Adjust the vortex mixer to gently shake/rock the vortex adapter without spilling or cross-contaminating samples (see *Vortex setup* on page 11 for instructions).

4. Add 100 µL Wash Solution 1 and 55 µL 100% ethanol, mix, and incubate ≤2 min at room temp

Since some RNA is released from the Binding Beads during TURBO DNase digestion, this step allows RNA to rebind the Binding Beads.

a. Remove the Processing Plate or reaction tubes from the vortex adapter.

b. Add 100 µL Wash Solution 1 and then 55 µL 100% ethanol to each sample.

c. Pipette up and down ~10 times to mix.

d. Incubate 2 min at room temperature, including mixing time.

5. Capture the Binding Beads for 1–3 min at room temp

a. Return the Processing Plate or reaction tube to the magnetic stand and capture the Binding Beads for 1–3 min at room temperature. A well defined bead pellet should be visible.

b. Carefully remove the supernatant without disturbing the beads.

6. Wash the RNA-bound Binding Beads with 300 µL Wash Solution 2

a. Remove Processing Plate or reaction tube from magnetic stand.

b. Add 300 µL of Wash Solution 2, and pipet up and down a minimum of 5 times to disperse the beads.

c. Return the Processing Plate or reaction tube to the magnetic stand and capture the Binding Beads for 1 min at room temperature.

d. Carefully remove the supernatant without disturbing the beads.

e. Proceed to section *E. Final Wash and RNA Elution From the Binding Beads*.

E. Final Wash and RNA Elution From the Binding Beads

1. Wash the RNA-bound Binding Beads a final time with 300 µL Wash Solution 2

a. Remove Processing Plate or reaction tube from magnetic stand.

b. Add 300 µL of Wash Solution 2, and pipet up and down a minimum of 5 times to disperse the beads.

c. Return the Processing Plate or reaction tube to the magnetic stand and capture the Binding Beads for 1 min at room temperature.

d. Carefully remove the supernatant without disturbing the beads. Ensure that no residual Wash Solution 2 remains.

2. Air dry the Binding Beads for 5 min at room temp

a. With the Processing Plate or reaction tube still on the magnetic stand, open the lid(s) and allow the Binding Bead pellets to air dry for 5 min at room temperature. Do not exceed 5 min or the beads may overdry, making it difficult to resuspend the RNA.

b. After drying, remove the Processing Plate or reaction tube from the magnetic stand.

3. Add 20 µL (Processing Plate) or 40 µL (single-tube) Elution Buffer

a. Briefly vortex the Elution Buffer before use.

b. ***Processing Plate method:*** Add 20 µL of Elution Buffer to each well containing Binding Beads.
Single-tube method: Add 40 µL of Elution Buffer to each sample.

4. Mix thoroughly by pipetting and incubate 2 min at room temp

a. Pipette up and down ≥10 times to disperse the Binding Beads without creating bubbles. Thorough bead dispersal is important for optimum RNA yield. If any bubbles do form, pierce them with a clean pipette tip.

b. Incubate the beads in the Elution Buffer for 2 min at room temperature.

5. Capture the Binding Beads for 2–5 min at room temp

Return the Processing Plate or reaction tube to the magnetic stand and capture the Binding Beads for a maximum of 5 min at room temperature.

NOTE

When working in reaction tubes (not in a Processing Plate) during this final Binding Bead capture, it is important that the magnet in the magnetic stand is touching or nearly touching the bottom of the tubes. If necessary, you should manually hold (or otherwise manipulate) the base of the reaction tube so that it is closer to, or touching the magnet. This results in a tighter Binding Bead pellet, making it possible to transfer the eluted RNA without transferring any of the Binding Beads.

6. Collect the purified RNA and transfer it to an Elution Tube

a. Once the Binding Beads have formed a tight pellet and the Elution Buffer containing the RNA is transparent, carefully transfer each RNA-containing supernatant to a 0.5 mL non-stick RNase-free Elution Tube, provided in the kit.
If the captured Binding Bead pellet is disturbed during sample transfer and beads are carried into the Elution Tube, centrifuge the tubes at 10,000 x g for 3 min at room temperature before downstream analysis and applications.

b. Keep RNA samples on ice during analysis, or store at –80°C.

F. Assessing RNA Yield and Integrity

Expected RNA yield

RNA yield varies greatly depending on many factors such as tissue type; health of the organism; and sample handling during dissection, storage, and partitioning. Figure 2 on page 3 shows empirical yield data from several mouse tissues obtained at Ambion using the MELT Total Nucleic Acid Isolation System Kit.

Assessing RNA yield by UV absorbance

NOTE

The on-bead TURBO DNase digestion and subsequent clean-up remove small DNA fragments that can confound RNA concentration measurements. If you haven't included the TURBO DNase digestion in your procedure, RNA yield determinations derived from UV absorbance may be skewed.

The concentration and purity of RNA can be determined by reading the absorbance of an aliquot of the preparation in a spectrophotometer at 260 nm and 280 nm. Be sure to zero the spectrophotometer using the Elution Buffer provided with the kit.

We recommend the Nanodrop ND-1000A UV-Vis Spectrophotometer (www.nanoambion.com). With the NanoDrop Spectrophotometer, the user simply pipets 1.5–2 µL of sample onto the measurement pedestal; no dilutions or cuvettes are necessary. The NanoDrop Spectrophotometer performs all UV/Vis spectrophotometric analyses carried out by traditional spectrophotometers.

An A_{260} of 1 is equivalent to 40 µg RNA/mL

The concentration (µg/mL) of RNA is therefore calculated by multiplying the A_{260} x dilution factor (if sample was diluted) x 40 µg/mL.

Assessing RNA yield with RiboGreen

If a fluorometer or a fluorescence microplate reader is available, Invitrogen's RiboGreen fluorescence-based assay for RNA quantitation is a convenient and sensitive way to measure RNA concentration. Follow the manufacturer's instructions for using RiboGreen. When using RiboGreen, it is important to note that significant amounts of DNA in the RNA samples will affect the RiboGreen quantitation. The RiboGreen signal intensity from DNA is roughly 2-fold more than the signal intensity for the same mass of RNA.

Assessing RNA integrity with the Agilent 2100 bioanalyzer

The Agilent 2100 bioanalyzer used in conjunction with an RNA LabChip Kit provides a powerful and sensitive method to assess RNA integrity. To use this system, follow the instructions for RNA analysis provided with the RNA LabChip Kit. The 28S/18S rRNA ratio is typically used as an indicator of RNA integrity. RNA isolated with the MELT Total Nucleic Acid Isolation System Kit should typically have a 28S/18S rRNA ratio of 1.0 or greater.

Agilent offers a software algorithm that was developed to extract total RNA integrity information from a sample's bioanalyzer electrophoretic trace. The RNA Integrity Number (RIN) is determined using information from both the rRNA bands, as well as information contained outside the rRNA peaks (potential degradation products) to provide a full picture of RNA integrity. Search for "RIN" in the Life Sciences/Chemical section of Agilent's website for more information:

www.chem.agilent.com

IV. Troubleshooting

A. Poor RNA Quality

Poor quality tissue before starting

The quality and handling of the tissue prior to beginning the MELT procedure will have a significant impact on the integrity of RNA that can be obtained. It is important to procure tissue samples as quickly as possible from the host. If immediate processing is not possible, snap-freeze tissue samples in liquid nitrogen immediately after obtaining them to inactivate endogenous RNases before they can damage cellular RNA.

The MELT procedure requires that tissue samples be partitioned into small fragments for optimal sample liquefaction. We provide detailed instructions for partitioning both fresh and frozen tissue in sections II.B and II.C starting on page 12. If you still have questions about tissue handling after reading those instructions, consider contacting our Technical Services Department for more information.

The sample type is incompatible with the MELT systems

The following tissue types are incompatible with the MELT System:

- Tissues that contain very high levels of ribonuclease
- Extremely hard or fibrous tissues
- Adipose tissue
- Tissues that have been stored in RNA*later* or RNA*later*-ICE

Too much starting material

Starting with more than 10 mg of tissue can significantly slow the MELT digestion reaction, compromising RNA integrity. Once tissue is immersed in the MELT digestion master mix, it is important that the digestion proceeds rapidly so that RNases are quickly exposed to the MELT reagents and thereby inactivated before they have an opportunity to degrade cellular RNA.

Since it is often not feasible to weigh tissue samples without causing RNA degradation, a common practice is to estimate the weight of input material. For many sample types, such as brain, liver, kidney, and thyroid, using 1–2 mg more than the maximum recommended input amount of 10 mg will not affect RNA quality. For some tissues, such as heart and small intestine, however, the 10 mg limit must be scrupulously observed. If a particular sample type yields poor quality RNA, and you estimate that sample input was near the 10 mg limit, then use less material in subsequent RNA isolations. Large tissue samples can, of course be split and processed in multiple MELT reactions.

Try using a few 1–3 mg pieces of tissue rather than a single ≤10 mg piece

With some tissue types, digestion may be improved by using a few small pieces of tissue with a combined weight of ≤10 mg rather than a single ≤10 mg tissue sample. Rapid tissue digestion is important for high yields of pure RNA, and increasing the surface area exposed to the digestion reagents by using smaller tissue fragments may have an important effect on RNA yield and quality.

Tissue fragments remain in the MELT digestion reaction even after 15 min

Vigorous shaking is required for good tissue digestion

For rapid tissue digestion/disruption, the samples must be mixed vigorously. We have found that the motion of the Vortex Adapter-60 with the microcentrifuge tube foam rack is important for the recovery of quality RNA using the MELT System.

Check that digestion conditions were met in section III.B starting on page 16

- Be sure that the MELT Cocktail is added to the reaction.
- The MELT digestion reaction works best at room temperature (22–25°C).
- Shake very vigorously, but not so fast that the MELT digestion mixture completely turns to foam.

Too much tissue was used, or the tissue is incompatible with the MELT System.

The following situations indicate that too much tissue has been added or that it is incompatible with the MELT system.

- The tissue is not completely digested within 10 min and it is clear that an additional 5 min of digestion, as suggested in step III.B.3 on page 16, will not completely digest the remaining tissue.
- Tissue fragments remain even after 15 min of agitation in the MELT digestion mix.
- Some tissues are simply too tough to be digested in the MELT System, and they are not liquefied after 15 min.

In all such cases, quality RNA may still be isolated if the RNA is purified immediately. Proceed directly to step III.C.3 on page 18, centrifuging the reaction tubes for 10,000 x g for 3 min (it is very important to remove undigested cellular material). Without disturbing the tissue pellet, transfer the MELT lysate to the Binding Bead mix and continue with the RNA purification procedure.

B. Low RNA Yield

Binding Beads were unintentionally lost

Any loss of the beads during the RNA purification procedure will result in the loss of RNA and decreased overall yield. Avoid aspirating the Binding Beads when removing supernatant from the captured beads. To prevent aspiration of Binding Beads, reduce the aspiration speed if possible, or attach a long flexible pipette tip (first remove filter) to the vacuum hose. When removing the supernatant, use the side of the well opposite the bead pellet as a guide to remove liquid from the well without disturbing the captured bead pellet.

Binding Beads were not fully resuspended/dispersed

- Make sure that the Binding Beads are fully resuspended before using them at the start of the procedure (step III.C.1 on page 18).
- Make sure that the Binding Beads are fully resuspended in the Elution Buffer to efficiently elute the RNA.
- Do not overdry the beads before treating with TURBO DNase (optional) or eluting the RNA. If you suspect that the beads were overdried, you may be able to partially rectify this problem by prolonging the mixing and incubation of Binding Beads in Elution Buffer to rehydrate the beads and facilitate elution.

C. DNA Contamination

The on-bead TURBO DNase digestion must be incubated at 22–25°C

For optimum DNA removal, equilibrate the non-enzymatic reagents for the on-bead TURBO DNase treatment to room temperature (22–25°C) before use. (Store the TURBO DNase at –20°C except just before use, when it can be stored on ice at the lab bench).

If the ambient temperature in your lab is below 20°C, then we recommend incubating the digestion at 37°C with gentle agitation (if possible).

Carryover of wash solution to the on-bead TURBO DNase digestion

For optimal TURBO DNase digestion conditions, ensure that all of the Wash Solution 1 and 2 are removed in steps III.C.6 and 7 on page 20, prior to air drying the beads.

V. Appendix

A. DNA Purification from MELT Lysates

The MELT Total Nucleic Acid Isolation System is optimized for RNA isolation; however, with a slight modification to the RNA purification procedure, DNA can also be recovered from MELT lysates. We have isolated DNA from the following mouse tissue types.

• Liver	• Brain	• Thyroid
• Tail	• Heart	• Lung
• Kidney	• Small Intestine	• Spleen

Note that lung and spleen required long (>20 min) MELT digestion times that may not be suitable for isolation of highly intact RNA.

Expected DNA yield

As for RNA, DNA yield can vary from species to species, among developmental stages, and with tissue collection and handling methods. Using good handling techniques, expected DNA yield from this procedure is approximately 1 µg of DNA/mg of tissue.

Necessary reagents not provided with the kit

In addition to the reagents provided in the kit, you will need:

- 100% isopropanol, ACS reagent grade or equivalent
- (Optional) RNase A, (P/N AM2270), for RNA removal

1. Preheat Nuclease-free Water to 70°C for DNA elution

Nuclease-free Water at 70°C is used to elute the DNA at the end of the procedure (step 8 below). Aliquot 20 µL Nuclease-free Water per sample, for the Processing Plate method, or 40 µL per sample for the single-tube method, plus ~10% overage, into a nuclease-free container and begin heating it to 70°C.

2. Digest samples as described in section III.B

Digest tissue samples following the instructions for RNA isolation in section III.B starting on page 16.

Once the MELT digestion is complete, centrifuge the reaction tubes for 3 min at >10,000 x g to clarify the lysates. This clarifying spin is necessary for optimum bead handling of highly viscous tissue lysates. Experienced users may find that it can be skipped if lysates are not viscous (aspirate lysate with a micropipettor to evaluate its viscosity).

When transferring the clarified MELT lysate in the next step, do not disturb the debris at the bottom of the tube, even though it may not be easily visible.

3. Mix lysate with nucleic acid binding components and incubate 3 min at room temp

a. Mix lysate with the nucleic acid binding components in the order shown in Table 4. Note that reagent amounts are shown for binding reactions that use the entire 100 µL MELT lysate, and from only 50 µL of the lysate for users who want to isolate RNA from half the lysate (see section II.A starting on page 10), and DNA from the other half.

NOTE

See the beginning of section II.A on page 10 for information on whether the Processing Plate method or the single-tube method is more appropriate for your application.

Cut away the Plate Seal to expose the number of wells in the Processing Plate that will be used during the purification. The Plate Seal will then protect unused wells during purification.

Table 4. Nucleic Acid Binding Mixture

Entire Lysate	Half Lysate	Component
50 µL	25 µL	Nuclease-free Water
50 µL	25 µL	Binding Solution (with β-ME, step III.A.1)
10 µL	10 µL	Binding Beads*
100 µL	50 µL	100% Isopropanol
100 µL	50 µL	MELT Lysate

* Mix the Binding Beads thoroughly by vortexing before dispensing.

b. Pipette the solution up and down ~10 times with a pipettor to mix.
   - Small clumps of Binding Beads may be visible; this does not affect the efficiency of the DNA purification process.
   - If bubbles form during the mixing process, use a clean pipette tip to pierce the bubbles and release any trapped beads.

c. Allow nucleic acids to bind to the beads for 2 min at room temperature.

4. Capture the beads and wash them twice with 300 µL Wash Solution 2

a. Transfer the Processing Plate or reaction tube to a magnetic stand. Allow the Binding Beads to be captured for 1–3 min (maximum 5 min) at room temperature. For optimum DNA yield, capture beads for 5 min on the magnetic stand.

b. With the plate or tube still positioned on the magnetic stand, carefully vacuum/aspirate/pipette as much of the solution as possible from the wells/tubes without disturbing the Binding Beads.

c. Remove the Processing Plate or reaction tube from the magnetic stand and add 300 µL Wash Solution 2.

d. Pipette up and down at least 5 times to disperse the beads.

e. Return the plate or tube to the magnetic stand and recapture the beads for 1–2 min.

f. With the plate or tube still positioned on the magnetic stand, carefully vacuum/aspirate/pipette as much of the solution as possible from the wells/tubes without disturbing the Binding Beads.

g. Repeat steps 4.a–f to wash a second time with Wash Solution 2.

h. ***If RNase digestion is not required,*** proceed to step 8 for a final wash and elution.

5. (Optional) Digest with RNase to remove RNA

a. With the Processing Plate or reaction tube still on the magnetic stand, open the lid(s) and allow the Binding Bead pellets to air dry for 5 min at room temp. Do not exceed 5 min or the beads may overdry, making it difficult to resuspend the DNA.

b. Use the table below to prepare an RNase master mix to treat all the samples in your experiment.

Table 5. On-bead RNase digestion master mix (one reaction)

Amount	Component
99 µL	Nuclease-free Water
1 µL	RNase A (1 mg/mL), Ambion P/N AM2270
100 µL	Volume per reaction

c. Remove the Processing Plate or reaction tube from the magnetic stand, and add 100 µL RNase digestion master mix to each sample.

d. Incubate for 15 min at room temperature with gentle agitation. Adjust the vortex mixer to gently shake/rock the vortex adapter without spilling or cross-contaminating samples (see *Vortex setup* on page 11 for instructions).

6. Rebind the DNA to the Binding Beads

a. Add the following to each sample:

Amount	Component
50 µL	Nuclease-free Water
50 µL	Binding Solution (with β-ME, step III.A.1)
100 µL	100% isopropanol

b. Pipette the solution up and down ~10 times to mix.

c. Allow DNA to bind to the beads for 2 min at room temperature.

7. Capture the beads and wash with 300 µL Wash Solution 2

Capture the Binding Beads and wash with 300 µL Wash Solution 2 as described in step 4 on page 28.

Continue to step 8 (next).

8. Wash the beads with 300 µL Wash Solution 2 and air dry

If RNase digestion (steps 5–7) was skipped, continue the procedure at this step.

a. Wash the Binding Beads a final time with 300 µL Wash Solution 2 as described in step 4 on page 28.

b. Air dry the Binding Beads for 5 min at room temperature.

9. Elute the DNA in 70°C Nuclease-free Water

a. Add 20 µL (Processing Plate) or 40 µL (single-tube) of 70°C Nuclease-free Water to each sample.

IMPORTANT

The 70°C elution temperature is critical for high DNA recovery.

b. Mix thoroughly by pipetting and incubate 2 min at room temperature.

c. Capture the Binding Beads for 5–10 min at room temperature.

d. Collect the purified DNA (in the supernatant) and transfer it to an Elution Tube.

B. Quality Control

Functional testing

At least 2 µg of RNA is obtained per mg of frozen mouse liver using the procedure outlined in section III starting on page 15. RNA is analyzed on an Agilent bioanaylzer.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with super-coiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

Protease testing

Meets or exceeds specification when a sample is incubated with protease substrate and analyzed by fluorescence.

C. Safety Information

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety goggles, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining the MSDS

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.

For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.