Product Information

CELLDATA RNAstorm™ FFPE RNA Extraction Kit

Catalog Number: CD501 (50 preps)

Kit Contents

Component	Size
99860-30ML: Deparaffinization Reagent	30 mL
99861: RNAstorm™ FFPE CAT5™ Reagent	5 mL
99862: RNAstorm™ FFPE Lysis Buffer	5 mL
99863: CELLDATA FFPE Binding Buffer	15 mL
99864: CELLDATA Wash Buffer	12 mL (Add 48 mL ethanol prior to use)
99865: DNase Buffer	5 mL
99866: RNAstorm™ FFPE Protease	600 uL
99867-600U: DNase I (lyophilized)	600 U (Reconstitute in 120 uL water prior to use)
99868-50: CELLDATA Spin Columns	50 each

Storage and Handling

Upon receipt, store protease solution and DNase I at 2-8°C. After reconstitution, DNase I solution should be stored at -20°C. Store other kit components at room temperature. Kit components are stable for at least 9 months from date of receipt when stored as recommended. The Binding Buffer contains the chaotropic salt guanidine hydrochloride, which is hazardous (see SDS). Use gloves and other appropriate laboratory protection when using this kit. Mixing bleach with guanidine hydrochloride can produce hazardous byproducts. DO NOT mix waste from this kit with bleach. Handle all kit components using universal laboratory safety precautions.

Product Description

Biopsies and surgical specimens are routinely preserved as formalin-fixed, paraffin embedded (FFPE) tissue blocks. While formaldehyde stabilizes tissue for storage and sectioning, it also forms extensive crosslinks and adducts with nucleic acids in the sample. Such modifications strongly interfere with molecular analysis methods and must be removed for efficient extraction of nucleic acids from FFPE tissue.

Existing methods rely primarily on heat to remove crosslinks and adducts, which is only partially effective and leads to additional fragmentation of labile nucleic acids such as RNA. In contrast, the catalytic technology used in the CELLDATA RNAstorm™ FFPE RNA Extraction Kit greatly accelerates the removal of formaldehyde damage and allows the use of milder conditions, resulting in markedly improved recovery of amplifiable RNA. Compared to other methods, this greatly enhances the chances of success in recovering higher yields of good quality nucleic acids suitable for a variety of applications, including next-gen sequencing, qPCR, microarray, or other gene expression analysis.

Evaluating FFPE RNA Extraction

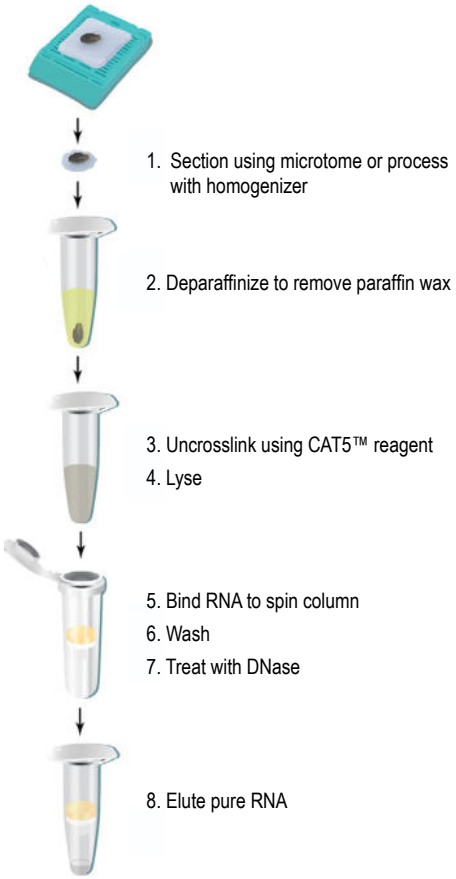
The following techniques may be used to evaluate the quality and quantity of your FFPE-derived RNA after extraction.

- **Quantity/Concentration:** The RNA recovery will depend primarily on the amount and integrity of the tissue sample, but with samples of good quality it is possible to recover greater than 1 ug of total RNA per sample. Measurements can be obtained using quantitative RT-PCR. Note that UV/Vis spectroscopy (e.g., NanoDrop®) may also be used but are particularly susceptible to contaminants such as cellular debris, proteins, salts, and detergents which absorb in the 200-280 nm range.
- **RNA Integrity:** Gel or capillary gel electrophoresis, such as the Agilent Bioanalyzer, expressed as a RIN number or DV_{200} percentage can be used to evaluate RNA integrity. Note that FFPE RNA will have a lower molecular weight profile compared to RNA from fresh samples, and will not show intact rRNA bands.
- **Amplifiability:** The standard method is quantitative RT-PCR expressed as a Ct number or as a relative or absolute amount of RNA.

Protocol Outline

The RNAstorm™ FFPE RNA extraction procedure involves the following steps:

- **Preparation of sections**: Paraffin sections are cut from a block using a microtome, or the tissue is processed using a grinder or homogenizer.
- **Deparaffinization**: The paraffin is removed from the sections.
- **Uncrosslinking and lysis**: The tissue is treated to release RNA from other cellular components and to remove formaldehyde-induced modifications.
- **DNase I treatment**: Contaminating genomic DNA is degraded using DNase I. This step is optional but highly recommended.
- **RNA isolation**: Cellular debris and other impurities are removed from the RNA. The RNA is first bound to a spin column in the presence of Binding Buffer, then washed using Wash Buffer. Pure RNA is finally eluted using water.



Materials required but not supplied

- Microtome for tissue sectioning
- Optional: Xylenes may be used in place of the provided deparaffinization reagent (see Option B in protocol)
- Ethanol (200 proof, molecular biology grade)
- Heat block set to 72°C
- An ice-filled container
- 1.5 mL microcentrifuge tubes (Eppendorf LoBind® tubes recommended)
- Microcentrifuge (12,000 x g minimum)
- RNase-free water for DNase I reconstitution and final RNA elution step

Before you begin

Prepare the following buffers

- CELLDATA Wash Buffer: Add 48 mL of 200 proof ethanol to the bottle and mix well. Mark the ethanol added box on the label.
- DNase I: Reconstitute the lyophilized DNase I by adding 120 uL of RNase-free water. Using a pipette, mix gently to ensure the DNase is fully reconstituted (do not vortex, which can denature DNase). Briefly centrifuge the tube to collect contents at bottom. Store in aliquots at -20°C and avoid freeze/thaw cycles.

Prepare the tissue

The RNAstorm™ kit can be used with FFPE sections between 5-10 um thick. For each isolation, 1 to 4 sections should be used. The tissue should have an approximate total surface area between 10 and 100 mm^2. Tissue sections may be scraped off of slides using a razor blade and collected in a microcentrifuge tube for processing. Alternatively, an equivalent amount of tissue may be ground or crushed using a tissue grinder/homogenizer or a small mortar and pestle.

Detailed RNA isolation protocol

Option A (recommended): Deparaffinization using included reagent

Note: This recommended procedure relies on the included Deparaffinization Reagent, which is efficient and non-toxic. A fume hood is not necessary to perform this step. An alternative deparaffinization protocol using xylenes is included below (Option B).

A1. Place the desired number of sections in a 1.5 mL microcentrifuge tube.

A2. Add 500 uL of Deparaffinization Reagent.

A3. Invert the tube several times to mix, then centrifuge briefly to bring sample to the bottom of the tube.

A4. Incubate the tube in a heat block at 72°C for 3 minutes, then cool to room temperature.

A5. Add 80 uL of RNAstorm™ FFPE CAT5™ Reagent to the tube containing deparaffinized tissue sections.

A6. Invert the tube several times to mix, then centrifuge for 1 minute at 16,000 x g. At the bottom of the tube, a clear aqueous phase should form containing the tissue, along with a bright yellow upper organic phase containing the Deparaffinization Reagent and paraffin. If tissue appears to be present in the upper organic phase, mix gently by inverting the tube (do not vortex), and then centrifuge again until all tissue is contained in the lower aqueous phase.

A7. Using a pipette, carefully remove most of the upper organic phase (approximately 100 uL can remain). Discard the upper organic phase.

A8. Centrifuge the tube briefly, then proceed to step 1.

Option B: Deparaffinization using xylenes

Note: Xylene fumes are toxic and should not be inhaled. Perform these steps in a suitable fume hood.

B1. Place the desired number of sections in a 1.5 mL microcentrifuge tube.

B2. In a fume hood, add 1 mL of xylenes and close the tube lid. Invert the tube several times to mix, then centrifuge at 16,000 x g for 5 minutes.

B3. Remove all liquid, being careful not to disturb the pellet.

B4. Add 1 mL of ethanol, then invert the tube to mix. Centrifuge at 16,000 x g for 2 minutes.

B5. Remove and discard all liquid, being careful not to disturb the pellet.

B6. Repeat steps B4 and B5, for a total of two ethanol washes.

B7. Let the tube stand open at room temperature for 10 minutes for the residual ethanol to evaporate completely. Alternatively, a centrifugal evaporator may be used for quick drying of the samples.

B8. Add 80 uL of RNAstorm™ FFPE CAT5™ Reagent to the tube containing deparaffinized tissue sections and invert the tube several times to mix gently (do not vortex).

B9. Proceed to step 1.

1. Uncrosslinking and lysis

1.1 Incubate the tube containing tissue in a heat block at 72°C for 30 minutes, then place on ice for 1 minute.

1.2 Add 80 uL of RNAstorm™ FFPE Lysis Buffer to the tube, then add 10 uL of RNAstorm™ FFPE Protease. Invert the tube gently several times to mix, then centrifuge briefly to collect contents at bottom of the tube.

1.3 Incubate the tube in a heat block at 72°C for 2 hours.

1.4 Place the tube on ice for 3 minutes.

1.5 Centrifuge the tube for 15 minutes at 16,000 x g. A pellet will form containing cellular debris, while the RNA will remain in solution. The pellet may not be visible, but this will not affect the yield and quality of the obtained RNA.

1.6 Using a pipette, carefully transfer as much of the supernatant as possible to a new tube, without disturbing the pellet.

Note: If using Deparaffinization Option A, a thin organic layer may still be present, but will not interfere with the isolation procedure. Bypass this organic layer by inserting the pipette tip along the wall of the tube.

2. Begin RNA isolation

2.1 Add 150 uL of CELLDATA FFPE Binding Buffer to the supernatant from step 1.6 and then add 450 uL ethanol. Mix well by inverting the tube several times.

2.2 Promptly transfer contents from the tube to a spin column.

2.3 Centrifuge for 1 minute at 16,000 x g. Discard the flow-through.

3. DNase I treatment (recommended)

Note: This step ensures that any contaminating genomic DNA is degraded. To skip DNase I treatment, proceed to step 4.1.

3.1 Mix 120 uL of RNase-free water, 120 uL of CELLDATA FFPE Binding Buffer and 360 uL of ethanol in a separate tube, for a total volume of 600 uL.

3.2 Add 300 uL of this mixture to the column. Centrifuge for 30 seconds at 16,000 x g and discard the flow-through.

3.3 Mix 70 uL of DNase Buffer with 2 uL of reconstituted DNase I, and add this mixture directly to the center of the membrane of the spin column. Let stand for 15 minutes at room temperature.

3.4 Add the remaining 300 uL of the CELLDATA FFPE Binding Buffer/ethanol mixture (prepared in Step 3.1) to the column. Centrifuge for 30 seconds at 16,000 x g and discard the flow-through.

4. Continue RNA isolation

4.1 Add 500 uL of CELLDATA Wash Buffer to a CELLDATA Spin Column and centrifuge for 30 seconds at 16,000 x g. Discard the flow-through.

4.2 Wash again by repeating step 4.1.

4.3 Dry the spin column by placing it back into an emptied collection tube and centrifuging again for 5 minutes at 16,000 x g. Discard the flow-through.

4.4 Place the column in a clean 1.5 mL microcentrifuge tube.

4.5 Elute the pure RNA by adding 50 uL of RNase-free water to the center of the spin column membrane. Let stand for 1 minute. Centrifuge for 1 minute at 16,000 x g. Collect the eluted RNA from the centrifuge tube.

4.6 Optional: Repeat step 4.5 for a higher RNA yield but at a lower concentration.

4.7 Eluted RNA should be stored at -80°C.

Frequently Asked Questions (FAQs)

Question	Answer
Is any contaminating genomic DNA present in the RNA obtained using the RNAstorm™ kit?	Contamination from genomic DNA is a big concern because it can interfere with downstream applications. The RNAstorm™ kit includes an optimized DNase digestion step which removes contaminating genomic DNA without significantly affecting RNA yield. While this step is optional, it is highly recommended.
How much RNA can I expect to obtain from an FFPE sample?	The biggest variable that affects the total amount of RNA obtained is the quality of the sample itself (i.e. the type and amount of tissue, and the care taken in isolation and preservation of the sample). Using the RNAstorm™ kit, and assuming at least reasonable sample quality, amounts greater than 1 ug can be obtained.
Can RNA obtained using the RNAstorm™ kit be used in RNA-Seq?	Yes. Good quality libraries can be obtained, providing that the RNA is of sufficiently high quality. For Illumina sequencing, a DV200 of at least 30% is recommended, and samples should be used that provide at least 1 ug of RNA.
How should the tissue be prepared?	Use a microtome to obtain 5-10 um sections from FFPE samples. Sections thinner than 5 um may be used if they can be reliably cut. Sections thicker than 10 um are not recommended because they may not be fully digested. Also, no more than 5 sections (10 uM each) should be used for each extraction. Using too much tissue can lead to incomplete digestion and reduced yields.
Can I use tissue that is not paraffin-embedded?	Yes, tissue can be used which is not embedded in paraffin. In this case, we recommend mechanically grinding an amount of tissue equivalent to the recommended number of sections.
Can I use FFPE cores?	Yes, FFPE cores can be used. Because cores are not processed using a microtome, sample digestion tends to be more difficult and mechanical homogenization (e.g., using steel beads) is recommended if incomplete digestion is observed.
Which deparaffinization method do you recommend?	The RNAstorm™ kit includes a recommended Deparaffinization Reagent. Unlike other common methods (e.g., xylenes), the Deparaffinization Reagent is efficient, non-toxic and does not require the use of a fume hood. In our testing, the included reagent is at least as effective as xylenes at removing paraffin and purification of high quality nucleic acids.
What is the best way to quantitate RNA obtained from FFPE samples?	FFPE-derived RNA is much more challenging to quantitate accurately than RNA obtained from fresh samples. It is not enough to know the absolute amount of RNA that is present, but also whether the RNA will work in downstream applications, which depends on the following factors: • Fragment size distribution: A 5 ug sample (as measured by Qubit®) can be useless for RNA-Seq if it consists of fragments < 200 nt. • Chemical modification: For RNA obtained from formalin-fixed samples, various chemical adducts and crosslinks, including base modifications, base-base crosslinks, and base-protein crosslinks can make nucleic acid molecules inaccessible to enzymes and therefore inactive in downstream applications. • Contamination: Cellular debris, proteins, salts, and detergents used during purification can bias downstream assays. For example, UV/Vis-based methods such as NanoDrop® are particularly susceptible to contaminants which absorb in the 200-280 nm range. • Fluorescence-based methods such as Qubit® are liable to significant error. When working with low concentrations of DNA or RNA, dye-based detection may not be linear. One must also be mindful of contamination by genomic DNA in an RNA sample, because the dyes used for fluorescence quantitation are not entirely specific for FFPE-derived DNA or RNA. • Quantitative PCR is the preferred method for quantitation of heavily damaged and modified nucleic acids.
Should RIN numbers be used to determine quality of FFPE-derived RNA?	Although the RIN number can provide general information about the extent of sample fragmentation, it is not sensitive or predictable enough to be a useful indicator of downstream performance, especially for RNA-Seq. Very often, RIN numbers for FFPE-derived RNA will be between 2 and 3. Some of these samples will be useful for RNA-Seq, and others won't - the RIN will not tell you, however. A slightly better predictor of performance in RNA-Seq using Illumina sequencing is the DV200, which represents the percentage of RNA fragments longer than 200 nucleotides. The DV200 is also calculated based on Bioanalyzer data, but suffers from the same drawbacks as all Bioanalyzer-based methods, specifically high variability.
What do I need to know when extracting RNA from FFPE samples?	• Avoid methods based on organic solvents (TRIzol™) • Avoid harsh chaotropic salts (i.e. guanidinium) • Avoid detergents which impact downstream quantitation by UV and/or Qubit® (e.g., Triton™ X-100) • Do not rely on RIN to quantitate integrity of an FFPE-derived sample. Use DV200 instead. • Use a kit or method such as the RNAstorm™ kit that removes chemical modifications from formalin. Do not raise the temperature to 80°C or above. Even short times at this temperature will significantly lower integrity. • Be wary of Qubit® and NanoDrop® concentrations because of the possibility of contamination by organic molecules or DNA. • Use qPCR to quantitate your RNA, and always look carefully at melt curves to determine whether nonspecific amplification may have occurred.

Related Products

Catalog number	Product
CD502	CELLDATA DNAstorm™ FFPE DNA Extraction Kit
CD504	CELLDATA RNAstorm™ Fresh Cell and Tissue RNA Isolation Kit
41032	EMBER500™ RNA Prestain Loading Dye
31073	AccuBlue® Broad Range RNA Quantitation Kit
31028	AccuClear® Ultra High Sensitivity dsDNA Quantitation Kit
22028	RNase-X™ Decontamination Solution
31030	DNA Gel Extraction Kit
41003	GelRed® Nucleic Acid Gel Stain, 10,000X in water
41005	GelGreen® Nucleic Acid Gel Stain, 10,000X in water
41042	DNAzure® Blue Nucleic Acid Gel Stain
31022	Ready-to-Use 1 kb DNA ladder
31032	Ready-to-Use 100 bp DNA ladder
31042	Forget-Me-Not™ qPCR Master Mix
31043	Forget-Me-Not™ Universal Probe Master Mix
31077	EvaGreen® Plus Dye, 20X in water
31000	EvaGreen® Dye, 20X in water
41024-4L	Water, Ultrapure Molecular Biology Grade

Please visit our website at www.biotium.com for information on our products for RNA research and applications, including RNA extraction kits for fresh cells and FFPE tissues, RNA quantitation kits, and RNA gel stains.

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Application Note

Created: December 20, 2018

Dual DNA/RNA Extraction From a Single FFPE Sample

This protocol modification allows the extraction of both DNA and RNA from the same FFPE input tissue sample.

Materials required:

- 1 RNAstorm™ kit (CD201 or CD501)
- 1 DNAstorm™ kit (CD202 or CD502)

Method:

Begin by extracting the sample according to the RNAstorm™ kit protocol with the following modifications:

a. Perform step 3 (normally a 2 hour incubation) for only 30 minutes at 72°C. ***See note below regarding possible optimization of this step.***

b. Perform steps 4 and 5 of the RNAstorm™ protocol as directed, but do not discard the pellet in step 5.

c. Transfer the supernatant to a new tube as instructed in step 6.

d. Continue to incubate the supernatant for another 1.5 hours at 72°C (2 hours total including the initial 30 minutes), then proceed with step 7 of the RNAstorm™ protocol (add Binding Buffer) and all remaining steps as instructed.

e. Use the pellet from step b), which contains DNA, as input for step A5 (or B8, depending on deparaffinization choice) of the DNAstorm™ kit manual.

f. Continue with step A5 (or B8) of the DNAstorm protocol by adding 200 µL of CAT5™ Buffer to the pellet, then continue as instructed by the DNAstorm™ protocol.

***Note**: the initial incubation period can be adjusted depending on relative DNA and RNA yields. If the RNA yield is high but the DNA yield is low, reduce the incubation time in step 3 (no less than 15 mins). If the DNA yield is good but the RNA yield is low, increase the incubation time in step 3 (no more than 2 hours).*