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Product Information

DNAstorm™/RNAstorm™ 2.0 FFPE Combination Kit

Catalog Number: CD508

Kit Contents

Component	Size
99876-30ML: Dewaxing Solution	2 x 30 mL
99861: RNAstorm™ FFPE CAT5™ Reagent	5 mL
99862: RNAstorm™ FFPE Lysis Buffer	5 mL
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)
99869-12ML: DNAstorm™ FFPE CAT5™ Lysis Buffer	12 mL
99870: DNAstorm™ FFPE Proteinase K	1.2 mL
99871-600UL: RNase A	600 uL
99863: CELLDATA FFPE Binding Buffer	2 x 15 mL
99864: CELLDATA Wash Buffer	2 x 12 mL (add 48 mL ethanol to each bottle before use)
99868-50: CELLDATA Spin Columns	2 x 50 each

Unit Size: 50 assays

Storage and Handling

Upon receipt, store Proteinase K, DNase I, and RNase A solutions 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 FFPE Extraction Kits greatly accelerates the removal of formaldehyde damage and allows the use of milder conditions, resulting in markedly improved recovery of amplifiable RNA and DNA. This combination kit allows for the extraction of both RNA and DNA from the same sample. Compared to other methods, this kit 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 DNA and RNA Extraction

The following techniques may be used to evaluate the quality and quantity of your FFPE-derived DNA and RNA after extraction (see the FAQ section on pages 4-5 for more information).

- **Quantity/Concentration:** The DNA and 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 DNA and 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 is 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.
- **DNA Integrity:** Due to the wide size distribution of DNA isolated from FFPE tissue samples, we recommend using pulsed-field gel electrophoresis (PFGE) for evaluating DNA integrity. Methods based on capillary electrophoresis such as the Agilent Bioanalyzer® can also be used, but may not properly resolve high molecular weight fragments (greater than 10 kb) in better-quality samples.
- **Amplifiability:** The standard method is quantitative RT-PCR expressed as a Ct number or as a relative or absolute amount of DNA or RNA. For DNA amplification, view our EvaGreen® Dye and Forget-Me-Not EvaGreen® qPCR Master Mixes (see Related Products). Please note PCR inhibition from residual chemical modifications and DNA damage is common when high amounts of FFPE-extracted template DNA are used. For tips to address this issue, see the FAQ section on page 3 “Why does my extracted DNA fail to amplify properly?”.

Experimental Protocols

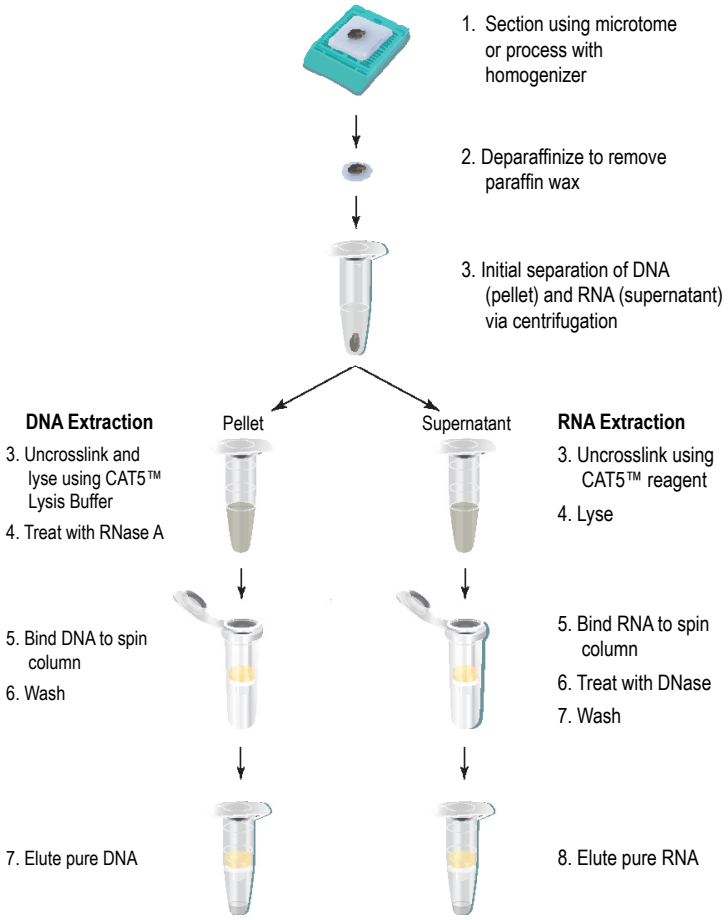
Materials required but not supplied

- Microtome for tissue sectioning
- Optional: Xylenes may be used in place of the provided Dewaxing Solution (see Option B in protocol)
- Ethanol (200 proof, molecular biology grade)
- Heat blocks set to 37°C, 56°C, 72°C, and 80°C.
- An ice-filled container appropriate for micro-centrifuge tubes
- 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
- DNA elution solution: We recommend using Tris or Tris-EDTA buffer at pH 8 (recommended) or nuclease-free water

Before you begin

Prepare the following buffers

- CELLDATA Wash Buffer: Add 48 mL of 200 proof ethanol to each bottle of CELLDATA Wash Buffer and mix well. Mark the ethanol added box on the product 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 and thaw cycles.



Prepare the tissue

This 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.

Deparaffinization protocols

Option A (recommended): Deparaffinization using included reagent

This recommended procedure uses the convenient Dewaxing Solution provided in the kit. Unlike xylenes, the Dewaxing Solution efficiently removes paraffin without a wash step, and does not need to be handled in a fume hood. An alternative protocol using xylenes is also provided below (Option B).

Note: Additional Dewaxing Solution may be purchased separately for dissolving larger amounts of paraffin. However, we do not recommend using excess input tissue because it may not be efficiently lysed in subsequent steps.

A1. Place 1 to 4 sections into a 1.5 mL microcentrifuge tube.

A2. Add 500 uL of Dewaxing Solution to the tube.

A3. Invert the tube several times to mix until the wax has dissolved.

Optional: If solid (white) wax appears to still be present, you may heat the tube for 1-5 minutes or longer in a heat block at 37°C or 72°C until the wax is transparent. Note that the tissue will remain solid at this stage.

A4. Centrifuge briefly to collect the tissue at the bottom of the tube.

A5. Carefully pipette off the Dewaxing Solution from the tissue. Use a fine tip to carefully remove as much residual solution from the bottom of the tube as you can without disturbing the tissue pellet.

A6. Place the tube with lid open in a heat block at 37°C for 10 minutes to dry the tissue.

A7. 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. Proceed to step 1.

1. Initial separation of RNA and DNA

1.1. 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).

1.2. Incubate the tube in a heat block at 72°C for 30 minutes, then place on ice for 1 min.

1.3. Add an additional 80 uL of RNAstorm™ FFPE Lysis Buffer to the tube, and 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.

1.4. Incubate in a heat block at 72°C for 30 minutes (see notes at end of protocol for potential optimization of this step).

1.5. Place tube on ice for 3 minutes.

1.6. Centrifuge tube for 15 minutes at 16,000 x g. A pellet will form, which contains DNA and cellular debris. The RNA will remain in the supernatant.

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

Note: Do not discard the original sample tube containing the pellet.

a. The tube containing the supernatant is the RNA sample tube that will be processed in step 2.

b. The tube containing the pellet is the DNA sample tube that will be processed in step 3.

2. RNA extraction

2.1. Uncrosslinking and lysis

2.1.1. Incubate the RNA sample tube (supernatant) for an additional 1.5 hours at 72°C.

Note: The total incubation time at 72°C after adding protease should be 2 hours (including the 30-minute incubation in step 1.4).

2.1.2. Remove the tube from the heat block and centrifuge briefly to collect the contents at the bottom of the tube.

2.1.3. Add 150 uL of CELLDATA FFPE Binding Buffer to the tube, and then add 450 uL ethanol. Mix well by inverting the tube several times.

2.1.4. Promptly transfer the contents of the tube to a spin column.

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

2.2. DNase I treatment of RNA (recommended)

This step ensures that any contaminating genomic DNA is degraded. To skip DNase I treatment, proceed to step 2.3.

2.2.1. 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.

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

2.2.3. Mix 70 uL DNase I 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.

2.2.4. Add the remaining 300 uL of the Binding Buffer/ethanol mixture (prepared in Step 2.2.1) to the spin column. Centrifuge for 30 seconds at 16,000 x g and discard the flow-through.

2.3. Continue RNA isolation

2.3.1. Add 500 uL of CELLDATA Wash Buffer to the spin column and centrifuge for 30 seconds at 16,000 x g. Discard the flow-through.

2.3.2. Wash the spin column again by repeating the previous step.

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

2.3.4. Place the column in a clean 1.5 mL tube.

2.3.5. Elute the pure RNA by adding 50 uL of RNase-free water to the center of the membrane of the spin column. Let stand for 1 minute. Centrifuge for 1 minute at 16,000 x g. The eluted RNA will be in the flow-through.

2.3.6. Optional: Repeat the elution step for a higher RNA yield but at a lower concentration.

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

3. DNA extraction

3.1. Uncrosslinking and lysis

3.1.1. To the pellet from step 1.7, add 200 uL of DNAstorm™ FFPE CAT5™ Lysis Buffer.

3.1.2. Add 20 uL of Proteinase K to the tube and mix briefly using a pipette, then centrifuge at 16,000 x g for 30 seconds.

3.1.3. Incubate the tube in a heat block at 56°C for 1 hour.

3.1.4. Move the tube to a heat block at 80°C and incubate for 4 hours.

3.1.5. Remove the tube from the heat block and allow the sample to cool to down room temperature.

3.1.6. Centrifuge the tube briefly to collect the contents at the bottom of the tube.

3.1.7. Using a pipette, carefully transfer as much of the supernatant as possible to a new tube, without disturbing the pellet. At this stage, the supernatant contains the DNA. Discard the pellet.

3.2. RNase treatment of DNA (recommended)

This step ensures that any contaminating RNA is degraded by incubating the sample with RNase A. To skip this step, proceed to step 3.3.

3.2.1. Add 10 uL of RNase A to the supernatant tube from step 3.1.7 and invert the tube gently to mix. Incubate at room temperature for 15 minutes.

3.3. Continue DNA isolation

3.3.1. To the tube with DNA from the previous step, add 200 uL of CELLDATA FFPE Binding Buffer, and then add 600 uL ethanol. Mix well by inverting the tube several times.

3.3.2. Promptly transfer 700 uL from the tube to a spin column.

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

3.3.4. Transfer the remaining content of the tube to the spin column and repeat centrifugation in step 3.3.3.

3.3.5. Add 500 uL of CELLDATA Wash Buffer to the spin column and centrifuge for 30 seconds at 16,000 x g. Discard the flow-through.

3.3.6. Wash again by repeating the previous step.

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

3.3.8. Place the column in a clean 1.5 mL tube.

3.3.9. Elute the DNA by adding 50 uL of Tris or Tris-EDTA buffer (pH 8) or nuclease-free water to the center of the membrane of the spin column. Centrifuge for 1 minute at 16,000 x g. The eluted DNA will be in the flow-through.

3.3.10. Optional: Repeat the elution step for a higher DNA yield but at a lower concentration.

3.3.11. Eluted DNA should be stored at -20°C.

Notes: The incubation period in step 1.4 can be adjusted depending on relative DNA and RNA yields:

a. If the RNA yield is high but the DNA yield is low, reduce the incubation time in step 1.4 to no less than 15 minutes.

b. If the DNA yield is good but the RNA yield is low, increase the incubation time in step 1.4 to no more than 2 hours.

c. The total incubation time with RNAstorm™ FFPE Protease at 72°C should be 2 hours.

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.
Is there any contaminating RNA in the DNA obtained using the DNAstorm™ kit?	Contamination from RNA is eliminated by performing an optional RNase digestion step immediately following the lysis step.
How much RNA and DNA can I expect to obtain from an FFPE sample?	The biggest variable that affects the total amount of RNA and DNA 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 DNAstorm™ kit, and assuming at least reasonable sample quality, amounts greater than 1 ug of RNA and 1 ug of DNA 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.
Can DNA obtained using the DNAstorm™ kit be used in next-generation sequencing?	Yes. Good quality libraries can be obtained, providing that the DNA is of sufficiently high quality.
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 4 sections (10 um each) should be used for each extraction. Using too much tissue can lead to incomplete digestion and reduced yields.
Can tissue that is not paraffin-embedded be used?	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 FFPE cores be used?	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?	CELLDATA FFPE Kits include a recommended Dewaxing Solution. The Dewaxing Solution removes wax without a separate wash step, is less hazardous than xylenes, and does not need to be handled in a fume hood.
How can I evaluate the integrity of the DNA I obtained?	Due to the wide size distribution of DNA isolated from FFPE tissue samples, we recommend using pulsed-field gel electrophoresis (PFGE). Methods based on capillary electrophoresis such as the Agilent Bioanalyzer can also be used, but may not properly resolve high molecular weight fragments (greater than 10 kb) in better-quality samples.
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.

Frequently Asked Questions (FAQs), continued

Why does my extracted DNA fail to amplify properly? I notice a lot of PCR inhibition and/or Ct values that make no sense.	PCR inhibition is often observed when high amounts of FFPE-extracted template DNA are used. The inhibition is usually not due to the presence of contaminants, but results from residual chemical modifications and damage in the DNA itself. Several simple adjustments to the PCR protocol can overcome this issue. First, the amount of template DNA should be decreased. Second, the amount of PCR polymerase should be increased by 2-4X. Third, the annealing and extension times should be extended. Fourth, the amount of dNTPs can be increased. An in-depth discussion of this issue is found in Dietrich et al. (2013), PLoS ONE 8(10): e77771.

Related Products

Cat. No.	Product
CD504	CELLDATA RNAstorm™ Fresh Cell and Tissue RNA Isolation Kit
CD506	CELLDATA RNAstorm™ 2.0 FFPE RNA Extraction Kit
CD507	CELLDATA DNAstorm™ 2.0 FFPE DNA Extraction 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
41011	GelRed® Prestain Plus 6X DNA Loading Dye
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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