Preparing FFPE Samples for Analysis Using the SOLiD™ SAGE™ Kit

Part Number MAN0003612 Rev. A

Introduction

This instruction sheet provides a protocol for extracting total RNA from formalin-fixed, paraffin-embedded (FFPE) tissue samples for use with the SOLiD™ SAGE™ Kit (Part no. 4452811) or SOLiD™ SAGE™ Kit with Barcoding Adaptor Module (Part no. 4443475). This method uses the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Part no. AM1975).

The total RNA obtained from this protocol may be used directly in the SOLiD™ SAGE™ workflow, as described in the *SOLiD™ SAGE™ Kit Guide* or *SOLiD™ SAGE™ Kit with Barcoding Adaptor Module Guide*.

Materials Required

- Microtome
- Razor blades
- Forceps
- RNase AWAY™ Reagent (Invitrogen Part no. 10328011)
- RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Applied Biosystems or Invitrogen Part no. AM1975)
- 1.5 mL microcentrifuge tubes (e.g., Applied Biosystems AM12400, AM12450; for initial digestion)—these do not have to be nuclease-free
- 100% xylene, ACS grade or higher quality
- 100% ethanol, ACS grade or higher quality
- Adjustable pipettors and RNase-free tips
- Microcentrifuge capable of at least 10,000 x g
- Incubators or heat blocks (deep well preferred) set at 50°C, 80°C, and 95°C
- ***Optional:*** Non-stick tubes (e.g., Applied Biosystems Part no. AM12450), for long-term storage of recovered nucleic acid
- ***Optional:*** Centrifugal vacuum concentrator

For RNA analysis

- RNA quantification method, such as the Qubit® Fluorometer (Invitrogen Part no. Q32866 or Q32867) with a Qubit™ RNA Assay Kit (Invitrogen Part no. Q32852 or Q32855) or UV absorbance on a NanoDrop® spectrophotometer
- RNA qualification method, such as a denaturing agarose gel or the Agilent 2100 Bioanalyzer™ Instrument.

Amount of FFPE Sample Needed

SOLiD™ SAGE™ library preparation typically requires 5–10 µg of high-quality total RNA, though higher amounts (up to 20 µg) may enhance library robustness. While the expected RNA yield from FFPE samples can vary greatly, we recommend starting with **four RecoverAll™ purifications per FFPE sample** to obtain the recommended quantity of total RNA.

Note: Lower amounts of RNA input may be used, but may require additional amplification cycles in the PCR check step of the SOLiD™ SAGE™ workflow.

FFPE Sample Preparation

Follow the steps below to slice 10–12-µm sections from a paraffin block using a microtome. Alternatively, you can core a ≤ 35-mg sample from the interior of the block.

1. Thoroughly clean the microtome, new razor blades, and forceps. Replace the microtome blade (if necessary) and clean.

2. Treat all microtome cutting surfaces with RNase AWAY™ Reagent and allow to air dry for 2 minutes.
3. Adjust the microtome to make slices that are 10–12 µm thick. Remove and discard 3–4 slices from the block to be sure that you are getting a slice that has not been exposed to the atmosphere during storage. The tissue should be visible in the center of the wax slice; if not, remove a few more test slices.
4. Cut ≤80 µm of slices (i.e., up to eight 10-µm slices) from the block and collect them in a 1.5-mL Eppendorf tube. Repeat this procedure three more times, collecting each group of slices in a separate tube.
5. Proceed to RecoverAll™ Purification.

Purification Using the RecoverAll™ Kit

Process the four sample tubes in parallel as described in this section.

Isolate total RNA using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE. For detailed protocol instructions, as well as safety information and additional guidelines, see the user manual provided with the RecoverAll™ kit. This instruction sheet contains a basic protocol for RNA purification using the kit.

Note: For safety and biohazard guidelines, refer to the "Safety" section in the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE protocol. For every chemical, read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Precautions

To avoid RNase contamination, we recommend cleaning the lab bench and pipettors with RNase AWAY™ Reagent. Wear gloves and use RNase-free pipette tips for all procedures.

Before Starting

Prepare the wash solutions from the concentrates provided in the RecoverAll™ kit:

1. Add 42 mL of ACS grade 100% ethanol to the bottle labeled Wash 1 Concentrate. Mix well.
2. Add 48 mL of ACS grade 100% ethanol to the bottle labeled Wash 2/3 Concentrate. Mix well.
3. Cap the wash solution bottles tightly to prevent evaporation.
4. Mark the labels to indicate that the ethanol has been added.

The final solutions are referred to as "Wash 1" and "Wash 2/3" in the following procedure.

RecoverAll™ Procedure

Treat with Xylene to remove paraffin

1. Add 1 mL 100% xylene to each sample.

   WARNING: Xylene is a toxic substance. Read the manufacturer's MSDS. Handle it only in a well ventilated area using personal protection equipment. Dispose of xylene waste according to applicable regulations.
2. Vortex briefly to mix.
3. Centrifuge briefly to bring any tissue that is stuck to the sides of the tube down into the xylene.
4. Heat the sample for 3 minutes at 50°C to melt the paraffin.
5. Centrifuge the sample for 2 minutes at room temperature and maximum speed to pellet the tissue.
6. ***Optional:*** If the sample does not form a tight pellet, recentrifuge for an additional 2 min. If a tight pellet still does not form, then proceed with caution in the following step.
7. Remove the xylene without disturbing the pellet. Discard the xylene according to applicable regulations.

Ethanol wash

8. Add 1 mL of 100% ethanol (room temperature) to the sample and vortex to mix. The tissue should turn opaque.
9. Centrifuge the sample for 2 minutes at room temperature and maximum speed to pellet tissue.
10. Remove and discard the ethanol without disturbing the pellet. The ethanol will contain trace amounts of xylene, and must be discarded accordingly.
11. Repeat steps 8–10 above to wash a second time with 1 mL of 100% ethanol.
12. Briefly centrifuge again to collect any remaining drops of ethanol. Remove as much residual ethanol as possible without disturbing the pellet.
13. Dry in a centrifugal vacuum concentrator at 40–45ºC for ≤20 minutes or 37–40ºC for 20–40 minutes. You may also air dry the pellet for 45 minutes at room temperature, though larger tissue sections may not dry completely.

Digest with Protease

14. Add 200 µL Digestion Buffer to each sample.
15. Add 4 µL Protease to each sample.
16. Swirl the tube gently to mix and immerse the tissue. If the tissue sticks to the sides of the tube, use a pipette tip to push it into the solution, or briefly centrifuge to bring the tissue down into the solution.
17. Incubate the sample in heat blocks for 15 minutes at 50°C, then 15 minutes at 80°C to clarify the sample.

Note: Extending the incubation at 80°C more than 2 minutes may result in RNA degradation.

Transfer sample to Filter Cartridge

18. In a separate tube, combine 2.3 mL 100% ethanol with 1 mL Isolation Additive (enough for four samples, plus overage).
19. Add 790 µL of the Isolation Additive/ethanol mixture to each sample and pipette up and down.
20. For each sample, place a Filter Cartridge in one of the Collection Tubes supplied in the kit.
21. Pipet up to 700 µL of each sample mixture onto the Filter Cartridge and close the lid. Avoid pipetting large pieces of undigested tissue onto the Filter Cartridge.
22. Centrifuge at 10,000 x g (typically 10,000 rpm) for 30 sec to pass the mixture through the filter.
23. Discard the flow-through, and re-insert the Filter Cartridge in the same Collection Tube.
24. Repeat steps 20–23 until all the sample mixture has passed through the filter.

Wash

25. Add 700 µL of Wash 1 to the Filter Cartridge.
26. Centrifuge for 30 sec at 10,000 x g to pass the mixture through the filter.
27. Discard the flow-through, and re-insert the Filter Cartridge in the same Collection Tube.
28. Add 500 µL of Wash 2/3 to the Filter Cartridge.
29. Centrifuge for 30 sec at 10,000 x g to pass the mixture through the filter.
30. Discard the flow-through, and re-insert the Filter Cartridge in the same Collection Tube.
31. Spin the assembly for an additional 30 sec to remove residual fluid from the filter.

Treat with DNase

32. In a separate tube, combine 27 µL 10X DNase Buffer, 18 µL DNase, and 225 µL nuclease-free water (enough for four samples, plus overage).
33. Add 60 µL of the DNase mix to the ***center*** of each Filter Cartridge.
34. Cap the tube and incubate for 30 minutes at room temp (22–25°C).

Final wash

35. Add 700 µL of Wash 1 to the Filter Cartridge.
36. Incubate for 30–60 sec at room temperature.
37. Centrifuge for 30 sec at 10,000 x g.
38. Discard the flow-through, and re-insert the Filter Cartridge in the same Collection Tube.
39. Add 500 µL of Wash 2/3 to the Filter Cartridge.
40. Centrifuge for 30 sec at 10,000 x g.
41. Discard the flow-through, and re-insert the Filter Cartridge in the same Collection Tube.
42. Repeat steps 39-41 to wash a second time with 500 µL of Wash 2/3.
43. Centrifuge the assembly for 1 minute at 10,000 x g to remove residual fluid from the filter.

Elute

44. Transfer the Filter Cartridge to a fresh Collection Tube.
45. Apply 60 µL of room-temperature Elution Solution or nuclease-free water to the center of the filter, and close the cap.
46. Allow the sample to sit at room temperature for 1 min.
47. Centrifuge for 1 minute at maximum speed to pass the mixture through the filter. The eluate contains the RNA.
48. Pool the elutions into a single tube. Store at –20°C or colder. To store your sample for an extended period of time, transfer the eluate to a non-stick tube to prevent loss of nucleic acid.

RNA Quantification and Qualification

You can quantitate the total RNA using a Qubit® Fluorometer with a Qubit™ RNA Assay Kit or UV absorbance on a NanoDrop® spectrophotometer.

You can assess the quality of the RNA by agarose gel electrophoresis (visit www.ambion.com/techlib/append/supp for a protocol) or by analysis on the Agilent® 2100 Bioanalyzer™ Instrument. On the Bioanalyzer™ Instrument, the RNA should have a RNA Integrity Number (RIN) of >7.0. On a denaturing gel, the RNA should have distinct 28S and 18S ribosomal RNA bands.

Proceed to SOLiD™ SAGE™ Library Preparation

Following analysis of the total RNA, proceed with the SOLiD™ SAGE™ protocol as described in the *SOLiD™ SAGE™ Kit Guide* or *SOLiD™ SAGE™ Kit with Barcoding Adaptor Module Guide*.

Trademarks

For Research Use Use Only. Not intended for any animal or human therapeutic or diagnostic use.

Notice to Purchaser: Please refer to the Ambion RecoverAll™ Kit, , SOLiD™ SAGE™ Kit, and/or SOLiD™ SAGE™ Kit with Barcoding Adaptor Module protocols and manuals for limited label license or disclaimer information.

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