Applied Biosystems SOLiD™ 3 System

SOLiD™ SAGE™ Guide

SAGE™ Tag Preparation | Templated Bead Preparation | Instrument Operation

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Experienced Users Protocol

Note

The following protocol is designed for experienced users of this kit. It omits the detailed instructions, cautions, and notes aimed at the new user. If you are performing the procedure for the first time or are uncertain of any of the steps, follow the more detailed procedures in this manual.

Preparing RNA and Beads

See pages 5–9 for a detailed protocol.

1. Warm the Lysis/Binding Buffer to room temperature to dissolve any precipitate or cloudiness.
2. Isolate 1–10 µg of high-quality total RNA using TRIzol® Reagent or comparable method. Select for non-poly(A) RNA if desired (page 27)

   Optional: Treat total RNA with DNase I and RNaseOUT™ as described on page 7 and resuspend in 450 µL of Lysis/Binding Buffer.
3. Resuspend the Dynabeads® Oligo(dT) EcoP and transfer 100 µL of beads to an RNase-free 1.5-mL nonstick or low-bind microcentrifuge tube.
4. Wash the 100 µL of beads *twice* with 200 µL of Lysis/Binding Buffer.
5. Add 200 µL of Lysis/Binding Buffer to the isolated total RNA (*omit this step* for RNA treated with DNase I and RNaseOUT™)
6. Place the prepared Dynabeads® on the magnetic stand and remove the liquid.
7. Add the RNA in Lysis/Binding Buffer to the beads.
8. Mix the beads and the RNA by slowly rotating the tube on a rocking platform or gentle vortexing the tube intermittently for 15–30 minutes.
9. Wash the beads *three times* with 200 µL of Wash Buffer A.
10. Wash the beads *once* with 200 µL of Wash Buffer B.
11. Prepare 210 µL of 1X First-Strand Buffer by diluting 42 µL of 5X First-Strand Buffer provided in the kit in 168 µL of DEPC-treated water
12. Wash the beads *once* with 200 µL of 1X First-Strand Buffer.

cDNA Synthesis

See pages 10–12 for a detailed protocol.

1. For each reaction, prepare the following on ice.

5X First-Strand Buffer	18.0 µL
RNaseOUT™ Recombinant Ribonuclease Inhibitor	1.0 µL
0.1 M DTT	9.0 µL
dNTP Mix (10 mM each)	4.5 µL
DEPC-treated Water	54.5 µL
Total Volume	87.0 µL

2. Place the beads on the magnetic stand, remove the liquid, and add the reaction mix. Mix gently and pulse spin to collect.
3. Incubate at 42°C for 3 minutes.
4. Add 3 µL of SuperScript® III Reverse Transcriptase. Mix and incubate at 45°C for 1–2 hours, mixing gently every ~15 minutes.

cDNA Synthesis continued on next page

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cDNA Synthesis, continued

cDNA synthesis continued from previous page

5. Chill the reaction on ice for 2 minutes, then add the following:

5X Second Strand Buffer	80 µL
DEPC-treated water	215 µL
dNTP Mix (10 mM each)	15 µL
E. coli DNA Ligase	5 µL
E. coli DNA Polymerase	20 µL
E. coli RNase H	5 µL

6. Mix, then incubate at 16°C for 2–3 hours. Mix gently every ~15 minutes.
7. Place the beads on ice and add 45 µL of 0.5 M EDTA to stop the reaction.
8. Warm the Wash Buffer C bottle in a water bath at 37°C to dissolve the precipitate.
9. Place the beads on the magnetic stand, remove the liquid, and add 500 µL of warm Wash Buffer C. Mix gently.
10. Incubate at 72–75°C for 12–15 minutes to inactivate the polymerase.
11. Wash the beads *three times* with 200 µL of Wash Buffer D.
12. **Optional:** Remove 5 µL of beads and store at 4°C to QC the cDNA synthesis reaction.

Stopping Point: If you do not have enough time to complete the *Nla* III digestion, you may store the beads in Wash Buffer D at 4°C overnight.

Nla III Digestion

See page 13 for a detailed protocol.

1. Prepare 410 µL of 1X Buffer 4 per reaction by diluting 41 µL of 10X Buffer 4 in 369 µL of DEPC-treated water.
2. Wash the beads once with 200 µL of 1X Buffer 4.
3. Transfer the beads to a new nonstick or low-bind microcentrifuge tube. If necessary, gently scrape off the beads from the old tube using a pipette tip.
4. Rinse the old tube with 200 µL of 1X Buffer 4 and transfer to the beads.
5. In a separate RNase-free tube, prepare the following mix per reaction.

10X Buffer 4	20 µL
100X BSA	4 µL
DEPC-treated Water	166 µL
Total Volume	190 µL

6. Place the beads on the magnetic stand, remove the liquid, and add the reaction mixture. Mix gently, then pulse spin to collect.
7. Add 10 µL of *Nla* III. Mix gently, then pulse spin to collect.
8. Incubate at 37°C for 2 hours. Mix gently every ~15 minutes.
9. After incubation, place the beads on the magnetic stand, remove the liquid, and wash the beads *once* with 200 µL of warm Wash Buffer C.
10. Wash the beads *twice* with 200 µL of Wash Buffer D.
11. **Optional:** Remove 5 µL of beads and store at 4°C to QC the *Nla* III digestion.

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Adapter A Ligation

See page 14 for a detailed protocol.

1. In a separate tube, prepare 210 µL of 1X Ligase Buffer per reaction by diluting 42 µL of 5X Ligase Buffer in 168 µL of DEPC-treated water.
2. In another RNase-free tube, prepare the following mix per reaction.

5X Ligase Buffer	5 µL
Adapter A	1.5 µL
DEPC-treated water	16 µL
Total volume	22.5 µL

3. Wash the beads *once* with 200 µL of 1X Ligase Buffer.
4. Place the beads on the magnetic stand, remove the liquid, and add the 22.5-µL ligase reaction mix from Step 1.
5. Incubate at 50°C for at least 4 minutes and then at room temperature for 10 minutes. Then place on ice to chill.
6. Add 2.5 µL of T4 DNA Ligase (5 U/µL). Mix gently, then pulse spin.
7. Incubate at 16°C for 2 hours up to 16 hours. Mix gently every ~15 minutes for at least the first 2 hours.
8. After incubation, add 200 µL of warm Wash Buffer C directly to the reaction. Mix gently, then pulse spin to collect.
9. Wash the beads *three times* with 200 µL of Wash Buffer D.

*Eco*P15I Digestion

See pages 15–16 for a detailed protocol.

1. Prepare 210 µL of 1X Buffer 3 per reaction by diluting 21 µL of 10X Buffer 3 in 189 µL of DEPC-treated water.
2. Wash the beads once with 200 µL of 1X Buffer 3.
3. Place the beads on the magnetic stand, remove the liquid, and add the following reagents in order. Prepare a master mix for multiple reactions.

10X Buffer 3	10 µL
DEPC-treated water	68 µL
10X ATP	10 µL
100X BSA	1 µL
Sinefugin	0.4 µL

4. Add 10 µL of *Eco*P15I to the tube. Mix by mild vortexing.
5. Incubate at 37°C for 2 hours to overnight. Mix gently every ~15 minutes for at least the first 2 hours.

   Do not discard the supernatant! This contains your SAGE tags.
6. Place the tube on a magnetic stand and carefully transfer the supernatant containing the SAGE tags to a new RNase-free 1.5-mL microcentrifuge tube.
7. To ensure that you transfer all the SAGE tags, add 100 µL of LoTE to the beads. Remove from the magnet, mix, pulse spin, place the tube on the magnet and transfer this 100 µL to the tube containing the SAGE tags.
8. **Optional:** Add another 100 µL of LoTE to the beads and store the beads at 4°C for additional QC analysis.
9. Remove 5 µL of the SAGE tags for use as a no-ligase control.

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Ethanol Precipitation

See page 16 for a detailed protocol.

1. Add 200 µL of UltraPure™ Phenol:Chloroform:Isoamyl Alcohol to the SAGE tags.
2. Mix by high-speed vortexing, and spin at 10,000 × *g* for at least 5 minutes.
3. Transfer the top 200 µL aqueous phase to a new RNase-free microcentrifuge tube, and add the following:

Glycogen	2 µL
3M Na Acetate	20 µL
100% Ethanol	~650 µL

4. Mix thoroughly and place on dry ice or at –80°C for at least 30 minutes.
5. Centrifuge at ≥14,000 × *g* for 30 minutes.
6. Wash the pellet at least once with 500 µL of 80% ethanol.
7. Centrifuge at ≥14,000 × *g* for 5 minutes.
8. Carefully invert the sample tube at an angle on a clean kimwipe or paper towel and allow the ethanol to drain.
9. Air dry the pellet for ~30 minutes or until the pellet is translucent.

Adapter B Ligation

See page 17 for a detailed protocol.

1. Prepare the following Adapter B mix per reaction. (Scale the complete ligation mix, including T4 DNA Ligase, to 30 µL if necessary to dissolve the pellet.)

DEPC-treated water	12 µL
5X Ligase Buffer	4 µL
Adapter B	2 µL

2. Fully resuspend the pellet in the Adapter B mix. Use gentle pipetting and/or incubation at 37°C in a water bath to dissolve the pellet.
3. Add 2 µL of T4 DNA Ligase and incubate at 16°C for 2–16 hours (*i.e.*, overnight if necessary).
4. Store the SAGE tags at –20°C until use.

Template Qualification and Quantification

See pages 18–21 for detailed instructions.

Determine the quality of the SAGE tags by PCR using the reagents provided in the kit, followed by gel electrophoresis. Template should appear as a 100-bp band. No-template and no-ligase control lanes should contain no bands.

Determine template quantity as described in Appendix B of the **Applied Biosystems SOLiD™ 3 System Library Preparation Guide**.

The SOLiD™ DNA fragment library workflow requires 150–240 pg of sample. We recommend generating at least 400–500 pg of tags per SOLiD™ SAGE™ reaction. (Perform PCR scale-up and gel purification if necessary.)

Kit Contents and Storage

Kit Modules

The SOLiD™ SAGE™ Kit (catalog no. 4443475) is shipped in three separate modules, which should be stored as listed below. Sufficient components are provided for constructing 8 libraries.

Module A

Shipped on ice. **Store components at 4°C.**

Item	Amount
Dynabeads® Oligo(dT) EcoP, 5 mg/mL	1 mL
Lysis/Binding Buffer	10 mL
Wash Buffer A	12 mL
Wash Buffer B	7 mL
Wash Buffer C	12 mL
Wash Buffer D	14 mL
DEPC-treated Water	12 mL
LoTE	3 mL
3 M Na Acetate	1 mL

Module B

Shipped on dry ice. **Store components at –20°C.**

Item	Amount
5X First-Strand Buffer	1 mL
0.1 M DTT	100 µL
dNTP mix (10 mM each dNTP)	1 mL
RNaseOUT™ Recombinant Ribonuclease Inhibitor	30 µL
SuperScript® III Reverse Transcriptase	50 µL
5X Second-Strand Buffer	700 µL
E. coli DNA Ligase	100 µL
E. coli DNA Polymerase	200 µL
E. coli RNase H	100 µL
10X Buffer 4	1 mL
100X BSA	100 µL
Adapter A	50 µL
Adapter B	50 µL
5′ AMP Primer	90 µL
3′ AMP Primer	90 µL
EDTA 0.5 M	450 µL
Glycogen 20 mg/mL	180 µL
Platinum® PCR Supermix High Fidelity	3 × 1 mL
T4 DNA Ligase, 5 U/µL	90 µL
5X Ligase Buffer	1 mL

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Module B, continued

Item	Amount
10X ATP	225 µL
10X Buffer 3	450 µL
DNase I, Amplification Grade, 1 U/µL	25 µL
10X DNase I Buffer	1 mL
*Eco*P15I, 10 U/µL	80 µL
Sinefungin	20 µL
GAPDH Forward Primer, 100 ng/µL	50 µL
GAPDH Reverse Primer, 100 ng/µL	50 µL
EF1 alpha Forward Primer, 100 ng/µL	50 µL
EF1 alpha Reverse Primer, 100 ng/µL	50 µL

Module C

Shipped on dry ice. **Store at –80°C.**

Item	Amount
Nla III	3 × 45 µL

Dynabeads® Oligo(dT) EcoP Sequence

The following sequence is bound to each Dynabead® bead.

```
5′-CTGATCTAGAGGTACCGGATCCCAGCAGTTTTTTTTTTTTTTTTTTTTTTTT-3′
```

Adapter Sequences

The adapters are modified with an amino group at the 3′ end to prevent self-ligation. Adapter A includes an *Eco*P15I recognition site for binding of the Type III restriction endonuclease.

Adapter A (100 ng/µL)

```
      5′-CTGCCCCGGGTTCCTCATTCTCTCAGCAGCATG-3′
3′–amine-GACGGGGCCCAAGGAGTAAGAGAGTCGTC–Phos-5′
```

Adapter B (100 ng/µL)

```
      5′–CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGAT–3′
3′–amine-GGTGATGCGGAGGCGAAAGGAGAGATACCCGTCAGCCACTANN–Phos-5′
```

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Primer Sequences

The table below lists the sequence and concentration of the primers included in this kit.

Primer	Sequence	Concentration
5′ AMP Primer	5′–CCACTACGCCTCCGCTTTCCTCTCTATG–3′	300 ng/µl
3′ AMP Primer	5′–CTGCCCCGGGTTCCTCATTCT–3′	300 ng/µl
EF1 alpha Sense Primer	5′-CATGTGTGTTGAGAGCTTC-3′	100 ng/µl
EF1 alpha Anti-sense Primer	5′-GAAAACCAAAGTGGTCCAC-3′	100 ng/µl
GAPDH Forward Primer	5′-TTAGCACCCCTGGCCAAGG-3′	100 ng/µl
GAPDH Reverse Primer	5′-CTTACTCCTTGGAGGCCATG-3′	100 ng/µl

Note

It is normal to have some leftover reagents in the kit after constructing eight SOLiD™ SAGE™ libraries. These reagents are supplied in excess to ensure that you have enough reagents to perform your experiments.

Product Qualification

The Certificates of Analysis provide detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to www.invitrogen.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

Additional Materials

! Microcentrifuge Tubes

We strongly recommend using RNase-free nonstick or low-bind microcentrifuge tubes to prevent the beads from sticking to the tube walls—*e.g.*, Ambion Nonstick Microcentrifuge Tubes, 1.5-mL, catalog no. AM12450, *or* Eppendorf DNA LoBind 1.5-mL PCR Tubes, Brinkman cat. no. 022431021.

Note: Eppendorf DNA LoBind tubes are required for the SOLiD™ sequencing workflow (following SAGE™ tag generation)

Required Materials

The following materials must be supplied by the user:

- DynaMag™-Spin or DynaMag™-2 Magnet (ordering information on page 31)
- TRIzol® Reagent, or comparable RNA isolation method (ordering information on page 31)
- RNase-free nonstick or low-bind 1.5-mL microcentrifuge tubes (see note on microcentrifuge tubes, above)
- Automatic pipettors
- Sterile, aerosol-resistant pipette tips
- Microcentrifuge for 1.5-mL tubes
- Vortex mixer
- Thermocycler
- Water baths, heat blocks, and/or incubators
- 100% ethanol
- 80% ethanol
- Phenol:Chloroform:Isoamyl Alcohol (ordering information on page 31)
- Isopropanol (100%)
- Laminar flow hood at two separate locations, for preparing different PCR reactions (see **PCR Guidelines and Precautions** on page 18)
- E-Gel® 4% or comparable agarose gel and associated gel electrophoresis materials and buffers (ordering information on page 31)
- PCR purification kit for purifying 100-bp SOLiD™ SAGE™ tags (*e.g.*, the PureLink™ PCR Micro Kit or equivalent; ordering information on page 31)
- Additional Platinum® PCR SuperMix High Fidelity may be needed, depending on how many PCRs you perform (ordering information on page 31)

Materials for Non-Poly(A) Enrichment

The following materials are needed if you are enriching for non-poly(A) RNA and then poly(A) tailing this fraction for subsequent SOLiD™ SAGE™ analysis, as described on pages 27–28 (ordering information on page 31):

- RiboMinus™ Eukaryote Kit for RNA-Seq
- Dynabeads® Oligo(dT)$_{25}$
- RiboMinus™ Concentration Module
- NCode™ miRNA First-Strand cDNA Synthesis Kit

Description of the System

SOLiD™ SAGE™ Technology

The SOLiD™ SAGE™ kit provides a method for analyzing the genome-wide expression levels of both traditional poly(A) and non-poly(A) transcripts. The system uses Serial Analysis of Gene Expression (SAGE™) to generate a library of 27-bp "tags" for all the transcripts in a cell, followed by SOLiD™ (Sequencing by Oligonucleotide Ligation and Detection) sequencing of the tags and downstream mapping to RefSeq mRNA and genome databases.

SAGE™ is a method developed by Velculescu *et al.*, 1995 and St. Croix *et al.*, 2000 and modified by Saha *et al.*, 2002 for obtaining a digital genome-wide expression profile of the genes involved in normal and disease processes. Unlike microarrays, SAGE™ enables the user to analyze every transcript in a cell without any prior knowledge of the transcript. This digital quantification method is unaffected by PCR or cloning bias, and because each tag sequence represents one copy of a transcript, it has increased dynamic range, reproducibility, and accuracy over analog expression analysis systems (*i.e.*, systems that rely on an indirect method of quantitation, such as array-based hybridization) (Velculescu & Kinzler, 2007).

The original SAGE™ method generates a 10–14 bp unique sequence tag for each transcript in the genome, while the more recent Long SAGE™ method generates 21-bp tags. The SOLiD™ SAGE™ system employs a modified version of the protocol that generates a longer, 27-bp tag per transcript, using *Eco*P15I digestion (Matsumura *et al.*, 2005; Matsumura *et al.*, 2003; Raghavendra & Rao, 2005). The longer sequence allows for the identification of more unique transcripts, while the novel adapters ligated to the ends of each tag are specifically designed for downstream sequencing by Applied Biosystems' SOLiD™ platform.

Previous SAGE™ methods required week-long protocols involving the ligation of tags into longer sequences, followed by cloning, transformation, and DNA isolation before sequencing. The SOLiD™ SAGE™ protocol has a greatly streamlined workflow that eliminates all downstream concatenation and cloning steps, and enables direct sequencing of the individual tags following emulsion PCR. Use of the SOLiD™ platform allows for massive parallel sequencing of 2–4 million RefSeq mapped tags per SOLiD™ SAGE™ reaction on a single slide (octet well). The system also requires smaller amounts of tag template (150–240 pg).

For more details on SAGE™, please refer to published reviews (Hu & Polyak, 2006; Velculescu *et al.*, 2000). For a glossary of terms used in this manual, please see page 4.

Applications

The SOLiD™ SAGE™ kit can be used to:

- Obtain a comprehensive, digital gene expression profile of a specific tissue or cell type
- Identify novel genes
- Characterize and compare transcriptomes
- Reduce the number of matches to multiple unigene clusters identified by traditional SAGE™ techniques
- Provide more sequence information (27 bp) per transcript for enhanced mapping to genomic DNA

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Description of the System, Continued

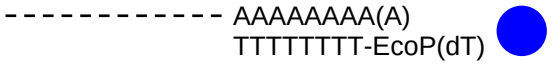
Principles

SOLiD™ SAGE™ is based on two major principles:

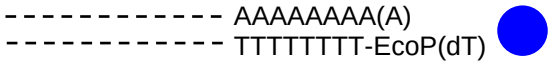
- A sequence tag cleaved from the 3′-most *Nla* III restriction site in each transcript contains sufficient information to uniquely identify the transcript.
- The expression level of the transcript can be quantified by the number of times a particular tag is observed (one tag = one transcript).

System Summary

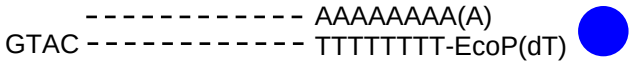
1. **RNA Binding:** Bind purified total RNA to Dynabeads® Oligo(dT) EcoP magnetic beads. The beads capture poly(A) RNA directly from total RNA.



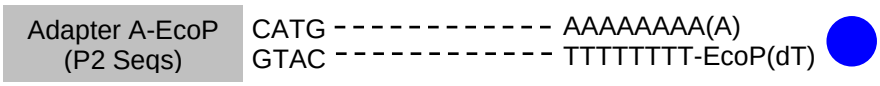
2. **cDNA Synthesis:** Synthesize double-stranded cDNA from the RNA on the beads using SuperScript® III Reverse Transcriptase and *E. coli* DNA polymerase. Performing all the enzymatic steps in one tube enhances the efficiency of cDNA synthesis.



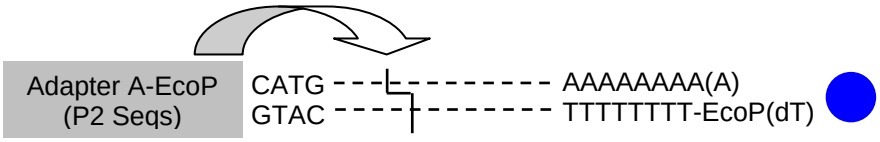
3. ***Nla* III Digestion:** Digest the double-stranded cDNA with *Nla* III, a sequence-specific restriction endonuclease that cleaves ~99% of all human transcripts in RefSeq. *Nla* III is used as an anchoring enzyme, because *Nla* III sites are known to occur approximately every 250 bp.



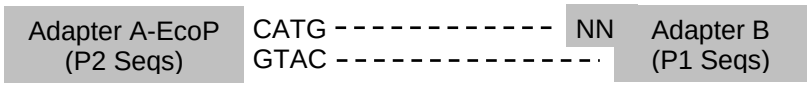
4. **Adapter A Ligation:** Adapter A contains a cohesive 4-bp overhang complementary to the *Nla* III-digested cDNA, an *Eco*P15I restriction enzyme recognition site at the 3′ end, and a priming site for PCR amplification (P2).



5. ***Eco*P15I Digestion:** *Eco*P15I is a Type III restriction endonuclease used as the tagging enzyme. *Eco*P15I binds to a recognition sequence in the adapter adjacent to the CATG site and cleaves the cDNA ~27 bp downstream from the adapter, releasing a tag with a 2-bp overhang. The tag consists of 33 bp of adapter sequence and 27 bp of unique sequence from a single transcript.



6. **Adapter B Ligation:** Adapter B contains the other PCR priming site (P2) and SOLiD™ sequencing initiation sites.



7. **PCR Check:** Purify and PCR amplify if necessary. Proceed to emulsion PCR and SOLiD™ sequencing, followed by analysis using SOLiD™ SAGE™ software.

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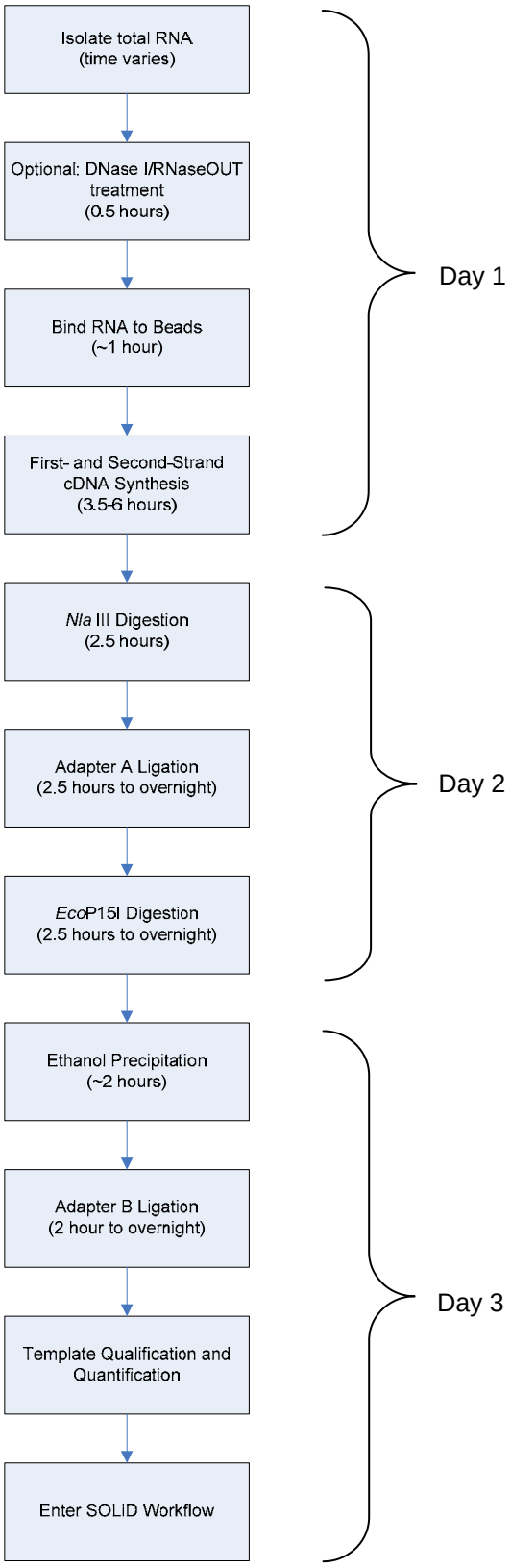
Workflow

A workflow and one possible timeline for SOLiD™ SAGE™ tag generation is provided at the right. Note that there are several incubation steps that may be performed overnight if desired. You may also store the beads overnight following cDNA synthesis.



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Glossary

The different terms used in this manual are defined below:

Tag: 27-bp sequence from a given cDNA containing a 3′ proximal *Nla* III recognition site. One tag = one transcript.

Anchoring Enzyme: A restriction endonuclease with a 4-bp recognition site that cleaves almost all transcripts at least once. *Nla* III is used as the anchoring enzyme in this kit.

Tagging Enzyme: A Type III restriction endonuclease that cleaves at a defined distance downstream from the recognition site. *Eco*P15I is used as the tagging enzyme in this kit.

Adapters: Annealed oligonucleotides (33 bp and 41 bp in length) containing a Type III restriction site at the 3′ end, cohesive overhangs complementary to the *Nla* III recognition site, and priming sites for PCR amplification.

General Molecular Biology Techniques

You must be familiar with standard molecular biology techniques to successfully use the SOLiD™ SAGE™ kit. For protocols and guidance on RNA isolation, cDNA synthesis, DNA ligations, and restriction enzyme analysis, refer to *Molecular Cloning: a Laboratory Manual* Sambrook *et al.*, 1989 or *Current Protocols in Molecular Biology* Ausubel *et al.*, 1994.

Procedure

Using Dynabeads®

Introduction

The SOLiD™ SAGE™ protocol uses Dynabeads® for many of the steps in the workflow. Below are standard instructions for using the beads.

! Key Points to Remember

- Do not allow Dynabeads® to dry out. During all wash steps, after removing liquid from the beads, add liquid immediately.
- Use only mild vortexing or finger flicking to mix. We do not recommend pipetting the beads up and down, as the DNA-bead mixture may become quite sticky.
- Never freeze Dynabeads®.
- When removing liquid from Dynabeads®, avoid touching the magnetized bead pellet with the pipette tip. This will disturb the pellet.

Mixing Dynabeads®

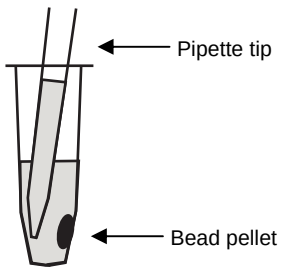
Use a vortex mixer at a low-speed setting of ~2 or 3. Higher vortex speeds will damage the beads.

Resuspending Dynabeads®

To resuspend Dynabeads®, vortex briefly on a very low setting (~2 or 3). Then perform a quick pulse spin on a microcentrifuge to collect the beads.

Washing Dynabeads®

Use the following procedure to remove and add liquid to Dynabeads® whenever the instructions call for washing the beads.



1. Place the tube containing the beads in the magnet and let stand for at least 60 seconds. During this time, the beads will concentrate into a tight pellet along the surface of the tube wall. (If the supernatant appears cloudy near the bottom of the well after a pellet has formed, you can pipet it slowly up and down without the touching the beads to help clear it.)
2. Open the tube without displacing it from the rack or disturbing the bead pellet and carefully extract the liquid volume with a pipette tip *without touching the bead pellet*. Angle the pipette tip away from the bead pellet to avoid contact, as shown in the figure.
3. After the liquid has been removed, remove the tube from the rack and quickly and gently resuspend the beads in the next solution. ***Do not let the beads dry out.***
4. Mix the resuspended beads gently by mild vortexing (setting 2 or 3), then perform a brief pulse spin to collect the contents.

Isolating Total RNA

Items Needed

Provided in the kit

- DNase I, Amplification Grade
- 10X DNase I Buffer
- DEPC-treated water
- RNaseOUT™ Ribonuclease Inhibitor

Provided by the user:

- TRIzol® Reagent, or comparable purification method (see page 31 to order)
- Method for determining total RNA quality
- Method for determining total RNA quantity

Recommended Amount and Isolation Method

We recommend starting with 1–10 µg of high-quality total RNA, isolated from tissues or cells using TRIzol® Reagent (mRNA may also be used).

Note that TRIzol® Reagent preserves the non-poly(A) fraction in the total RNA sample. If you want to poly(A)-tail and profile this population separately, be sure to use a total RNA isolation method that preserves this fraction.

General Handling of RNA

When working with RNA:

- Wear latex gloves while handling reagents, materials, and RNA samples to prevent RNase contamination.
- Use RNase-free microcentrifuge tubes.
- Use disposable, individually wrapped, sterile plasticware for all procedures.
- Use aerosol-resistant pipette tips.
- Use proper microbiological aseptic technique.
- Dedicate a separate set of pipettes, buffers, and enzymes for RNA work, including cDNA synthesis.

You may use RNase *Away*™ Reagent, a non-toxic solution available from Invitrogen (Catalog no. 10328-011) to remove RNase contamination from surfaces. For further information on controlling RNase contamination, see Ausubel, *et al.*, 1990, Unit 4 or Sambrook, *et al.*, 1989, Chapter 7.

Profiling Non-Poly(A) RNA Using SOLiD™ SAGE™

You can use the SOLiD™ SAGE™ kit to profile non-poly(A) RNA. First, you must enrich the non-poly(A) RNA in a sample, and then add a poly(A) tail to it. See page 27 for details.

Note: It is not clear what percentage of small RNA has *Nla* III sites.

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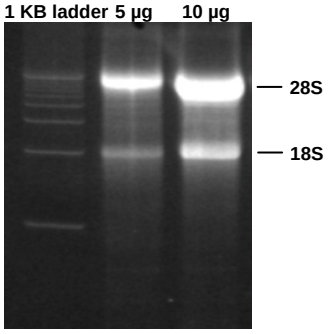
Determining Total RNA Quality

High-quality total RNA is essential for SAGE library construction. Total RNA quality should be analyzed using a bioanalyzer (such as the Agilent 2100 bioanalyzer with an RNA LabChip®) or by agarose gel electrophoresis. On the bioanalyzer, the RNA should have a RNA Integrity Number (RIN) of >7.0. On a 1% agarose gel or denaturing gel, the RNA should have discreet 28S and 18S ribosomal RNA bands. For RNA isolated using TRIzol® Reagent, the 28S band should be >1.5 times the intensity of the 18S band, and mRNA will appear as a smear from 0.5 to 12 kb.

If your RNA does not meet these criteria, repeat the RNA isolation procedure. Be sure to follow the recommendations listed above to prevent RNase contamination.



An example of total RNA isolated from peripheral blood mononuclear cells and analyzed on a 1% E-Gel®.

Determining Total RNA Yield

Total RNA can be quantitated using UV absorbance at 260 nm or a kit such as the Quant-iT™ RiboGreen® RNA Assay Kit (see page 31 for ordering information).

Optional: DNase I and RNaseOUT™ Treatment of the Sample

Following isolation and quantitation, we recommend treating the total RNA with DNase I, Amplification Grade, and RNaseOUT™, both provided in the kit, to eliminate any contaminating genomic DNA and RNases.

1. Warm the Lysis/Binding Buffer to room temperature to dissolve any precipitate or cloudiness.
2. Add the following to 1–10 µg of isolated total RNA to an RNase-free microcentrifuge tube on ice:

Total RNA (1–20 µg)	X µL
10X DNase I Buffer	5 µL
DNase I, Amplification Grade	2.5 µL
RNaseOUT™ Ribonuclease Inhibitor	1 µL
DEPC-treated water	to 50 µL

3. Incubate at room temperature for 15 minutes.
4. Add 450 µL of room-temperature Lysis/Binding Buffer and proceed to **Binding RNA to Dynabeads®**, next page.

Binding RNA to Dynabeads®

Items Needed

Provided in the kit

- Dynabeads® Oligo(dT) EcoP
- Lysis/Binding Buffer
- DEPC-treated Water
- 5X First-Strand Buffer
- Wash Buffer A
- Wash Buffer B

Provided by the user:

- Total RNA, purified by the method of your choice, 1–10 µg
- Water bath or heat block at 37°C
- DynaMag™-Spin or DynaMag™-2 Magnet (see page 31 to order)
- RNase-free 1.5-mL nonstick or low-bind microcentrifuge tubes (see note below)

Important

Before proceeding, see the guidelines for handling and mixing the Dynabeads® on page 5. Do not allow Dynabeads® to dry out and only vortex on a very low setting.

Preparing the Beads

To prepare the beads, wash them twice with Lysis/Binding Buffer as described below.

1. Warm the Lysis/Binding Buffer to room temperature to dissolve any precipitate or cloudiness.
2. Resuspend the Dynabeads® Oligo(dT) EcoP from the kit by mild vortexing (setting 2 or 3).
3. Transfer 100 µL of the resuspended beads to an RNase-free 1.5-mL nonstick or low-bind microcentrifuge tube.
4. Wash the beads *twice* with 200 µL of room-temperature Lysis/Binding Buffer from Step 1, following the protocol for **Washing Dynabeads®** on page 5.

   Always remember to:

   - Let the beads stand in the magnet until they form a tight pellet.
   - Angle the pipette tip away from the beads as shown in the illustration.
   - Do not let the beads dry out (add liquid immediately).
   - Vortex on a very low setting (2 or 3) to avoid damaging the beads.

After washing the beads, proceed to **Binding the RNA**, next page.

Continued on next page

Binding RNA to Dynabeads®, Continued

Binding the RNA

1. In an RNase-free microcentrifuge tube, add 200 µL of Lysis/Binding Buffer to 1–10 µg of total RNA (isolated as described on page 6).

   Note: ***Omit this step*** for RNA treated with DNase I and RNaseOUT™ as described on page 7.
2. Place the tube containing the prepared Dynabeads® (from Step 5, previous page) on a magnetic stand for at least 60 seconds, until the beads have formed a tight pellet.
3. With the tube on the magnet, remove and discard the liquid, being careful not to disturb the bead pellet.
4. Remove the tube from the magnet and add the RNA to the beads.
5. Mix the beads and the RNA sample by slowly rotating the tube on a rocking platform or gently vortexing the tube intermittently on a low setting for 15–30 minutes at room temperature.

Proceed to **Washing the Bound RNA**.

Washing the Bound RNA

1. In a separate RNase-free tube, prepare 210 µL of 1X First-Strand Buffer by diluting 42 µL of 5X First-Strand Buffer provided in the kit in 168 µL of DEPC-treated water, also provided in the kit (note that 200 µL is required; the volume includes overage for ease of pipetting).
2. Wash the beads (from Step 5, above) *three times* with 200 µL of Wash Buffer A, following the protocol for **Washing Dynabeads®** on page 5.
3. Wash the beads once with 200 µL of Wash Buffer B.
4. Wash the beads once with 200 µL of 1X First-Strand Buffer from Step 1.

With the beads resuspended in 1X First-Strand Buffer, proceed immediately to **First-Strand cDNA Synthesis**, next page.

First-Strand cDNA Synthesis

Items Needed

Provided in the kit

- 5X First-Strand Buffer
- RNaseOUT™
- 0.1 M DTT
- dNTP Mix
- SuperScript® III Reverse Transcriptase
- DEPC-treated Water

Provided by the user:

- Thermal cycler or water bath at 42–45°C
- Ice
- Magnetic stand

First-Strand Reaction Mix

For each first-strand cDNA synthesis reaction, prepare the following on ice. Prepare a master mix for multiple reactions.

5X First-Strand Buffer	18.0 µL
RNaseOUT™ Recombinant Ribonuclease Inhibitor	1.0 µL
0.1 M DTT	9.0 µL
dNTP Mix (10 mM each)	4.5 µL
DEPC-treated Water	54.5 µL
Total Volume	87.0 µL

First-Strand cDNA Synthesis

1. Place the tube containing the beads (from Step 6, previous page) on the magnetic stand, and remove and discard the liquid.
2. Add 87 µL of the First-Strand Reaction Mixture, prepared above, to the beads. Vortex gently to mix, then pulse spin to collect the contents.
3. Incubate at 42°C for 3 minutes.
4. Add 3 µL of SuperScript® III Reverse Transcriptase to the tube. Mix gently and incubate at 42°C for 1–2 hours. Mix every ~15 minutes by mild vortexing followed by a pulse spin to collect the tube contents.

   Note: Longer incubation times (up to 2 hours) typically result in more product.
5. Chill the first-strand reaction on ice for 2 minutes and proceed to **Second-Strand cDNA Synthesis,** next page.

Second-Strand cDNA Synthesis

Items Needed

Provided in the kit

- 5X Second-Strand Buffer
- dNTP Mix
- *E. coli* DNA Ligase
- *E. coli* DNA Polymerase
- *E. coli* RNase H
- EDTA 0.5 M
- Wash Buffer C
- Wash Buffer D
- 10X Buffer 4
- DEPC-treated Water

Provided by the user:

- Thermal cycler, water bath, or heat block at 16°C, 37°C, and 72–75°C
- Ice
- Magnetic stand

Second-Strand cDNA Synthesis

1. To the tube containing the first-strand reaction on ice (Step 5, previous page), add the following second-strand reagents. Prepare a master mix for multiple reactions.

5X Second Strand Buffer	80 µL
DEPC-treated water	215 µL
dNTP Mix (10 mM each)	15 µL
E. coli DNA Ligase	5 µL
E. coli DNA Polymerase	20 µL
E. coli RNase H	5 µL

2. Mix by mild vortexing, then incubate at 16°C for 2–3 hours. Mix every ~15 minutes by mild vortexing followed by a pulse spin.
3. Warm the Wash Buffer C bottle in a water bath at 37°C to dissolve any precipitate.
4. After incubation, place the tube containing the beads on ice and add 45 µL of 0.5 M EDTA to stop the reaction.
6. Place the tube on a magnetic stand and remove and discard the liquid.
7. Remove the tube from the magnet and add 500 µL of warm Wash Buffer C. Mix gently by mild vortexing.
8. Incubate at 72–75°C for 12–15 minutes to completely inactivate the polymerase and any trace exonuclease activity.
9. Wash the beads *three times* with 200 µL of Wash Buffer D.
10. **Optional:** Remove 5 µL of resuspended beads and store in an RNase-free nonstick or low-bind tube to QC the cDNA synthesis reaction, if necessary. Store the 5-µL sample at 4°C. See **Optional PCR Check** on page 29.

Stopping Point: If you do not have enough time to complete the *Nla* III digestion, you may store the beads in Wash Buffer D at 4°C overnight.

Proceed to **Washing of Beads from cDNA Synthesis**, next page.

Continued on next page

Washing of Beads from cDNA Synthesis

The following wash procedure is designed to remove all traces of *E. coli* DNA polymerase prior to *Nla* III digestion. (The exonuclease activity in the DNA Polymerase could impact downstream reactions.)

1. In a separate RNase-free tube, prepare 410 µL of 1X Buffer 4 per reaction by diluting 41 µL of 10X Buffer 4 in 369 µL of DEPC-treated water (volume includes overage).
2. Wash the beads once with 200 µL of 1X Buffer 4.
3. Transfer the beads to a new, RNase-free nonstick or low-bind microcentrifuge tube. If necessary, gently scrape off the beads from the old tube using a pipette tip.
4. Rinse the old tube once with 200 µL of 1X Buffer 4 and transfer the contents to the new tube containing the beads.

Proceed to ***Nla* III Digestion**, next page.

Nla III Digestion

Items Needed

Provided in the kit

- 10X Buffer 4
- 100X BSA
- *Nla* III
- Wash Buffer C
- Wash Buffer D
- DEPC-treated Water

Provided by the user:

- Water bath or incubator set at 37°C
- Magnetic stand

Important

Nla III is extremely sensitive to warm temperatures. Do not keep the enzyme at room temperature or +4°C for long periods. Use immediately upon removal from –80°C and return the enzyme to –80°C as soon as possible.

Digesting the cDNA with *Nla* III

Follow the procedure below to cleave the cDNA with *Nla* III:

1. In a separate RNase-free tube, prepare the following mix per reaction. Prepare a master mix for multiple reactions.

10X Buffer 4	20 µL
100X BSA	4 µL
DEPC-treated Water	166 µL
Total Volume	190 µL

2. Place the tube containing the beads (from Step 4, previous page) on a magnetic stand and remove and discard the liquid.
3. Add the 190-µL reaction mixture from Step 1 to the beads. Mix gently by mild vortexing, and pulse spin to collect the contents.
4. Add 10 µL of *Nla* III to the tube. Mix gently by mild vortexing, and pulse spin to collect the contents.
5. Incubate at 37°C for 2 hours. Mix gently every ~15 minutes by flicking the tube with a finger or mild vortexing.

   Note: Warm the bottle of Wash Buffer C in a water bath at 37°C to dissolve any precipitation.
6. After incubation, place the beads on the magnetic stand, remove and discard the liquid, and wash the beads once with 200 µL of warm Wash Buffer C.
7. Wash the beads *twice* with 200 µL of Wash Buffer D.
8. **Optional:** Remove 5 µL of resuspended beads and reserve to QC the *Nla* III digestion, if necessary. Store the 5-µL sample at 4°C. See **Optional PCR Check** on page 29.

Proceed to **Adapter A Ligation**, next page.

Adapter A Ligation

Introduction

In this procedure, you ligate the Adapter A to the cDNA. The adapter also contains the recognition site for the Type III restriction enzyme, *Eco*P15I.

Adapters A and B

Adapter A contains a Type III restriction endonuclease (*Eco*P15I) site at the 3′ end, cohesive overhangs complementary to the *Nla* III recognition site, and priming sites for PCR amplification.

Items Needed

Provided in the kit

- T4 DNA Ligase
- 5X Ligase Buffer
- Adapter A
- Wash Buffer C
- Wash Buffer D
- DEPC-treated Water

Provided by the user:

- Water baths, thermocyclers, or heat blocks set at 16°C, 37°C, and 50°C
- Ice
- Magnetic stand

Ligating Adapter A to the cDNA

1. In a separate RNase-free tube, prepare 210 µL of 1X Ligase Buffer per reaction by diluting 42 µL of 5X Ligase Buffer in 168 µL of DEPC-treated water (volume includes overage).
2. In another RNase-free tube, prepare the following mix per reaction. Prepare a master mix for multiple reactions.

5X Ligase Buffer	5 µL
Adapter A	1.5 µL
DEPC-treated water	16 µL
Total volume	22.5 µL

3. Wash the beads (from Step 8, page 13) once with 200 µL of 1X Ligase Buffer.
4. Place the tube on a magnetic stand until the beads have formed a pellet. Remove and discard the liquid.
5. Add the 22.5-µL ligase reaction mix from Step 1 to the tube.
6. Incubate at 50°C for at least 4 minutes and then at room temperature for 10 minutes. Then place the tube on ice to chill.
7. Add 2.5 µL of T4 DNA Ligase (5 U/µL). Mix gently by finger flicking or mild vortexing, and pulse spin to collect the contents.
8. Incubate at 16°C for 2 hours up to 16 hours. Mix gently every ~15 minutes for at least the first 2 hours.

   Note: While incubating, warm the bottle of Wash Buffer C in a water bath at 37°C to dissolve any precipitation.
9. After incubation, add 200 µL of warm Wash Buffer C directly to the reaction. Mix gently, then pulse spin to collect.
10. Wash the beads *three times* with 200 µL of Wash Buffer D. Be careful to remove all trace volume between washes.

Proceed directly to ***Eco*P15I Digestion**, next page.

*Eco*P15I Digestion

Introduction

In this procedure, you cleave the Adapter A ligation products from the beads with the Type III restriction enzyme *Eco*P15I to release the adapter with a short tag of the cDNA.

Cleavage Site

*Eco*P15I digestion generates a 25-bp cut on the top strand and a 27-bp cut with a 5′ "NN" overhang on the bottom strand. The overhang allows the hybridization/ligation of Adapter B, which contains a mix of sequences with complementary 2-base overhangs as well as a SOLiD™-specific sequence.

Items Needed

Provided in the kit

- *Eco*P15I
- 10X ATP
- 10X Buffer 3
- 100X BSA
- Sinefugin
- LoTE
- Glycogen
- 3M Na Acetate
- DEPC-treated water

Provided by the user:

- Water bath or incubator at 37°C
- **Optional:** Phase Lock Gel™ tubes (available from 5 PRIME, www.5prime.com)
- Phenol:Chloroform:Isoamyl Alcohol (see ordering information on page 31)
- Dry ice or –80°C storage
- 100% Ethanol
- 80% Ethanol

Important

Do not discard the supernatant following *Eco*P15I digestion!

In this step, *Eco*P15I digestion removes the SAGE™ tags from the beads; the supernatant contains the tags.

Cleaving the Tags with *Eco*P15I

1. Prepare 210 µL of 1X Buffer 3 per reaction by diluting 21 µL of 10X Buffer 3 in 189 µL of DEPC-treated water.
2. Wash the beads (from Step 10, page 14) *once* with 200 µL of 1X Buffer 3.
3. Place the tube on a magnetic stand until the beads have formed a pellet. Remove and discard the liquid.
4. Add the following reagents in order to the tube. Prepare a master mix for multiple reactions.

10X Buffer 3	10 µL
DEPC-treated water	68 µL
10X ATP	10 µL
100X BSA	1 µL
Sinefugin	0.4 µL

5. Add 10 µL of *Eco*P15I to the tube. Mix by mild vortexing.

Protocol continued on the next page

Continued on next page

Cleaving the Tags with *Eco*P15I, continued

Protocol continued from the previous page

6. Incubate at 37°C for 2 hours to overnight. Mix gently every ~15 minutes for at least the first 2 hours.

   Do not discard the supernatant! This contains your SAGE tags.

7. Place the tube on a magnetic stand and carefully transfer the supernatant containing the SAGE tags to a new RNase-free 1.5-mL microcentrifuge tube.
8. To ensure that you transfer all the SAGE tags, add 100 µL of LoTE to the beads. Remove the tube from the magnet, mix by mild vortexing, and pulse spin to collect. Place the tube back on the magnet and transfer this 100 µL to the tube containing the SAGE tags.
9. **Optional:** Add another 100 µL of LoTE to the beads and store the beads at 4°C for additional QC analysis.
10. Remove 5 µL of the liquid containing the SAGE tags for use as a no-ligase control. See **Determining Template Quality and Quantity** on page 18.

Proceed to **Ethanol Precipitation**, below.

Ethanol Precipitation

Note: Phase Lock Gel™ tubes may be used for the following procedure.

1. Add 200 µL of UltraPure™ Phenol:Chloroform:Isoamyl Alcohol to the tube containing the SAGE tags.
2. Mix by high-speed vortexing, and spin at 10,000 × *g* for at least 5 minutes.
3. Transfer the top 200 µL aqueous phase to a new RNase-free microcentrifuge tube, and add the following:

Glycogen	2 µL
3M Na Acetate	20 µL
100% Ethanol	~650 µL

4. Mix thoroughly and place on dry ice or at –80°C for at least 30 minutes.
5. Centrifuge at ≥14,000 × *g* for at least 30 minutes.
6. Wash the pellet at least once with 500 µL of 80% ethanol.
7. Centrifuge at ≥14,000 × *g* for 5 minutes.
8. Carefully invert the sample tube at an angle on a clean kimwipe or paper towel and allow the ethanol to drain.
9. Air dry the pellet for ~30 minutes or until the pellet is translucent.

Proceed to **Adapter B Ligation**, next page.

Adapter B Ligation

Introduction

In this procedure, you ligate Adapter B to the tags.

Items Needed

Provided in the kit

- DEPC-treated water
- 5X Ligase Buffer
- Adapter B
- T4 DNA Ligase

Provided by the user:

- Water bath, thermocycler, or heat block at 16°C and 37°C

Adapter B Ligation

1. Prepare the following Adapter B mix per reaction. Note that the complete ligation mix (including T4 DNA Ligase) may be scaled up to 30 µL if the pellet does not fully resuspend. Prepare a master mix for multiple reactions.

DEPC-treated water	12 µL
5X Ligase Buffer	4 µL
Adapter B	2 µL

2. Completely resuspend the pellet from **Ethanol Precipitation**, Step 9, in the Adapter B mix. Use gentle pipetting and/or incubation at 37°C in a water bath to dissolve the pellet.

   Important: Sample may be slightly cloudy, but be sure to completely resuspend the full pellet.

3. Add 2 µL of T4 DNA Ligase and incubate at 16°C for 2–16 hours (*i.e.*, overnight if necessary).
4. Store the SAGE tags at –20°C until use. Minimize freeze-thaws.

Proceed to **Template Quantification and Qualification**, next page.

Determining Template Quality

Template Qualification

The quality of the SAGE tags can be determined by PCR using the reagents provided in the kit, followed by visualization of the 100-bp tags by gel electrophoresis.

Items Needed

Provided in the kit

- 5′ AMP Primer
- 3′ AMP Primer
- Platinum® PCR SuperMix High Fidelity

Provided by the user:

- Thermal cycler
- Ice
- 4% Agarose Gels (*e.g.*, E-Gel® agarose gels, see ordering information on page 31)
- DNA standards (*e.g.*, Low DNA Mass™ Ladder, see ordering information on page 31)

Platinum® PCR SuperMix High Fidelity

Platinum® PCR SuperMix High Fidelity, included in the kit, contains anti-*Taq* DNA polymerase antibody, Mg^{++}, dNTPs, and a blend of recombinant *Taq* DNA polymerase and *Pyrococcus* species *GB-D* thermostable polymerase. The anti-*Taq* antibody inhibits polymerase activity at room temperature, providing an automatic "hot start" in PCR for improved specificity and yield. *Pyrococcus* species *GB-D* polymerase possesses a proofreading ability by virtue of its 3′ to 5′ exonuclease activity. Mixture of the proofreading enzyme with *Taq* DNA polymerase increases fidelity approximately six times over that of *Taq* alone.

PCR Guidelines and Precautions

PCR is a powerful technique capable of amplifying trace amounts of DNA, and all appropriate precautions should be taken to avoid cross-contamination, including:

- Set up the tagged template in a separate location—*i.e.*, PCR or laminar flow hood—from the control reactions to prevent cross contamination
- Assemble all amplification reactions on ice in a DNA-free environment
- Use sterile automatic pipettors and aerosol-resistant barrier pipette tips
- Avoid contamination of the PCR SuperMix with the primers or template DNA used in individual reactions.
- Analyze PCR products in an area separate from the rest of the tag-generation protocol.

Important

It is very important to purify and analyze post-PCR products in a room or area that is separate from the rest of the protocol, to avoid cross-contamination.

Continued on next page

Template Evaluation by PCR

1. Set up the following reactions **on ice** in RNase-free microcentrifuge tubes or a PCR plate. Use tag template from **Adapter B Ligation,** page 17, Step 4 and the no-ligase negative control from ***Eco*P15I Digestion,** page 16, Step 10.

	Experimental Template	No-template Control	No-ligase Control
5´ Amp Primer	0.5 µL	0.5 µL	0.5 µL
3´ Amp Primer	0.5 µL	0.5 µL	0.5 µL
Platinum® PCR SuperMix High Fid.	48 µL	49 µL	48 µL
SAGE tag template (20 µL total)	1 µL	—	—
No-ligase negative control	—	—	1 µL
Total reaction volume	50 µL	50 µL	50 µL

2. Cap reaction vessels and load in thermal cycler.
3. Amplify using the following cycling parameters. Collect tagged template at 5, 10, 15, and 20 cycles.

Temperature	Time	Cycles
95°C	2 minutes	1
95°C 55°C 72°C	30 seconds 1 minute 1 minute	20 total (collect template at 10, 15, and 20)
72°C	5 minutes	1

4. Hold at 4°C. When ready, proceed to running the gel.

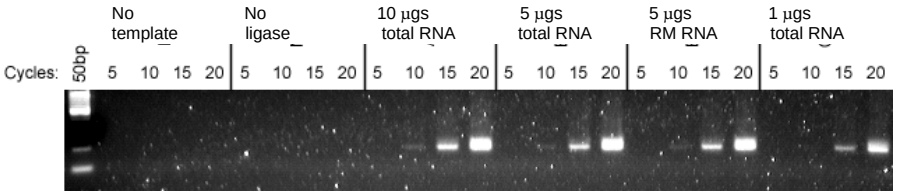
Gel Analysis of Quality

Run the PCR products on a 4% agarose gel with a DNA mass ladder. You can make a rough estimate of the quantity of SAGE tag template by using a ladder with bands of known quantity (*e.g.,* the Low DNA Mass™ Ladder, see below), as described in **Estimating Template Quantity from PCR Results,** next page.

SAGE Tag Template: The SAGE tagged template should appear as a clear 100-bp band. The cycle number that provides the optimal amplification will vary from sample to sample. Bands of lower molecular weight may indicate adapter self-ligation products or primer dimers.

No-template and No-ligase Controls: The negative controls should not contain any contaminating amplified product of the size of the tags.

Example Gel:



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Low DNA Mass™ Ladder

The Low DNA Mass™ Ladder, available separately from Invitrogen (catalog no. 10068-013), can be used for the quantification of SAGE tags. It is composed of an equimolar mixture of six blunt-ended DNA fragments of 2000, 1200, 800, 400, 200, and 100 bp. Electrophoresis of 4 µL of the Low DNA Mass Ladder results in bands containing 200, 120, 80, 40, 20, and 10 ng of DNA, respectively.

Analyzing the PCR Controls

Evaluate the presence of any contamination using the negative control reactions as follows:

- A 100-bp band in *both* the no-template control and no-ligase control indicates contamination of reagents during the PCR set-up. Repeat the PCR reactions as described on the previous page using fresh reagents for PCR.
- A 100-bp band in the no-ligase control and no band in the no-template control indicates contamination of the template. Discard the tagged template and redo the SOLiD™ SAGE™ protocol using fresh RNA sample and new, sterile plasticware.

Estimating Template Quantity from PCR Results

To estimate the quantity of the SAGE tags using the Low DNA Mass™ Ladder, determine the cycle number at which a 100-bp band appears in the template lane, and compare its band intensity to that of the most comparable band in the DNA Mass Ladder. Then back-calculate from the cycle number to provide a rough estimate of the quantity of tagged template. For example:

1. A 5 µL aliquot from the original 50-µL PCR reaction was run on a gel and determined to consist of 10 ng of DNA after 10 cycles
2. 10 ng/5 µL = 2 ng/µL of amplified SAGE tags in the 50-µL PCR reaction
3. 2 ng/µL ÷ 1024 (*i.e.*, 2^{10}, doubling of template per cycle for 10 cycles) = ~2 pg/µL starting concentration of SAGE tags in the PCR reaction
4. 2 pg/µL × 50 = ~100 pg/µL starting concentration of SAGE tags in ligation reaction (template diluted 1:50 in PCR)
5. 100 pg/µL × 20 µL ligation rxn = ~2000 pg of total SAGE tags in the library

Preparing for Emulsion PCR and SOLiD™ Sequencing

Introduction

After analyzing the quality of the SAGE tags, proceed with quantification, PCR scale-up (if necessary), and purification.

Quantification of Template for the SOLiD™ Workflow

For template quantification instructions, see Appendix B of the **Applied Biosystems SOLiD™ 3 System Library Preparation Guide**—SOLiD™ 3 System Library Quantitation. This appendix provides detailed instructions for quantifying your tag library by quantitative PCR (qPCR) using the SOLiD™ TaqMan® Gene Expression Assay or SYBR® Green Assay.

A bioanalyzer may also be used for quantification, but higher amounts of DNA are required and usually PCR-amplified products are used.

Purification of the Template

Prior to emulsion PCR (ePCR), purification of the tags is required. The template may be purified by a PCR purification kit that can distinguish the 100-bp tags from spurious 70–80 bp artifacts, such as primer dimers or adapter self-ligations. (*e.g.*, the PureLink PCR Micro kit from Invitrogen; see page 31 for ordering information).

Note:

- If PCR scale-up is performed, gel purification of the tags may be necessary if extra bands appear (see below and next page).
- The template may have to be re-quantitated after purification.
- If using the PureLink™ kit, see note below.

Note

If you are using the PureLink™ PCR Micro Kit, add 10.6 mL of isopropanol (100%) to the 15 mL of Binding Buffer provided in the kit for a final concentration of 42.5% isopropanol. This higher concentration increases recovery of the 100-bp SAGE tag. Elute in 20 µL.

PCR Scale-up If Necessary

In general, the SOLiD™ DNA fragment library sequencing workflow requires 150–240 pg of tags. We recommend starting with at least 400–500 pg of tags per SOLiD™ SAGE™ reaction (*i.e.*, each reaction is sufficient for at least two sequencing runs).

If, based on DNA quantification, you determine that you do not have enough template, you can scale up the reaction using 5–15 cycles of PCR. Follow the protocol provided in **Template Evaluation by PCR** on page 19, using the Platinum® PCR SuperMix High Fidelity provided in the kit and 1–4 µL of the ligation reaction as template. Run the PCR products on a gel to determine purity.

Important Note: Do not over-amplify the template, as this can result in the generation of higher molecular-weight products. Titrate the PCR cycles to the optimal number that generates the purest 100-bp fragment, and then gel-purify the PCR product if necessary. You may also use a sub-peak cycle number and perform multiple 50-µL PCRs, and then pool the samples together.

Continued on the next page

Gel Purification of PCR Scale-up

If PCR scale-up results in extra high or low bands, perform a gel purification using a 10% TBE gel or equivalent, as follows:

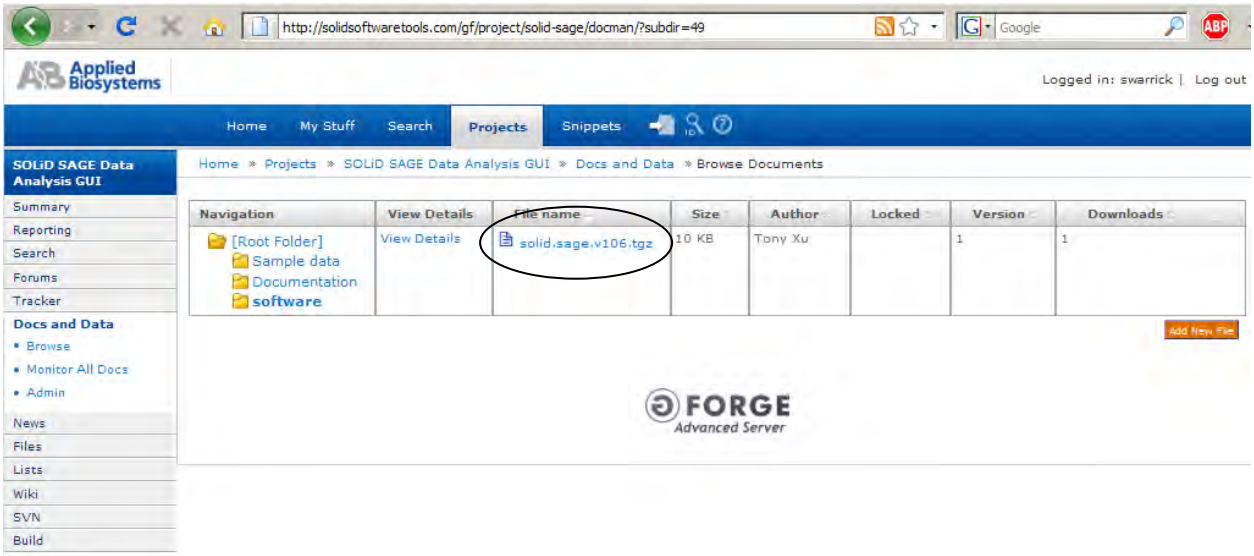
1. Add 5X TBE sample loading buffer to the template and load onto a 10% TBE gel. Include a DNA ladder in a separate well.
2. Run the gel such that the 100-bp fragment can be easily isolated; stain and visualize the gel.
3. Make a small hole in the bottom of a 0.5-mL tube with an 18-guage needle. Cut out the 100-bp band with a razor blade and place it into the tube. Place this smaller tube in a 1.5–2.0-mL tube and centrifuge at ≥14,000 × *g* for 3 minutes.
4. Add ~500 µL of a mix of LoTE and acetate (9:1 LoTE and 10 M NH_4OAc) to the pulverized fragments in the tube. Add this mix to the 1.5-mL microcentrifuge tube containing the gel pieces. Make sure that all gel pieces are covered with the buffer. Mix well by vortexing.
5. Incubate at 65°C for at least 2 hours to elute the DNA from the gel. If you do not have enough time to complete the elution, you may incubate at 4°C overnight followed by 65°C for 15 minutes.
6. Transfer the LOTE mix into a Costar SpinX column (or equivalent) fitted in a centrifuge tube and centrifuge at ≥14,000 × *g* for 5–10 minutes. Switch the direction of the tube halfway through the cycle.
7. To each 300 µL of eluate, add 133 µL of 7.5 M ammonium acetate, 3 µL of glycogen, and 1090 µL of 100% ethanol. Mix well and incubate the tube on dry ice for 10–20 minutes.
8. Centrifuge at maximum speed in a microcentrifuge at 4°C for 30 minutes.
9. Wash with 80% ethanol, air dry, and resuspend in ~10ul.

SOLiD™ SAGE™ Analysis Software

SOLiD™ SAGE™ Analysis Software

After you have sequenced the tags on the SOLiD™ system, you are ready to analyze the sequence data. The SOLiD™ SAGE™ analysis software runs on the Linux platform and is available as a free download from the SOLiD™ project page (registration required):

http://solidsoftwaretools.com/gf/project/solid-sage



What It Does

The software combines and compares relevant reference sequence information from RefSeq or similar databases with the SOLiD™ SAGE™ tag sequence data, identifies tags, and tabulates tag abundances.

The software can generate the following output files:

- A mapping summary file that lists each tag, its frequency of occurrence, its GenBank Identifier (GI) number, and a brief description of the identified gene
- A results file that provides more detailed information, including SOLiD™ sequencing read IDs and mismatches
- A file comparing the tags in two different samples
- A file calculating the abundances of repeat reads

System Requirements

SOLiD™ SAGE™ analysis software can run on the same Linux system that is used for your SOLiD™ instrument. Requirements include:

- Unix platform
- Corona_Lite software (also available for free download from http://solidsoftwaretools.com)
- Perl Tk
- Database of reference sequences (*e.g.*, RefSeq database)
- ≥2 gigabytes memory—varies depending on the size of the reference genome, selected tag length, and selected number of allowed mismatches

Appendix

Troubleshooting

Introduction

RNA quality is the key factor that will affect the outcome of your results using the SOLiD™ SAGE™ kit. Please review the information provided in the table below to troubleshoot your experiments.

Problem	Cause	Solution
28S and 18S bands are not observed after isolation of total RNA, or RIN value is <7.0	RNA is degraded due to RNase activity	Follow the guidelines on page 6 to avoid RNase contamination. Use fresh sample (tissue or cells) for RNA isolation.
	Too little RNA loaded on the gel for analysis	Be sure to load at least 250 ng of RNA for analysis.
No bands seen after cDNA synthesis when analyzed by PCR	Poor quality of RNA or RNA is degraded	Check the quality of the RNA as described on page 7. Use extreme care while handling RNA samples to prevent RNase contamination (see page 6).
	cDNA synthesis reagents not working	Perform cDNA synthesis with a total RNA control sample to ensure that the cDNA synthesis reagents are working properly.
Clumping of beads	Robust cDNA synthesis results in entangling of the double-stranded DNA in the beads	Perform 2–4 additional wash steps with Wash Buffer D using low-to-medium vortexing to disperse the beads.
	Wash Buffer C precipitate	Keep the solution warm to prevent precipitation, which results in bead clumping.
	Improper mixing and washing of beads	Be sure to thoroughly mix the beads after every reaction and during all the wash steps. Intermittent mixing of the beads during incubation of enzymatic reactions will increase the efficiency and robustness of each reaction.
Loss of beads	Beads stick to sides of tube	Use nonstick or low-bind microcentrifuge tubes. Be sure to scrape the sides of the microcentrifuge tube to remove any beads that are sticking to the tubes. Minimize pipetting steps.

Continued on next page

Problem	Cause	Solution
Inefficient adapter ligation	CATG overhangs on the adapters and *Nla* III digested cDNA may be cleaved by the exonuclease activity of *E. coli* DNA polymerase	Thoroughly wash and heat the beads with Wash Buffer C to remove any traces of exonuclease activity.
	Adapter amount is not optimal	Optimize the adapter concentration if the amount of your starting sample is not in the range of 1–10 µg total RNA.
Incomplete *Eco*P15I digestion	Improper reaction conditions	Be sure to perform the *Eco*P15I incubation at 37°C for at least 2 hours for optimal digestion, mixing the reaction occasionally. If you have saved the beads as specified in **Cleaving the Tags with *Eco*P15I**, page 16, Step 9, you may re-digest them with *Eco*P15I.
Presence of non-specific bands in PCR reactions	Contamination of PCR tubes or reagents	Be sure set up the PCR reactions in separate locations to prevent contamination from template or cross-contamination of reagents. Include negative controls to help you evaluate your results.
	Formation of primer-dimers or non-specific binding of primers resulting in amplification of unrelated products	Set up the PCR reaction on ice to prevent primer-dimer formation.
Presence of higher molecular weight bands	Robust PCR can result in higher molecular weight products	Reduce the amount of template or reduce the number of PCR cycles.
Presence of lower molecular weight bands	Adaptor self-ligation or primer dimers	Increase the amount of template and/or check the quality of your starting material and re-purify if necessary Confirm that you are carefully performing all wash steps Set up PCR reactions on ice

Continued on next page

Problem	Cause	Solution
Low yield of tags	Reduced activity of *Nla* III	Be sure to keep the enzyme at –80°C at all times. Use the enzyme immediately upon removal from –80°C and immediately return to –80°C.
	Loss of DNA during precipitation	Be careful not to lose the DNA pellet when precipitating and washing.

Preparing Non-Poly(A) RNA for SOLiD™ SAGE™ Analysis

Overview

This section provides basic steps for isolating non-poly(A) RNA for analysis using SOLiD™ SAGE™.

This procedure uses kits and components that are available separately from Invitrogen. See ordering information on page 31.

Items Needed

Provided in the kit

- Lysis/Binding Buffer
- DEPC-treated Water
- 3M Na Acetate

Provided by the user (see ordering information on page 31):

- RiboMinus™ Eukaryote Kit for RNA-Seq
- Dynabeads® Oligo(dT)$_{25}$
- RiboMinus™ Concentration Module
- NCode™ miRNA First-Strand cDNA Synthesis Kit
- Magnetic stand
- Dry ice or –80°C storage
- 100% Ethanol
- 80% Ethanol

Step 1: RiboMinus™ Depletion of rRNA

Use the RiboMinus™ Eukaryote Kit for RNA-Seq (catalog no. A10837-08) for the following steps:

1. Follow the protocol provided with the RiboMinus™ Eukaryote Kit for RNA-Seq for removing rRNA from your sample.
2. To the ~530 µL of rRNA-depleted supernatant, add 470 µL of Lysis/Binding Buffer provided in the SOLiD™ SAGE™ kit.

Step 2: Binding Poly(A) RNA to Dynabeads®

Use Dynabeads® Oligo(dT)$_{25}$ (catalog no. 610-02) to bind and remove the poly(A) RNA in the sample. **The non-poly(A) fraction will remain in the supernatant.** (You may also use the Dynabeads® Oligo(dT) EcoP provided in this kit, but this will reduce the number of SAGE reactions you can perform.)

1. Follow the procedures for **Binding RNA to Dynabeads®** starting on page 8, ***except*** when **Binding the RNA** (page 9), rotate/incubate for 30–60 minutes in Step 5 to fully bind the poly(A) RNA.

   ****Do not discard the supernatant.**** The supernatant contains your non-poly(A) RNA.
2. Carefully transfer the supernatant (~1000 µL) to a new RNase-free tube. (Do *not* proceed to washing the beads.)

Continued on next page

Step 3: Ethanol Precipitation

Ethanol precipitate the supernatant containing your non-poly(A) RNA:

1. Combine the following:

Supernatant containing non-poly(A) RNA	1000 µL
3M Na Acetate	100 µL
100% Ethanol	2750 µL

2. Mix thoroughly and place on dry ice or at –80°C for at least 15 minutes.
3. Centrifuge at ≥14,000 × *g* for 40 minutes.
4. Wash the pellet at least once with 500 µL of 80% ethanol.
5. Centrifuge at ≥14,000 × *g* for 15 minutes.
6. Carefully invert the sample tube at an angle on a clean kimwipe or paper towel and allow the ethanol to drain.
7. Air dry the pellet for ~30 minutes or until the pellet is translucent.

Step 4: Concentrate the Non-poly(A) RNA

Use the RiboMinus™ Concentration Module (catalog no. K1550-05) for the following steps:

1. Resuspend the pellet in 100 µL of DEPC-treated water, 100 µL of ethanol, and 100 µL of Binding Buffer (L3) from the RiboMinus™ Concentration Module.
2. Follow the procedure in the RiboMinus™ Concentration Module for concentrating the RiboMinus™ RNA, and elute in 25 µL of DEPC-treated water.

Step 5: Poly(A) Tail the RNA

Use the NCode™ miRNA First-Strand cDNA Synthesis Kit (catalog no. MIRC-10) for the following steps:

1. Poly(A) tail the 25 µL of non-tailed RNA using the **Poly(A) Tailing of miRNA** procedure in the NCode™ manual, ***except*** scale the reaction to 50 µL as follows:

Component	**Volume**
Non-poly (A) RNA	25 µL
5X miRNA Reaction Buffer	10 µL
25 mM $MnCl_2$	5 µL
Diluted ATP	1 µL
Poly A Polymerase (see **Note** above)	1.25 µL
DEPC-treated water	to 50 µL

2. Mix gently and centrifuge the tube briefly to collect the contents.
3. Incubate the tube in a heat block or water bath at 37°C for 15 minutes.
4. Proceed to **Binding RNA to Dynabeads®** starting on page 8, and follow the regular SOLiD™ SAGE™ workflow.

Optional PCR Check for cDNA Synthesis and *Nla* III Digestion

Note

If you are having problems generating the 100-bp tag, you can perform the following PCR check of aliquots preserved from the cDNA synthesis reaction and/or *Nla* III digestion to help isolate the problem.

Verifying cDNA Synthesis and *Nla* III Digestion by PCR

The following procedure can be used to check the cDNA synthesis reaction (pages 10–12) and *Nla* III digestion (page 13) using the 5–µL aliquots preserved from those reactions.

The PCR primers (GAPDH and EF) used in the following procedure are designed for human RNA sample, but have also been found to work with mouse and rat. Note that this reaction may not detect subtle problems with the cDNA reaction and/or *Nla* III digestion (*e.g.*, loss of enzyme activity during storage).

1. Set up four 50-µL PCR reactions as shown below on ice in RNase-free PCR tubes.

Reagents	GAPDH/ cDNA Template	GAPDH/ *Nla* III Template	EF/ cDNA Template	EF/ *Nla* III Template
GAPDH Forward Primer	2 µL	2 µL	--	--
GAPDH Reverse Primer	2 µL	2 µL	--	--
EF Sense Primer	--	--	2 µL	2 µL
EF Anti-sense Primer	--	--	2 µL	2 µL
Platinum® *Taq* SuperMix Hi Fi	45 µL	45 µL	45 µL	45 µL
cDNA Synthesis Template	0.5 µL	--	0.5 µL	--
Nla III Digestion Template	--	0.5 µL	--	0.5 µL
DEPC Water	to 50 µL	to 50 µL	to 50 µL	to 50 µL

2. Cap the tubes and spin to collect the contents.
3. Amplify using the following cycling parameters:

Temperature	Time	Cycles
95°C	2 minutes	1
95°C 55°C 72°C	30 seconds 30 seconds 1 minute	25–30
72°C	5 minutes	1

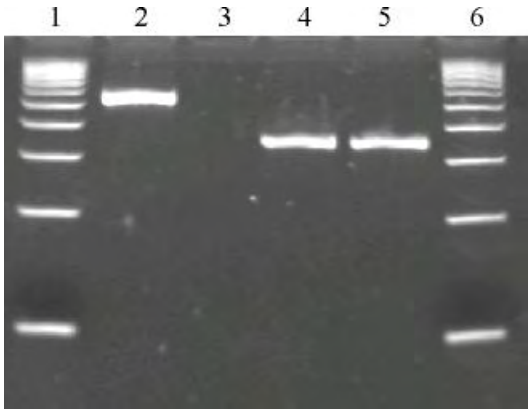
4. Maintain the reaction at 4°C after cycling.
5. Analyze 10 µL of the reaction using agarose gel electrophoresis (see following page for expected results).

Continued on next page

Optional PCR Check for cDNA Synthesis and *Nla* III Digestion, Continued

Expected Results

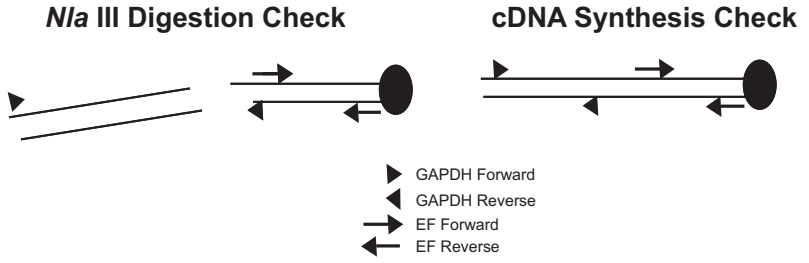
After electrophoresis, you should see a 540-bp band with the cDNA Synthesis template and GAPDH primers, no band with the *Nla* III template and GAPDH primers, and a 350-bp band with either template as well as the EF primers, as shown below. (Note that efficient *Nla* III digestion will result in loss of the GAPDH primer binding site.) If you do not observe any bands after cDNA synthesis with your RNA sample, please see the **Troubleshooting** section on page 24.



Legend for the gel:

An aliquot was removed after double-stranded cDNA synthesis using total RNA from peripheral blood mononuclear cells and was PCR amplified using gene-specific primers for GAPDH (lane 2) and EF (lane 4) as described on the previous page.
After *Nla* III digestion, the GAPDH primer binding sites are lost (lane 3), while the EF-1 primer binding sites are intact (lane 5).

Lanes 1 and 6: 100-bp ladder



Accessory Products

Additional Products

The following products for use with the kit are available separately from Invitrogen. To order, visit www.invitrogen.com or contact Technical Support (see page 33).

Product	Amount	Catalog no.
DynaMag™-Spin Magnet	1 magnet	123-20D
DynaMag™-2 Magnet	1 magnet	123-21D
TRIzol® Reagent	200 mL 100 mL	15596-018 15596-026
Phenol:Chloroform:Isoamyl Alcohol (25:24:1,v/v)	100 mL	15593-031
Quant-iT™ RiboGreen® RNA Assay Kit	1 kit	R11490
Low DNA Mass™ Ladder	50 apps	10068-013
EXPRESS SYBR® GreenER™ qPCR Supermix Universal	200 rxns 1000 rxns	11784-200 11784-01K
EXPRESS SYBR® GreenER™ qPCR Supermix with Premixed ROX	200 rxns 1000 rxns	11794-200 11794-01K
Quant-iT™ PicoGreen® dsDNA Assay Kit	2000 assays	P11496
E-Gel® 4% with Ethidium Bromide	18 pak	G5018-04
PureLink™ PCR Micro Kit	10 reactions 50 reactions	K3100-10 K3100-50
Platinum® PCR SuperMix High Fidelity	100 reactions 500 reactions	12532-016 12532-024
For Preparing Non-Poly(A) RNA for SOLiD™ SAGE™ Analysis:		
RiboMinus™ Eukaryote Kit for RNA-Seq	2 preps 8 preps	A10837-02 A10837-08
RiboMinus™ Concentration Module	6 preps	K1550-05
NCode™ miRNA First-Strand cDNA Synthesis Kit	10 reactions	MIRC-10
Dynabeads® Oligo(dT)$_{25}$	2 mL	610-02

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