Applied Biosystems

SOLiD™ Small RNA Expression Kit

Small RNA Library Preparation for SOLiD™ Sequencing

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Safety information

Note: For general safety information, see this Preface and Appendix B on page 25. When a hazard symbol and hazard type appear by a chemical name or instrument hazard, see the "Safety" Appendix for the complete alert on the chemical or instrument.

Safety alert words

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IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

CAUTION! – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING! – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER! – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

MSDSs

The MSDSs for any chemicals supplied by Applied Biosystems or Ambion are available to you free 24 hours a day. For instructions on obtaining MSDSs, see "MSDSs" on page 25.

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

How to use this guide

Text conventions

This guide uses the following conventions:

- **Bold** text indicates user action. For example:

  Type **0**, then press **Enter** for each of the remaining fields.
- *Italic* text indicates new or important words and is also used for emphasis. For example:

  Before analyzing, *always* prepare fresh matrix.
- A right arrow symbol (▸) separates successive commands you select from a drop-down or shortcut menu. For example:

  Select **File ▸ Open ▸ Spot Set**.

  Right-click the sample row, then select **View Filter ▸ View All Runs**.

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Two user attention words appear in Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below:

Note: – Provides information that may be of interest or help but is not critical to the use of the product.

IMPORTANT! – Provides information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

How to obtain support

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At the Applied Biosystems web site, you can:

- Access worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.
- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents.
- Download PDF documents.
- Obtain information about customer training.
- Download software updates and patches.

SOLiD™ Small RNA Expression Kit Protocol

Product information

Product description and background

Prepare small RNA samples for SOLiD™ sequencing

The SOLiD™ Small RNA Expression Kit (P/N 4397682) enables conversion of the small RNA in a sample into a double-stranded cDNA library that is compatible with the Applied Biosystems SOLiD™ System for next generation high-throughput sequencing. The cDNA library generated with the SOLiD Small RNA Expression Kit is ideally suited as input for the emulsion PCR step of SOLiD sample preparation.

The SOLiD System can sequence over 200 million DNA fragments per run, with high sensitivity and a wider dynamic range than microarray technology. This massive output provides a direct means to identify most, if not all, small RNA species present in the sample, enabling rapid discovery of unknown small RNAs, even those with low level expression, and providing an efficient way to perform quantitative expression analysis of small RNAs.

Small RNA has a large role in gene expression

Small noncoding RNAs are recognized as significant effectors of gene regulation in organisms spanning the evolutionary spectrum. Animals, plants, and fungi contain several distinct classes of small RNA, including microRNA (miRNA), short interfering RNA (siRNA), piwi-interacting RNA (piRNA), and repeat-associated siRNA (rasiRNA). These molecules are typically only ~18–40 nt in length; however, their effects on cellular processes are profound. They have been shown to play critical roles in developmental timing, cell fate, tumor progression, neurogenesis, transposon silencing, viral defense, and many other cellular processes. Small RNAs function in gene regulation by binding to their targets and modulating gene expression using mechanisms such as heterochromatin modification, translational inhibition, mRNA decay, and even nascent peptide turnover mechanisms.

Kit contents and storage conditions

The kit contains reagents for preparing amplified cDNA libraries from 10 samples of small RNA for use in SOLiD System sequencing.

Amount	Component	Cap	Storage
25 µL	Adaptor Mix A	purple	–20°C
25 µL	Adaptor Mix B	purple	–20°C
10 µL	Control RNA (1 µg/µL human placenta total RNA)	clear	–20°C
2 x 1.75 mL	Nuclease-Free Water	clear	any temp‡
35 µL	Hybridization Solution	green	–20°C
150 µL	2X Ligation Buffer	green	–20°C
25 µL	Ligation Enzyme Mix	green	–20°C
45 µL	10X RT Buffer	yellow	–20°C
20 µL	ArrayScript™ Reverse Transcriptase	yellow	–20°C
20 µL	RNase H, E coli	yellow	–20°C
550 µL	10X PCR Buffer I	white	–20°C
110 µL	AmpliTaq® DNA Polymerase	white	–20°C
470 µL	2.5 mM dNTP Mix	white	–20°C
50 µL each	SOLiD PCR Primers 1–10 (25 µM each forward/reverse primer)	blue	–20°C

‡ Store Nuclease-free Water at room temperature, 4°C, or –20°C.

Required materials not provided with the kit

Equipment

- General laboratory equipment, including microcentrifuge, pipettors, spectrophotometer, and barrier RNase-free tips
- (Optional but strongly recommended) Ambion® flashPAGE™ Fractionator System and accessories, to isolate small RNA from total RNA preparations
  - flashPAGE™ Fractionator Apparatus (P/N AM13100)
  - flashPAGE™ Pre-cast Gels (P/N AM10010)
  - flashPAGE™ Buffer Kit (P/N AM9015)
  - flashPAGE™ Reaction Clean-Up Kit (P/N AM12200)
  - Electro*Zap*™ Solution (P/N AM9785)
- NanoDrop ND-1000 spectrophotometer
- Agilent 2100 bioanalyzer with the Small RNA Chip Kit
- Thermal cycler with heated lid, capable of holding 0.2 mL tubes, for example, Applied Biosystems Veriti™ 96-Well Thermal Cycler, or GeneAmp® PCR System 9700
- Polyacrylamide gel electrophoresis equipment: for example, Bio-Rad Laboratories Mini-PROTEAN Tetra Electrophoresis System

Supplies

- RNase-free 0.2 mL thin-walled PCR tubes
- RNase-free 1.5 and 0.5 mL polypropylene microcentrifuge tubes
- Adjustable pipettors and RNase-free tips
- Qiagen MinElute® PCR Purification Kit (#28004, 28006)
- 21 gauge needle
- Ambion Spin Columns (P/N AM10065) or 0.45 µm filter centrifuge tube filters (for example, Costar Spin-X, manufactured by Corning, #8163)

Reagents

Nucleic acid cleanup and precipitation reagents

- 5 M Ammonium acetate (for example, Ambion P/N AM9070G, AM9071)
- Isopropanol (ACS reagent grade or equivalent)
- Glycogen coprecipitant (for example, Ambion P/N AM9510)
- TE Buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA; for example, Ambion TE, pH 8.0, P/N AM9858)

Polyacrylamide gel reagents

- Gel Loading Solution (Ambion P/N AM8556)
- 10 bp DNA Ladder (Invitrogen #10821–015)

  Note: It is important to use a ladder with 10 bp spacing.

- Reagents for preparation and electrophoresis of native polyacrylamide gels, see "Polyacrylamide gel instructions" on page 22
- SYBR® Gold Gel Stain (Invitrogen #S-11494)
- (Optional) Dark Reader Transilluminator, Clare Chemical Research (**www.clarechemical.com**)

  Dark Reader transilluminators use visible light, rather than UV light, in combination with proprietary filters to visualize fluorescently labeled nucleic acids.

Procedure overview

The procedure is based on Applied Biosystems Ligase-Enhanced Genome Detection (LEGenD™) technology (patent pending); an overview is shown in the workflow on page 6.

Hybridization and ligation to Adaptor Mix

First, the small RNA sample is hybridized with either Adaptor Mix A or Adaptor Mix B. These Adaptor Mixes are sets of oligonucleotides with a single-stranded degenerate sequence at one end and a defined sequence required for SOLiD sequencing at the other end. Each Adaptor Mix constrains the orientation of the RNA in the ligation reaction such that hybridization with Adaptor Mix A yields template for SOLiD sequencing from the 5' end of the sense strand, while hybridization with Adaptor Mix B yields template for sequencing the reverse complement (yielding sequence starting from the 3' end of the sense strand). This process is illustrated in Figure 1.

Note: To achieve higher confidence in the complete sequence of the larger species of small RNAs in a sample, prepare two separate small RNA libraries, using Adaptor Mix A for one reaction and Adaptor Mix B for the other.

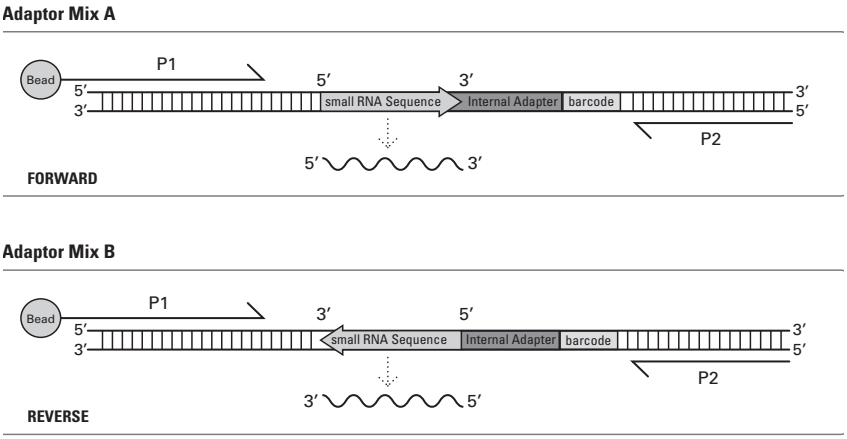


Figure 1 Emulsion PCR primer alignment on SOLiD™ Small RNA Expression Kit products

After hybridization, the adaptors are ligated to the small RNA molecules using Ligation Enzyme Mix, which is a mixture of an RNA Ligase and other components. Ligation requires an RNA molecule with a 5'-monophosphate and a 3'-hydroxyl end; therefore, most small RNAs can participate in this reaction, and intact mRNA molecules with a 5' cap structure are excluded.

Plant miRNAs have a 2'-O-methyl group at the 3' end (Yu et al. 2005). They can serve as template in the RNA ligation reaction, but the reaction efficiency is substantially reduced compared to RNA species with a 2'-OH at the 3' end.

Small RNAs with a 5'-triphosphate, such as endogenous siRNA from *C. elegans* (Pak and Fire 2007), can only participate in the ligation reaction after treatment with tobacco acid pyrophosphatase (TAP) to convert their 5' ends to 5'-monophosphates. Follow the manufacturer's instructions for using TAP.

Reverse transcription and RNase H digestion

Next, the small RNA population with ligated adaptors is reverse transcribed, to generate cDNA. Treatment with RNase H follows, to digest the RNA from RNA/cDNA duplexes and to reduce the concentration of unligated adaptors and adaptor by-products. At this point, reactions contain cDNA copies of the small RNA molecules in the sample.

cDNA Library amplification

To meet the sample quantity requirements for SOLiD sequencing, and to append the required terminal sequences to each molecule, the cDNA library is amplified using one of the supplied primer sets and 15–18 cycles of PCR. Limiting the cycle number minimizes the synthesis of spurious PCR products and better preserves the small RNA profile of the sample.

Ten sets of PCR primers are included in the kit; they are identical except for a 6 nt "barcode" sequence on the 3' (reverse) primer. The 5' PCR primer is identical in each set; its sequence corresponds to SOLiD emulsion PCR primer 1 (P1 in Figure 1 on page 4). The 3' reverse PCR primers are identical except for a 6 nt "barcode" sequence; the SOLiD emulsion PCR primer 2 sequence (P2) is on the 5' side of the barcode, and the adapter sequence is on the 3' side. (The barcode feature of the PCR primers is designed for future implementation of sequencing and analysis of multiplexed samples.)

Amplified library cleanup and size selection by PAGE

The final steps in the procedure involve cleanup and size selection of the amplified cDNA library to concentrate samples and remove PCR by-products. PCR products ~105–150 bp are isolated, corresponding to inserts derived from the small RNA population.

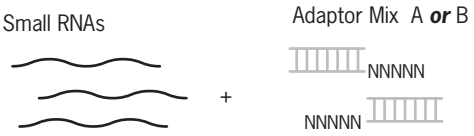
SOLiD sample preparation and sequencing (not included)

The amplified cDNA library generated with the SOLiD Small RNA Expression Kit is ready for attachment to beads at the emulsion PCR step of the SOLiD sample preparation workflow.

Workflow

Starting material

- Small RNA, purified using the flashPAGE™ Fractionator or PAGE, ***or***
- Total RNA that contains the small RNA fraction
  and
- Adaptor Mix A: for sequencing the 5' ends of small RNAs, ***or***
- Adaptor Mix B: for sequencing the reverse complement of the RNA



Hybridization and ligation to adaptor mix (8 hr)

On ice, mix RNA, Adaptor Mix, and Hybridization Solution.

↓

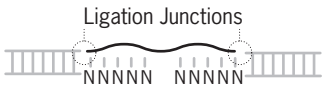
Incubate the sample at 65°C for 10 min, then at 16°C for 5 min.

↓

Add ligation reagents to each sample.

↓

Incubate the sample at 16°C for >8 hr in a thermal cycler.



Reverse transcription and RNase H digestion (1 hr)

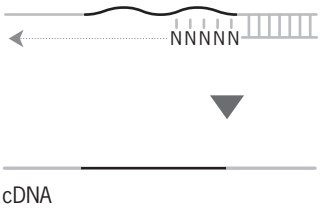
On ice, add 20 µL RT Master Mix to each sample.

↓

Incubate at 42°C for 30 min.

↓

Add 1 µL RNase H to 10 µL cDNA and incubate at 37°C for 30 min.



cDNA library amplification (1 to 1.5 hr)

Dispense PCR Master Mix into wells of a PCR plate or tubes.

↓

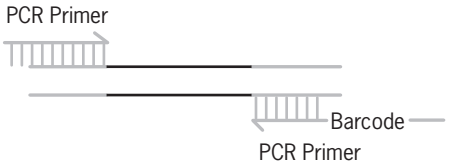
Add RNase H-treated cDNA to each reaction mix, then run the PCR.

↓

Run 5–10 µL PCR product on a native 6% polyacrylamide gel.

↓

Evaluate the PCR products.



Amplified library cleanup and size selection by PAGE (3 to 4 hr)



SOLiD™ Sample Preparation and Sequencing

SOLiD™ System: start at the “Templated Bead Preparation" section of the SOLiD™ System instructions (emulsion PCR)

Procedure

RNA sample type and amount

Size-selected small RNA is optimal

For use in this procedure, RNA must contain the small RNA fraction (<40 nt). For optimal results, use RNA that has been size selected for small RNA.

RNA samples vary widely in small RNA content, based on their source and the RNA isolation method. The proportion of small RNA is high enough in some tissues to allow efficient library preparation from total RNA (for example, the control RNA provided in this kit is total RNA isolated from placenta). Many tissues and most cell lines, however, contain a much smaller fraction of small RNA; Applied Biosystems recommends enrichment of these RNA samples for small RNA.

A survey by Agilent provides a guide for the relative proportion of miRNA of 40 different tissues (Tissot, 2008).

1. Obtain total RNA containing the small RNA fraction.

- Use Ambion FirstChoice® Total RNA, which is certified to contain miRNA and other small RNAs.
- Alternatively, isolate total RNA that includes the small RNA fraction using the Ambion *mir*Vana™ miRNA Isolation Kit (P/N AM1560) or *mir*Vana PARIS™ Kit (P/N AM1556). Follow the procedures for total RNA isolation.

2. Determine whether small RNA enrichment is needed.

Evaluate the small RNA content of your samples to determine whether small RNA enrichment is needed. Use an Agilent bioanalyzer with the Small RNA Chip.

- ***RNA samples that contain less than 0.5% small RNA***: enrichment for the ~18–40 nt RNA fraction is strongly recommended.
  - (Recommended) Use the Ambion flashPAGE™ Fractionator System and flashPAGE™ Clean-up Kit. Follow the instructions provided with the kit. Up to 100 µg total RNA can be loaded on the flashPAGE Fractionator.

    Expected recovery after flashPAGE fractionation and cleanup: From 5 µg total placenta RNA input, approximately 200 ng small RNA-enriched RNA. For <5 µg total RNA input, use glycogen as a coprecipitant during the flashPAGE Clean-Up Kit procedure.
  - Alternatively, use traditional denaturing PAGE to size fractionate small RNA.

- ***RNA samples that contain more than 0.5% small RNA*** (in the ~10–40 nt size range): these samples can generally be used without small RNA enrichment; however, for optimal results, Applied Biosystems recommends enrichment of all total RNA samples.

Note: When total RNA is used in the SOLiD Small RNA Expression Kit procedure, the resulting reaction products comprise a larger size range than those produced from small RNA-enriched samples.

Amount of RNA

A NanoDrop spectrophotometer is recommended for quantitating the concentration of your small RNA-enriched samples, because of the minimal volume required for accurate reads.

Table 1 RNA input recommendations

RNA Source	Amount
Small RNA fraction (size selected with the flashPAGE System or PAGE)	10–200 ng
Total RNA isolated from tissue or cultured cells	250–1000 ng
Control RNA (human placenta total RNA)	500 ng

Note: Cleanup or size selection of the positive control reaction products is necessary only if they will be sequenced.

Hybridization and ligation to adaptor mix

For the following hazards, see the complete safety alert descriptions in "Chemical alerts" on page 27.

CAUTION! CHEMICAL HAZARD. Hybridization Solution, 2× Ligation Buffer, and Ligation Enzyme Mix.

1. On ice, mix RNA, Adaptor Mix, and Hybridization Solution.

On ice, prepare the hybridization mix in 0.2 mL PCR tubes as shown in the following table. Mix well by gently pipetting up and down a few times, then centrifuge briefly to collect the solution at the bottom of the tube.

Hybridization mixture (8 µL total volume)	
Amount	**Component**
2 µL	Adaptor Mix A or B‡
3 µL	Hybridization Solution
1–3 µL	RNA sample (10–1000 ng)
to 8 µL	Nuclease-free Water

‡ Use Adaptor Mix A for SOLiD sequencing from the 5' ends of small RNAs. Use Adaptor Mix B for sequencing from the 3' ends. To sequence the small RNA in a sample from both the 5' and 3' ends, set up two ligation reactions, each with one Adaptor Mix.

2. Incubate the sample at 65°C for 10 min, then at 16°C for 5 min.

a. Place the reactions in a thermal cycler with a heated lid, programmed as shown, then start the run.

Adaptor hybridization incubation	
Temperature	**Time**
65 °C	10 min
16 °C	hold

b. Allow the sample to incubate at 16°C for 5 min.

c. Place the samples on ice and proceed immediately to the next step.

3. Add ligation reagents to each sample.

a. Add the RNA ligation reagents to each sample in the order shown.

Ligation reaction mix (20 µL final volume)	
Amount	**Component (add in order shown)**
10 µL	2× Ligation Buffer‡
2 µL	Ligation Enzyme Mix

‡ 2× Ligation Buffer is very viscous; pipette slowly to dispense it accurately.

b. Mix well by flicking the tube or slowly pipetting up and down a few times, then centrifuge it briefly.

4. Incubate the sample at 16°C for >8 hr in a thermal cycler.

Return the samples to the thermal cycler set to 16°C, and incubate them for at least 8 hr.

If possible, set the temperature of the thermal cycler lid to match the block temperature. Otherwise, incubate the reaction with the heated lid turned off, or do not cover the reaction tubes with the heated lid.

An 8–16 hr incubation is recommended for most samples, but less time may be sufficient if enough small RNA is present in the starting material. See Figure 2.

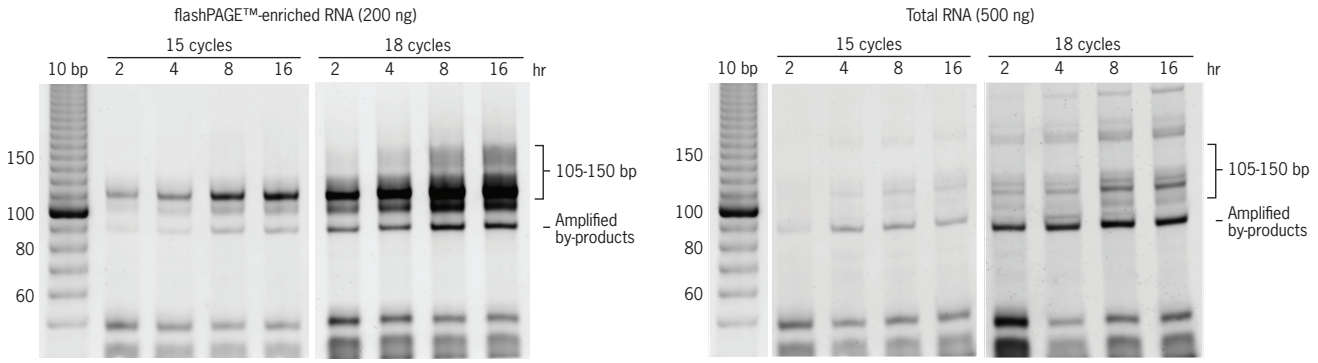


Figure 2 SOLiD™ Small RNA Expression Kit products: input, ligation time, and number of PCR cycles

Amplified libraries were prepared from 200 ng size-selected Control RNA (left panel) or 500 ng Control RNA (right panel), using the indicated ligation times and PCR cycles, run on a 6% native acrylamide gel, and stained with SYBR® Gold dye, as described in the SOLiD™ Small RNA Expression protocol. The leftmost lane in each panel contains 10 bp DNA ladder (Invitrogen).

Reverse transcription and RNase H digestion

For the following hazards, see the complete safety alert descriptions in "Chemical alerts" on page 27.

WARNING! CHEMICAL HAZARD. ArrayScript™ Reverse Transcriptase, RNase H, and 10× RT Buffer.

1. On ice, add 20 µL RT Master Mix to each sample.

   a. Place the sample(s) on ice and prepare a Reverse Transcription (RT) Master Mix on ice by combining the reagents in the order shown in the following table. Include 5–10% extra volume in the master mix to compensate for pipetting error.

RT Master Mix (20 µL per sample)	
Amount	**Component**
13 µL	Nuclease-free Water
4 µL	10X RT Buffer
2 µL	2.5 mM dNTP mix
1 µL	ArrayScript™ Reverse Transcriptase
20 µL	Total volume per reaction

   b. Add 20 µL RT Master Mix to each sample. ***Gently*** vortex to mix thoroughly and microcentrifuge briefly to collect the mixture at the bottom of the tube.

2. Incubate at 42°C for 30 min.

   Incubate at 42°C for 30 min to synthesize cDNA.

   Note: The cDNA can be stored at –20°C for a few weeks, at –80°C for long-term storage, or used immediately in the RNase H digestion (next).

3. Add 1 µL RNase H to 10 µL cDNA and incubate at 37°C for 30 min.

   a. Transfer 10 µL of the RT reaction mixture from the previous step (cDNA) to a fresh tube.

   b. Add 1 µL RNase H. Vortex gently to mix and microcentrifuge briefly to collect the mixture at the bottom of the tube.

   c. Incubate at 37°C for 30 min.

   Note: After the RNase H treatment, samples can be stored at –20°C for a few weeks or used immediately in the PCR (next).

cDNA library amplification

Pilot and large-scale PCRs

Because different sample types can contain substantially different amounts of small RNA, the number of PCR cycles needed to obtain enough DNA for SOLiD sequencing also varies. Perform a 50-µL trial PCR to determine the number of PCR cycles needed for a given sample type before proceeding to a set of three or more replicate 100-µL reactions (large-scale PCRs). Reaction products from the large-scale PCRs will be pooled to generate enough material for gel purification and subsequent SOLiD sequencing sample preparation.

Number of cycles recommended for trial PCRs

Most samples should be amplified for 15–18 cycles. For pilot experiments, use 15 PCR cycles. Increase the number of cycles to 18 for the large-scale PCRs if the amount of desired product seen in the pilot PCR is insufficient (Figure 2 on page 10).

SOLiD PCR Primer Sets

Ten different PCR primer sets for synthesis of SOLiD sequencing template are provided with the kit. The primer sets are identical except for a 6 nt barcode located near the middle of the primers (Figure 1 on page 4). ***For this procedure, use any of the provided SOLiD PCR Primers, but do not mix different samples together.***

cDNA library amplification procedure

For the following hazards, see the complete safety alert descriptions in "Chemical alerts" on page 27.

WARNING! CHEMICAL HAZARD. 10× PCR Buffer I, AmpliTaq DNA Polymerase.

1. Dispense PCR Master Mix into wells of a PCR plate or tubes.

   a. Prepare PCR Master Mix on ice by combining the reagents listed in the following table, in the order shown. ***Gently*** vortex the tube to mix thoroughly and microcentrifuge briefly to collect the mixture at the bottom of the tube.

PCR Master Mix (for a single reaction)

Trial PCR (50 µL)	Large-scale PCR‡ (100 µL)	Component (add in order shown)
38.9 µL	77.8 µL	Nuclease-free Water
5 µL	10 µL	10X PCR Buffer I
1 µL	2 µL	SOLiD PCR Primers (one set)§
4 µL	8 µL	2.5 mM dNTP Mix
0.6 µL	1.2 µL	AmpliTaq® DNA Polymerase
49.5 µL	99 µL	Total volume per reaction

‡ Run 3 or more replicate Large-Scale PCRs for each sample.
§ Use only one set of SOLiD PCR Primers (1–10) in each master mix.

b. Pipet PCR Master Mix for a single reaction into the wells of a PCR plate or 0.2 mL PCR tubes.

2. Add RNase H-treated cDNA to each reaction mix, then run the PCR.

a. **Trial PCR (50 µL)** – Add 0.5 µL RNase H-treated cDNA to each aliquot of PCR Master Mix.

Large-scale PCR (100 µL) – Add 1 µL RNase H-treated cDNA to each aliquot of PCR Master Mix.

IMPORTANT! Do not use >1 µL cDNA in a 50 µL PCR; more cDNA may inhibit the PCR.

b. Place the sample(s) in a thermal cycler with a heated lid and run the thermal profile shown in the table.

PCR Cycling Conditions

	Stage	Reps	Temp	Time
Denaturation (hold)	1	1	95°C	5 min
PCR (cycle)	2	15–18‡	95°C	30 sec
			62°C	30 sec
			72°C	30 sec
Final Extension	3	1	72°C	7 min

‡ The recommended number of PCR cycles is 15–18 but more or fewer cycles may be necessary depending on the small RNA content of the starting material. For the large-scale PCR, use the number of cycles identified in the trial PCR (see page 12).

3. Run 5–10 µL PCR product on a native 6% polyacrylamide gel.

Add 1 µL Gel Loading Solution (AM8556) to 5–10 µL of PCR product and run on a native 6% polyacrylamide gel (see "Polyacrylamide gel instructions" on page 22 for suggested gel compositions). Stain the gel with SYBR® Gold dye, following the manufacturer's instructions.

4. Evaluate the PCR products.

Figure 3 on page 14 shows results from reactions from various types of input RNA that were amplified using an appropriate number of PCR cycles. Expected results are as follows:

- The amplified products derived from small RNA (18–40 nt) migrate at ~108–130 bp. The size range of the desired products may be larger, depending on the small RNA profile of the sample source.
- Note that higher molecular weight bands at approximately 150 and 200 bp (derived from tRNA and larger species) are expected from reactions using total RNA as input, whereas these larger products are not expected from reactions using size-selected small RNA as input (see Figure 3).
- Self-ligated adaptors and their amplified products form a band at 89 bp. This band is typically present in all reactions.
- Occasionally another amplification artifact that migrates at 100 bp is present. (Avoid this when excising the gel piece in step 2 on page 17.)

Underamplified samples

Underamplified samples exhibit very little material in the ~108–130 bp size range (Figure 2 on page 10, right panel).

Overamplified samples

- Conversely, overamplified samples typically show a significant amount of material in the ~108–130 bp size range, plus a smear of reaction products larger than ~140–150 bp (Figure 2, left panel).
- Overamplified samples from total RNA input may also have a higher molecular weight ladder of bands that represent concatenated PCR products.

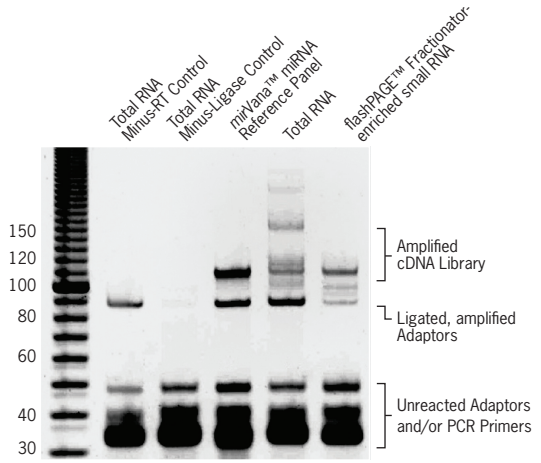


Figure 3 SOLiD™ Small RNA Expression Kit PCR Products

PCR products generated using the SOLiD Small RNA Expression Kit (using 15 PCR cycles) from the indicated control and experimental samples were run on a 6% native polyacrylamide gel stained with SYBR® Gold dye. Samples labeled "Total RNA" used 100 ng of the Control RNA provided with the kit (isolated from human placenta); indicated samples are minus-ligase and minus-reverse transcriptase controls. The other products were synthesized from 100 fmol of the Ambion® *mir*Vana™ miRNA Reference Panel v9.1 (P/N 4388891) and small RNA obtained by flashPAGE™ Fractionator enrichment of 5 µg of Control RNA. The leftmost lane contains 10 bp DNA Ladder (Invitrogen).

Amplified library cleanup

After PCR, the replicate large-scale amplification reactions are pooled and enriched to meet SOLiD sequencing template quantity and purity requirements. PCR by-products such as excess primers and adaptors are removed by a rapid glass fiber-filter column purification. Self-ligated adaptors and larger by-products are then separated from the PCR products derived from small RNA via gel electrophoresis, and the appropriate gel region is excised. The desired reaction products are eluted from the acrylamide, purified, and concentrated. These procedures are described here and in the next section.

Note: Do not heat samples at any step of this purification. It is important that the DNA duplexes remain annealed so that they migrate according to their size during the gel purification described in the next section.

1. Pool replicate PCRs.

Combine the replicate large-scale PCRs from each sample into a single tube. (Do not mix PCRs from different samples.)

2. Clean up the pooled PCRs using the MinElute® PCR Purification Kit.

Use the MinElute PCR Purification Kit (Qiagen) to remove unincorporated primers, enzymes, and salt from the pooled large-scale PCRs. Follow the manufacturer's instructions, with the following modifications.

- The kit may be supplied with Buffer PB (without pH Indicator) or Buffer PBI (with pH Indicator). Either buffer can be used as is; it is not necessary to add pH Indicator to Buffer PB before use.
- The loading volume of your sample will be larger than the capacity of the MinElute column reservoir (1.8 mL; 300 µL PCR + 1,500 µL Buffer PB/PBI). If you follow the microcentrifuge protocol, load the sample into the MinElute column in three 600-µL aliquots (discarding the flow-through after each centrifugation).
- Elute the DNA with 2 sequential applications of 10 µL EB.

Size selection of amplified library by PAGE

Before you start

- Prepare a 0.75 mm, native TBE, 6% polyacrylamide gel (see "Polyacrylamide gel instructions" on page 22). The size of the gel is not important; minigels (~60–100 cm^2) are typically the most convenient.

  Note: Gels cast in-house within a few hours of use typically provide better resolution than purchased pre-cast gels. Optimum gel resolution is important for good results.

- Prepare PAGE Elution Buffer; ~600 µL is needed for each sample.

PAGE Elution Buffer	
For 10 mL	**Component**
5 mL	TE Buffer pH 8 (10 mM Tris-HCl, pH 8, 1mM EDTA)
5 mL	5 M Ammonium acetate (2.5 M final conc.)

- Use a 21-gauge needle to puncture through the bottom-center of a 0.5 mL microcentrifuge tube for each sample. The gel pieces excised in step 2 below will be placed in these tubes, and the centrifugation in the subsequent step will shred the DNA-containing gel pieces for elution of the DNA.

Perform size selection of the amplified library

For the following hazards, see the complete safety alert descriptions in "Chemical alerts" on page 27.

WARNING! CHEMICAL HAZARD. 10× RNase III Reaction Buffer, RNase III.

1. Run samples on a 6% polyacrylamide gel and stain with SYBR® Gold dye.

   a. Add 4 µL Gel Loading Solution (AM8556) to the eluted DNA (20 µL) from step 2 in "Amplified library cleanup" on page 15.

   b. Load sample into 3 adjacent wells (8 µL each well) of a 6% native TBE polyacrylamide gel. (Do not overload; use more wells if necessary.)

   Include a well with the Invitrogen 10 bp DNA Ladder, or a similar ladder, as a marker.

   IMPORTANT! Use a ladder with 10 bp spacing.

   c. Run the gel at ~140 V (~30 min for a minigel) or until the leading dye front almost exits the gel.

   d. Stain the gel with SYBR® Gold dye following the manufacturer's instructions.

2. Excise the 105–150 bp material from the gel.

a. Excise the gel piece containing 105–150 bp DNA using a clean razor blade. If you are using a UV transilluminator to visualize the reaction products, work quickly to limit their exposure to UV radiation.

Figure 2 on page 10 shows an example of the reaction products from small RNA-enriched and total RNA. Most of the desired products migrate at 105–130 bp; however, excision of the gel containing up to 150 bp DNA ensures collection of the amplified products from the entire population of small RNA species.

Note: In addition to the expected 89 bp band formed by self-ligated adaptors, you may see a distinct band at 100 bp; this band represents a different PCR artifact. Avoid both bands if possible.

An additional band at ~150 bp is seen when total RNA is used as input for the SOLiD Small RNA Expression Kit procedure; avoid excising this band, which is derived from tRNA and larger species.

b. Place the gel piece in the 0.5 mL tube prepared with a hole in the bottom.

3. Shred the gel piece.

a. Place the 0.5 mL tube with the gel slice into a larger, 1.5 mL, nuclease-free microcentrifuge tube.

b. Microcentrifuge for 3 min at 13,000 x g to shred the gel.

c. Place the 1.5 mL tube containing the shredded gel piece on ice.

d. Inspect the 0.5 mL tube, and if any gel pieces remain, repeat the centrifugation step into a fresh 1.5 mL tube. Pool the gel pieces into a single collection tube using a pipet tip.

4. Elute the DNA in PAGE elution buffer.

a. Add 300 µL PAGE Elution Buffer to the shredded gel pieces.

b. Incubate the mixture overnight at room temperature, with gentle agitation.

c. Transfer the buffer, which contains eluted DNA, to a fresh tube, leaving the gel fragments behind.

Store the DNA on ice during the second elution (step e).

d. Add another 300 µL PAGE Elution Buffer to the shredded gel pieces.

e. Incubate the buffer and gel pieces for 1–2 hr at 37°C, with gentle agitation.

5. Remove the gel pieces from the sample using a filter spin column.

a. Combine the PAGE elution buffer from step 4c with the buffer plus gel slurry from step 4e.

b. Cut a pipet tip to make a larger opening and use it to transfer the combined PAGE elution buffer and gel slurry from each sample to an Ambion Spin Column (P/N AM10065).

c. Centrifuge the Spin Column at top speed for 5 min to remove gel pieces. The DNA is now in the flow-through.

   (Alternatively, you can use a 0.45 µm-filter spin column from another manufacturer for this step, following the manufacturer's instructions for the maximum centrifugation speed.)

6. Precipitate the DNA, then resuspend in 20 µL Nuclease-free Water.

a. Add 1/100 volume of glycogen and 0.7 volume isopropanol to each sample.

b. Mix thoroughly and incubate at room temperature for 5 min.

c. Centrifuge the sample at 13,000 x g for 20 min at room temperature.

d. Carefully remove and discard the supernatant and air dry the pellet.

e. Resuspend the DNA pellet in 20 µL Nuclease-free Water.

Note: Accurate quantitation of the DNA is important for the downstream SOLiD emulsion PCR titration step. The resuspension volume should yield DNA sufficiently concentrated for accurate measurements (~10 ng/µL).

7. Measure the DNA concentrations of all samples.

Quantitate the DNA in each sample on a NanoDrop ND1000 spectrophotometer (use 1.5 µL of your sample) and verify the size and quality using an Agilent bioanalyzer (use 1 µL).

8. Proceed to SOLiD sample preparation.

SOLiD Small RNA Expression Kit reaction products enter the SOLiD sample preparation workflow at the "SOLiD System Template Bead Preparation" stage, in which emulsion PCR is used to attach sequencing templates to beads. (See *Applied Biosystems SOLiD System User Guide*, P/N 4391578.)

Troubleshooting

No PCR products in the expected size range

The desired PCR products from the SOLiD™ Small RNA Expression Kit procedure are in the 105–150 bp size range. They should be easily visible when 5–10 µL of the PCR is run on a native acrylamide gel stained with SYBR® Gold at step 3 on page 13. Consider the following suggestion if no PCR products in the 105–150 bp size range are visible on the gel.

Run the positive control reaction

Especially if you are using the kit for the first time, try repeating the reaction with your experimental samples and include a parallel positive control reaction to make sure that the supplied reagents are working properly. .

To conduct the positive control reaction, use 500 ng of the supplied Control RNA in a SOLiD Small RNA Expression Kit reaction following the protocol in sections "Hybridization and ligation to adaptor mix" through "cDNA library amplification", starting on page 9. It is not necessary to gel purify the positive control reaction products unless you plan to carry the positive control reaction through SOLiD sequencing. Perform the positive control experiment using the same conditions as for your experimental samples:

- Mix 2 µL of the supplied Control RNA with 6 µL Nuclease-free Water to dilute the Control RNA to 250 ng/µL. Use 2 µL (500 ng) of the diluted Control RNA in the procedure.
- Hybridize and ligate to the same Adaptor Mix for 8 hrs.
- For the PCR ("cDNA library amplification" on page 12), set up a single 50 µL reaction using the same SOLiD Small RNA PCR Primer Set as for the experimental samples. Use **18** amplification cycles, and run 5 µL of the positive control PCR on an acrylamide gel (step 3 on page 13).

The expected result of the positive control reaction is shown in Figure 2 on page 10.

The positive control reaction worked, but experimental samples yield products 89 bp and smaller

PCR product that migrates at 89 bp represents an unwanted artifact caused by amplification of self-hybridized adaptors. If only the 89 bp product and/or low molecular weight nucleic acids (unreacted adaptors and PCR primers) are visible on the gel after PCR, there may not have been enough template in the reaction, or a reagent may have been mistakenly left out of the reaction.

The small RNA complement of the sample RNA is very low or the sample does not contain any small RNA.

- Applied Biosystems strongly recommends using 10–200 ng size-selected small RNA as input for the procedure, because it results in more efficient production of the desired products. The flashPAGE Fractionator System is recommended for size selection, but PAGE purification is an alternative method.

- Repeat the procedure with more input RNA, preferably size-selected RNA, or increase the number of PCR cycles at step 2 on page 13 to as many as 20.

  The number of PCR cycles should be minimized to maintain a more accurate profile of the small RNAs in a sample. If your application does not require strict quantitative profiling, the number of amplification cycles can be increased.

A key reagent was accidentally left out of the reaction.

Repeat the reaction and consider running a positive control reaction in parallel.

Input RNA was either degraded or otherwise could not participate in the reaction.

Repeat the procedure using RNA that is known to be intact by analysis on a denaturing agarose gel or on an Agilent bioanalyzer.

Supplemental Information

This appendix includes:

- Sequences of the SOLiD™ PCR primers included in the kit 21
- Polyacrylamide gel instructions 22
- Related products available from Applied Biosystems 23
- Quality control 23
- References 24

Sequences of the SOLiD™ PCR primers included in the kit

Ten sets of SOLiD PCR primers are provided in the kit. The concentration of each primer is 25 µM. The primer sequences are listed below.

5'-SOLiD™ PCR primer

The P1 adapter is the 5' PCR primer in each SOLiD PCR Primer Set. The sequence is shown in the 5' to 3' orientation:

CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGAT

3'-SOLiD™ PCR primers

The 3' PCR primers in each SOLiD PCR Primer Set are identical except for a 6 nt "barcode" sequence that is unique to each primer (shown in bold italics).

Sequences are shown in the 5' to 3' orientation.

Component	3' primer sequence (shown 5' to 3')
SOLiD PCR Primers 1	CTGCCCCGGGTTCCTCATTCTCT***AAGCCC***CTGCTGTACGGCCAAGGCG
SOLiD PCR Primers 2	CTGCCCCGGGTTCCTCATTCTCT***CACACC***CTGCTGTACGGCCAAGGCG
SOLiD PCR Primers 3	CTGCCCCGGGTTCCTCATTCTCT***CCCCTT***CTGCTGTACGGCCAAGGCG
SOLiD PCR Primers 4	CTGCCCCGGGTTCCTCATTCTCT***CATCGG***CTGCTGTACGGCCAAGGCG
SOLiD PCR Primers 5	CTGCCCCGGGTTCCTCATTCTCT***TCGTTG***CTGCTGTACGGCCAAGGCG
SOLiD PCR Primers 6	CTGCCCCGGGTTCCTCATTCTCT***GGGCAC***CTGCTGTACGGCCAAGGCG
SOLiD PCR Primers 7	CTGCCCCGGGTTCCTCATTCTCT***CCAGAC***CTGCTGTACGGCCAAGGCG
SOLiD PCR Primers 8	CTGCCCCGGGTTCCTCATTCTCT***CTCCGT***CTGCTGTACGGCCAAGGCG
SOLiD PCR Primers 9	CTGCCCCGGGTTCCTCATTCTCT***CCCTTC***CTGCTGTACGGCCAAGGCG
SOLiD PCR Primers 10	CTGCCCCGGGTTCCTCATTCTCT***GCGGTC***CTGCTGTACGGCCAAGGCG

Polyacrylamide gel instructions

6% Nondenaturing acrylamide gels

Gel mix

5 mL is enough gel solution for one minigel.

for 5 mL	Component
0.5 mL	10X TBE (for example, Ambion P/N AM9863)
0.75 mL	40% acrylamide (acryl:bis-acryl = 19:1; for example, Ambion P/N AM9022)
to 5 mL	deionized water

Stir at room temperature, then add:

50 µL	10% ammonium persulfate in dH_2O
5 µL	TEMED

Mix briefly after adding the last two ingredients, which catalyze polymerization, then pour the gel immediately.

Gel set up

- Follow the manufacturers instructions for the details of attaching gels to the running apparatus.
- Use 1✕ TBE as the gel running buffer.

IMPORTANT! Do not heat the samples before loading.

Electrophoresis conditions

Nondenaturing gels must be run slowly to avoid heat denaturation of the samples. Set the voltage to about 140 V, and run the gel for ~45 minutes.

Gel Loading Buffer

For this procedure, use Ambion Gel Loading Solution (P/N AM8556), as described in this protocol.

Related products available from Applied Biosystems

flashPAGE™ Fractionator AM13100	The Ambion® flashPAGE Fractionator is a specialized electrophoresis instrument for rapid PAGE-purification of small nucleic acids. Designed for use with flashPAGE Pre-Cast Gels and the optimized running buffers supplied in the flashPAGE Buffer Kit, the flashPAGE Fractionator purifies small nucleic acid molecules more quickly, easily, and efficiently than traditional PAGE purification.
flashPAGE™ Reaction Clean-Up Kit AM12200	The Ambion® flashPAGE Reaction Clean-Up Kit is a fast and convenient filter-based purification and concentration system for small nucleic acids obtained using the flashPAGE Fractionator. It is a rapid and simple alternative to overnight precipitation.
Ambion® electrophoresis reagents	Ambion® offers gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis. See **www.appliedbiosystems.com**.

Quality control

Functional testing

Amplified small RNA libraries were prepared following the procedure outlined in this document from 25 ng of the Control RNA with spike-in synthetic small RNA template. Two libraries were prepared, one with Adaptor Mix A and another with Adaptor Mix B. The ligation reactions were incubated for 2 hr. 15 amplification cycles were used in a 50 µL PCR. Products derived from both the Control RNA and the synthetic small RNA were detected by real-time PCR.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity

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

Nonspecific endonuclease activity

Meets or exceeds specification when a sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity

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

Protease testing

Relevant kit components are tested as follows:

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

References

Pak J, Fire A (2007) Distinct Populations of Primary and Secondary Effectors During RNAi in C. elegans. *Science* 315, 5809: 241–244.

Tissot, C (2008) Application Note: Analysis of miRNA content in total RNA preparations using the Agilent 2100 bioanalyzer. Agilent Technologies publication number 5989-7870EN. Available at **https://www.chem.agilent.com/Library/applications/5989-7870EN.pdf**

Yu B, Yang Z, Li J, Minakhina S, Yang M, Padgett RW, Steward R, Chen X (2005) Methylation as a Crucial Step in Plant microRNA Biogenesis. *Science* 307, 5711: 932–935.

Safety

This appendix includes:

- General chemical safety 25
- MSDSs 25
- Biological hazard safety 26
- Chemical alerts 27

General chemical safety

Chemical safety guidelines

To minimize the hazards of chemicals:

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

MSDSs

About MSDSs

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

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



Obtaining MSDSs

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

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.
- For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.

Biological hazard safety

General biohazard



WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at:

www.cdc.gov

Chemical alerts

For the definitions of the alert words **IMPORTANT**, **CAUTION**, **WARNING**, and **DANGER**, see "Safety alert words" on page v.

General alerts for all chemicals

Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Specific chemical alerts

WARNING! 2× Ligation Buffer may cause eye, skin, and respiratory tract irritation. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin.

WARNING! 10× PCR Buffer I causes eye, skin, and respiratory tract irritation. Exposure may cause nervous system depression. May be harmful if swallowed. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin.

WARNING! 10× RT Buffer contains dithiothreitol. Exposure may cause nervous system depression. May be harmful if swallowed. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin.

WARNING! AmpliTaq® DNA Polymerase may cause eye, skin, and respiratory tract irritation. May be harmful if swallowed. Avoid breathing vapor. Use with adequate ventilation.

WARNING! ArrayScript™ Reverse Transcriptase may cause eye, skin, and respiratory tract irritation. May be harmful if swallowed. Avoid breathing vapor. Use with adequate ventilation.

WARNING! Hybridization Solution may cause eye, skin, and respiratory tract irritation. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin.

WARNING! Ligation Enzyme Mix may cause eye, skin, and respiratory tract irritation. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin.

WARNING! RNase H may cause eye, skin, and respiratory tract irritation. May be harmful if swallowed. Avoid breathing vapor. Use with adequate ventilation.

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