Applied Biosystems

Applied Biosystems SOLiD™ 3 System

Library Preparation Guide

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Library Preparation Guide

For Research Use Only. Not for use in diagnostic procedures.

Part Number 4407413 Rev. B
02/2009

Contents

Preface

Safety information

Note: For important instrument safety information, refer to the *Applied Biosystems SOLiD™ 3 System Instrument Operation Guide* (PN 4407430). For general safety information, see this Preface and Appendix I, "Safety" on page 241. 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

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT, CAUTION, WARNING, DANGER**—implies a particular level of observation or action, as defined below:

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, results 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 245.

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**.

User attention words

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

For the latest services and support information for all locations, go to:

www.appliedbiosystems.com

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.

1 Introduction

Library preparation overview

Library preparation is the first step in which samples are adapted for SOLiD™ sequencing. During library preparation, forward and reverse adaptors are added to the ends of DNA inserts (see Figure 1).

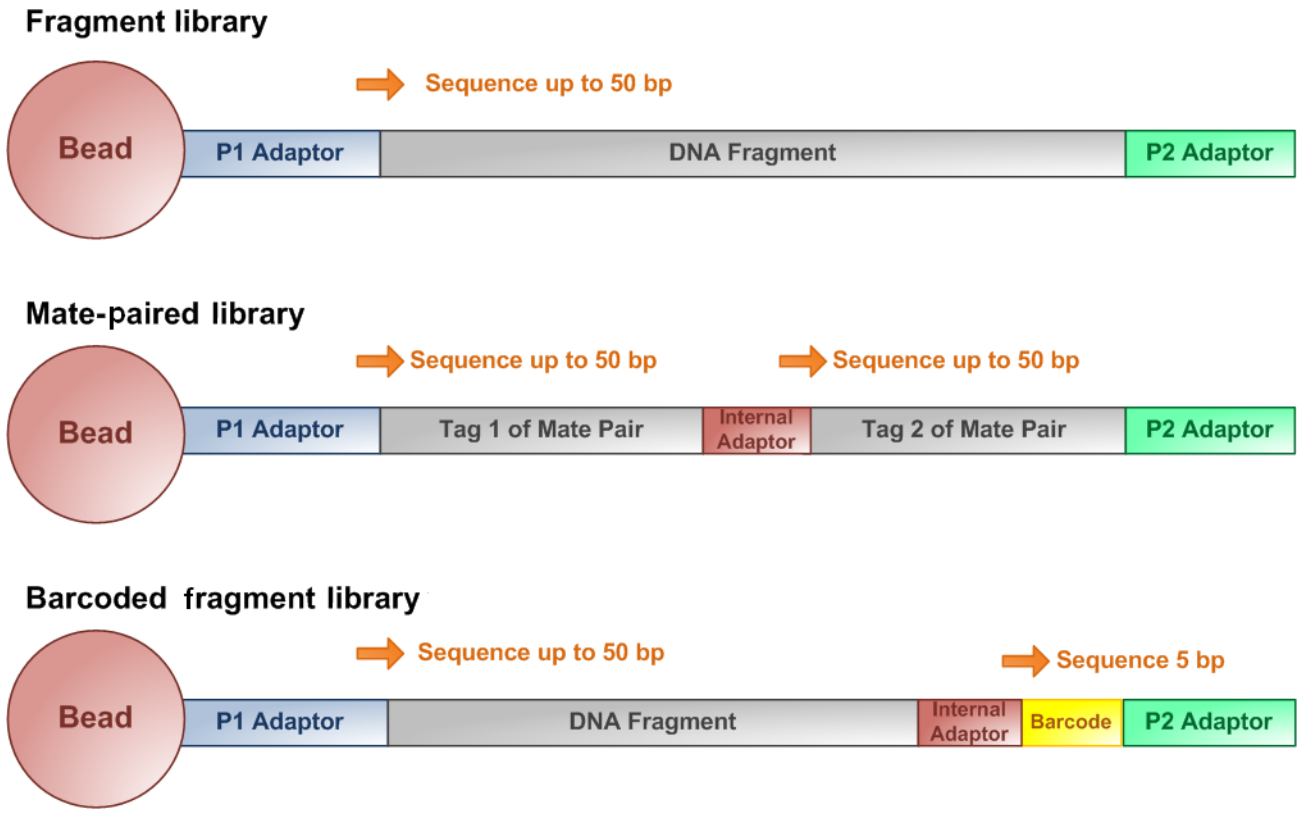


Figure 1 Fragment and mate-paired library construction.

Choose the appropriate library type

Table 1 Libraries that can be sequenced on the SOLiD™ 3 System

Library type	Features	Go to…
Fragment	• Adaptors on each end of sheared DNA insert (see Figure 1 on page 1) • Less input DNA required (10 ng to 20 µg) • Appropriate for sequence lengths ≤ 300 bp • Simpler library construction workflow • Higher recovery of unique molecules	Chapter 2, "Fragment Library Preparation" on page 3
Mate-paired	• Two DNA insert tags 600 bp to 6 kb apart (see Figure 1 on page 1) • More input DNA required (5 µg to 20 µg) • More even coverage of genome • Better ability for unique tag placement	Chapter 3, "Mate-Paired Library Preparation" on page 29
Barcoded fragment	• Same as fragment library, except with a barcode sequence located on one of the adaptors to enable multiplexed sequencing • Can be pooled for templated bead preparation	Chapter 4, "Barcoded Fragment Library Preparation" on page 111

The type of library used depends on the application and information needed. For deeper coverage of large and complex genomes (for example, human genomes), more DNA is required to prepare libraries. For smaller and less complex genomes (for example, microbial genomes), less DNA can be used, and shorter read lengths are adequate. For information about specific applications, go to the SOLiD System website:

http://solid.appliedbiosystems.com

or contact your field applications specialist.

2 Fragment Library Preparation

This chapter covers:

Overview

This chapter describes the method to generate a small-insert (100 to 110 bp) library. This method involves shearing DNA into small fragments and ligating P1 and P2 Adaptors (see Figure 2 and Figure 3).

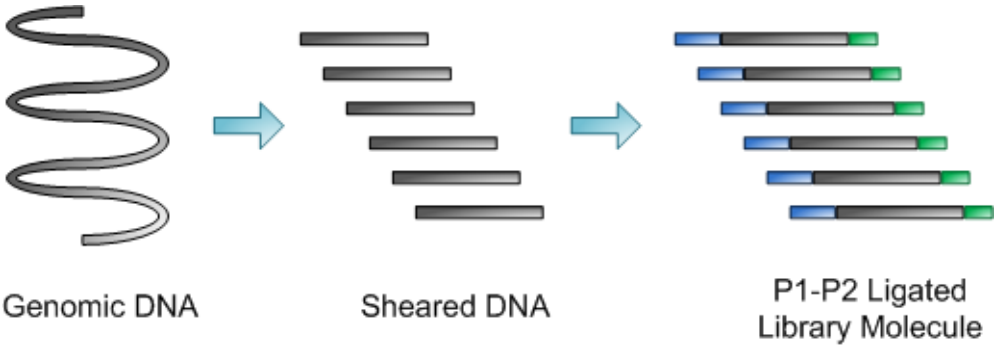


Figure 2 Basic fragment library preparation workflow overview.

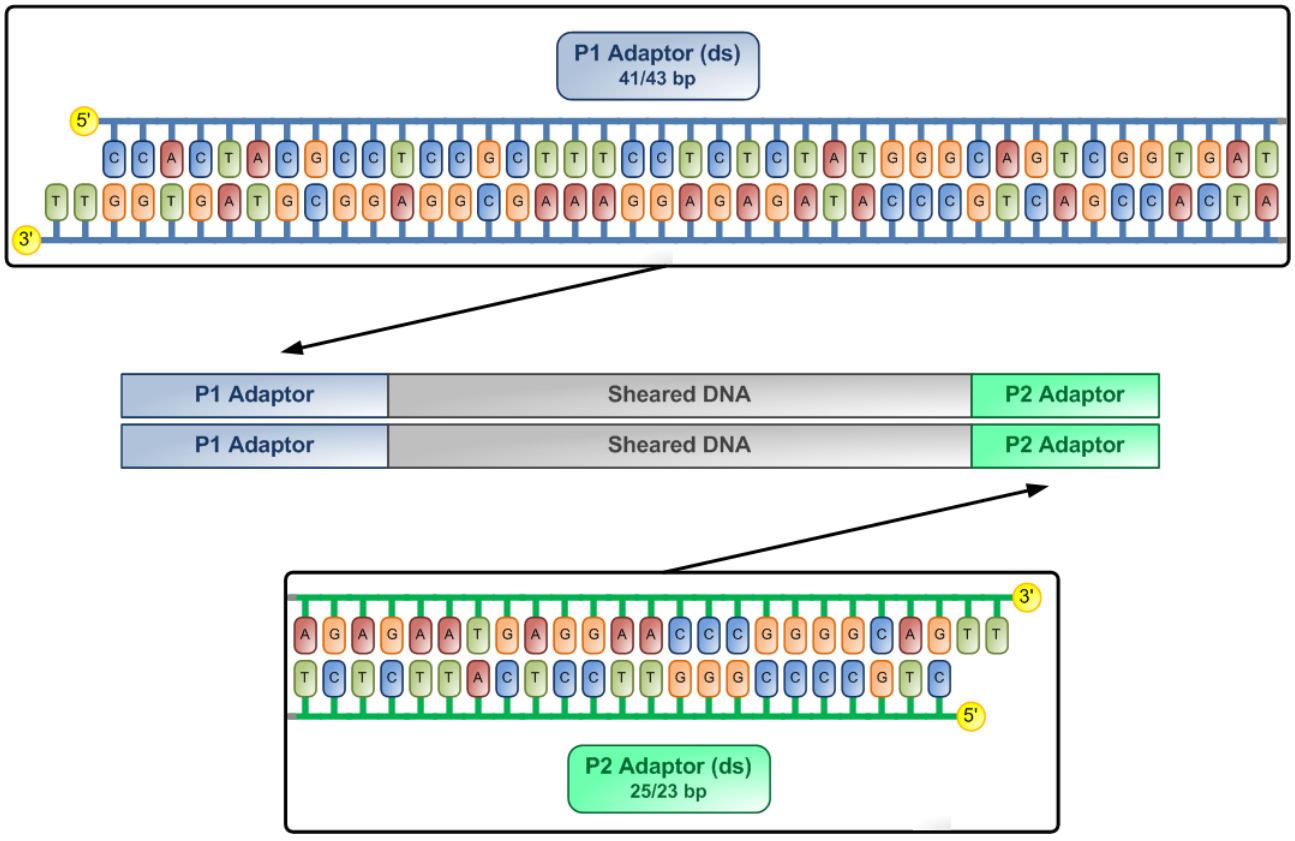


Figure 3 Fragment library design.

After P1 and P2 Adaptors are ligated to the sheared DNA, the library is amplified using primers specific to the P1 and P2 Adaptors (see Figure 4). Library PCR Primer 1 is a 3′-truncated version of the 5′ strand sequence of P1, while Library PCR Primer 2 is a 3′-truncated version of the 5′ strand sequence of P2. These primers can be used only for library amplification and not for alternative or modified library construction adaptor design, as they do not have 3′sequences compatible with the sequencing primers.

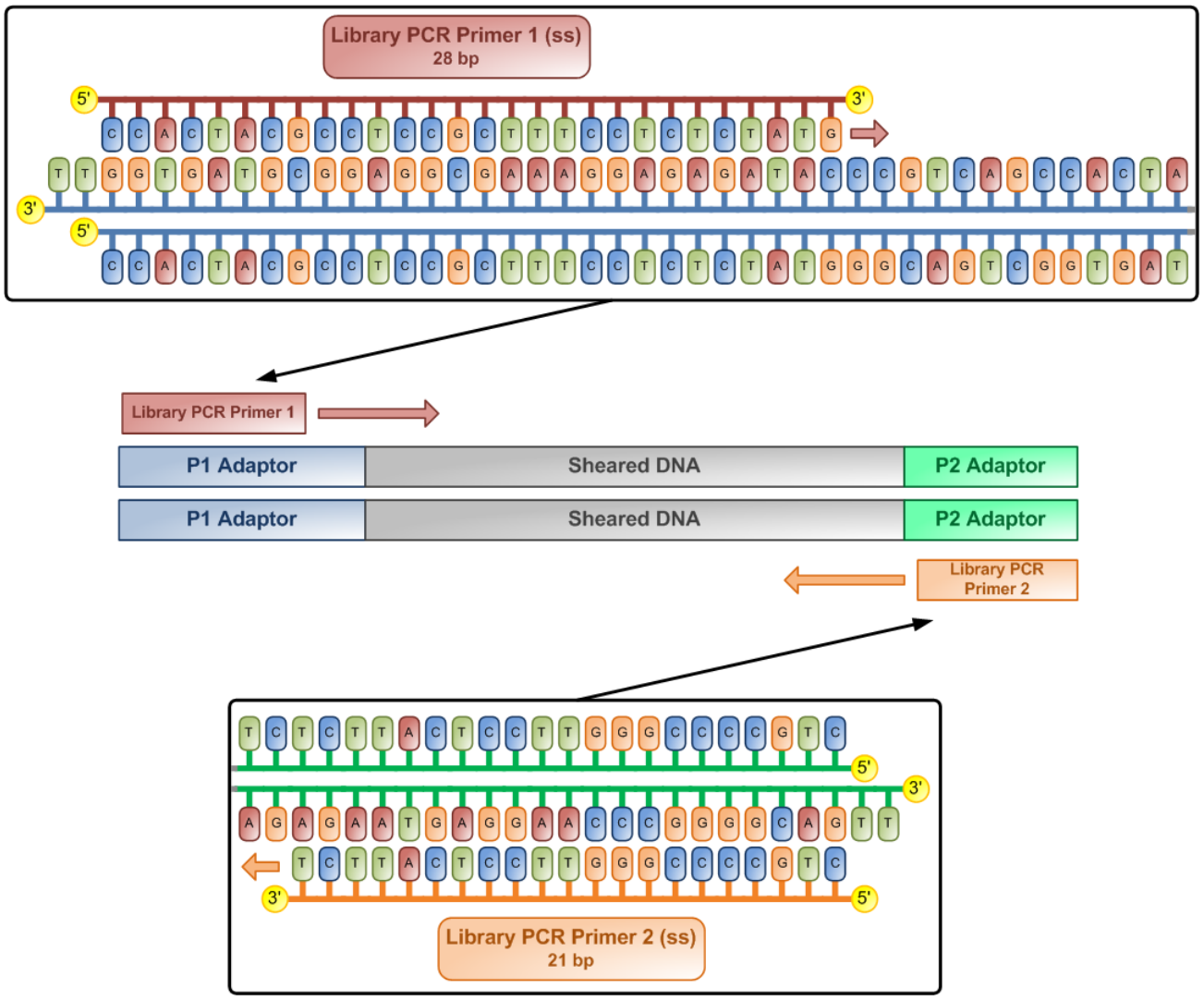


Figure 4 Fragment library amplification design.

This chapter is organized into two sections. Section 2.1 describes how to generate a small-insert library using gel-based size-selection. Section 2.2 describes how to generate a small-insert library without gel-based size-selection.

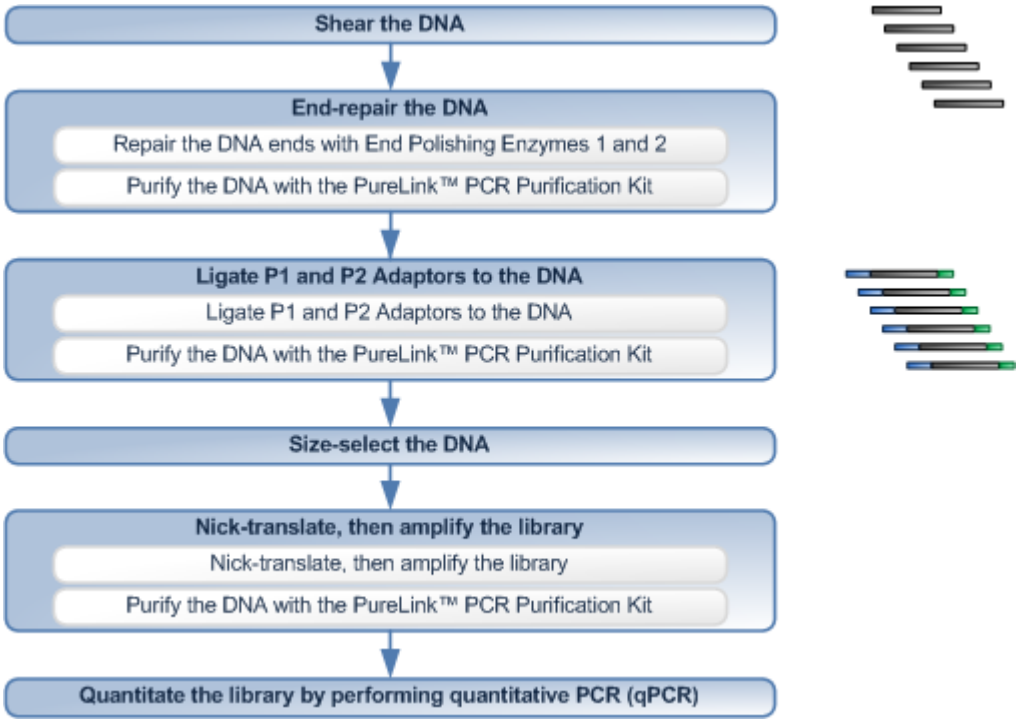
Section 2.1 Prepare a standard fragment library

This protocol is designed for 10 ng to 20 µg of genomic DNA or ligated PCR product. You should modify the protocol with any change in the starting amount of DNA. If you are constructing a targeted, resequencing library with small-sized PCR products (≤ 500 bp), then you must perform a PCR-product ligation step. For a concatenation protocol, contact your field applications specialist.

Materials and equipment required

See Appendix A on page 127 for a list of equipment, kits, and consumables necessary for this procedure.

Workflow



Shear the DNA

This step involves sonicating the input DNA into small fragments with a mean size of 100 to 110 bp by using a Covaris™ S2 System. The conditions have been tested for shearing 10 ng to 20 µg DNA in a total volume of 100 µL. For certain DNA samples, optimizing the shearing protocol may be necessary.

End-repair the DNA

End Polishing Enzyme 1 and End Polishing Enzyme 2 are used to convert DNA that has damaged or incompatible 5′-protruding and/or 3′-protruding ends to 5′-phosphorylated, blunt-ended DNA. The conversion to blunt-ended DNA results from 5′-to-3′ polymerase and the 3′-to-5′ exonuclease activities of End Polishing Enzyme 2. End Polishing Enzyme 1 and ATP are also included for phosphorylation of the 5′-ends of the blunt-ended DNA to allow for subsequent ligation.

Purify the DNA with the PureLink™ PCR Purification Kit

Sample purification is recommended with the PureLink™ PCR Purification columns supplied in the PureLink™ PCR Purification Kit. PureLink columns have a 40-µg capacity, but it may be necessary to use multiple columns during a purification step for higher yields.

Ligate P1 and P2 Adaptors to the DNA

P1 and P2 Adaptors are ligated to the ends of the end-repaired DNA. The P1 and P2 Adaptors are included in double-stranded form in the SOLiD™ Fragment Library Oligos Kit.

Size-select the DNA

The ligated, purified DNA is run on an E-Gel® 2% SizeSelect™ gel. The correctly sized ligation products (150 to 200 bp) are electrophoresed to the collection wells of the SizeSelect gel. The eluate in each collection well can be transferred directly to the nick translation reaction.

Nick-translate, then amplify the library

The eluates from the SizeSelect gel undergo nick translation and subsequently amplification using Library PCR Primers 1 and 2 and Platinum® PCR Amplification Mix. After amplification, PCR samples are purified with the PureLink™ PCR Purification Kit.

Quantitate the library by performing quantitative PCR (qPCR)

Quantitate the library by either the TaqMan® or SYBR® quantitative PCR (qPCR) method described in Appendix B, "SOLiD™ 3 System Library Quantitation" on page 155.

Tips

- Adjust microcentrifuge speeds and times according to the g-forces specified in the protocols. Applied Biosystems recommends the Eppendorf 5417R tabletop microcentrifuge.
- Perform all steps requiring 0.5-mL and 1.5-mL tubes with Eppendorf LoBind tubes.
- Thaw reagents on ice before use.

Shear the DNA

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. TRIS (Tris (hydroxymethyl)aminomethane).

1. Dilute the desired amount of DNA in 100 µL in 1X Low TE Buffer in a LoBind tube (see Table 2).

Table 2 Dilute the DNA for shearing

Component	Amount
DNA	10 ng to 20 µg
1X Low TE Buffer	Variable
Total	100 µL

2. Place a Covaris™ microTube into the loading station. Keep the cap on the tube and use a tapered pipette tip to slowly transfer the 100 µL of DNA sample through the pre-split septa. Be careful not to introduce a bubble into the bottom of the tube.

3. Shear the DNA using the following Covaris S2 System conditions:
   - Number of Cycles: **6**
   - Bath Temperature: **5** °C
   - Bath Temperature Limit: **30** °C
   - Mode: **Frequency sweeping**
   - Water Quality Testing Function: **Off**
   - Duty cycle: **20%**
   - Intensity: **5**
   - Cycles/burst: **200**
   - Time: **60 seconds**

IMPORTANT! Make sure that the water in the Covaris tank is filled with fresh deionized water to fill line level 12 on the graduated fill line label. The water should cover the visible glass part of the tube. Set the chiller temperature to between 2 to 5 °C to ensure that the temperature reading in the water bath displays 5 °C. The circulated water chiller should be supplemented with 20% ethylene glycol.

4. Place the Covaris microTube into the loading station. While keeping the snap-cap on, insert a pipette tip through the pre-split septa, then slowly remove the sheared DNA. Transfer the sheared DNA into a new 1.5-mL LoBind tube.

End-repair the DNA

Repair the DNA ends with End Polishing Enzyme 1 and End Polishing Enzyme 2

1. Combine and mix the following components in a 1.5-mL LoBind tube (see Table 3):

Table 3 Mix for end-repair of DNA

Component	Volume (µL)
Sheared DNA	100
5× End-Polishing Buffer	40
dNTP Mix, 10 mM	8
End Polishing Enzyme 1, 10 U/µL	2
End Polishing Enzyme 2, 5 U/µL	16
Nuclease-free water	34
Total	200

2. Incubate the mixture at room temperature for 30 minutes.

Purify the DNA with PureLink™ PCR Purification Kit

1. Add 4 volumes of Binding Buffer (B2) with 55% isopropanol to the end-repaired DNA.
2. Apply about 700 µL of end-repaired DNA in the Binding Buffer (B2) to the column(s). The maximum yield of DNA can be achieved if the amount of DNA loaded to a PureLink™ column is ≤ 5 µg. Use more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the PureLink column(s) back into the same collection tube(s).
6. Add 650 µL of Wash Buffer (W2) to wash the column(s).
7. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 50 µL of Elution Buffer (E1) to the column(s) to elute the DNA, then let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 1 minute.
11. Add the eluate from step 10 back to the column(s), then let the column(s) stand for 2 minutes. Repeat step 10.

12. If necessary, pool the eluted DNA.

13. If the starting DNA input amount is ≥ 500 ng, quantitate the purified DNA by using 2 µL of the sample on the NanoDrop® ND-1000 Spectrophotometer (see Appendix C, "Supplemental Procedures" on page 187). If the starting DNA input amount is < 500 ng, assume 70% recovery of input material after shearing.

STOPPING POINT. Store the purified DNA in Elution Buffer (E1) at 4 ºC, or proceed directly to "Ligate the P1 and P2 Adaptors to the DNA" on page 12.

Ligate P1 and P2 Adaptors to the DNA

Ligate the P1 and P2 Adaptors to the DNA

1. Calculate the amount of adaptor needed, *Y*, for the reaction based on the amount of DNA from the last purification step (for calculation details, see "Ligation of P1 and P2 Adaptors" on page 212). If DNA fragments were sheared using the standard protocol for fragment library preparation, the average insert size should be approximately 105 bp.

$$X\ \text{pmol/µg DNA} = 1\ \text{µg DNA} \times \frac{10^6\ \text{pg}}{1\ \text{µg}} \times \frac{1\ \text{pmol}}{660\ \text{pg}} \times \frac{1}{\text{Average insert size}}$$

$$Y\ \text{µL adaptor needed} = \#\ \text{µg DNA} \times \frac{X\ \text{pmol}}{1\ \text{µg DNA}} \times 30 \times \frac{1\ \text{µL adaptor needed}}{50\ \text{pmol}}$$

Example:

For 1 µg of purified end-repaired DNA with an average insert size of 105 bp

$$X\ \text{pmol/µg DNA} = 1\ \text{µg DNA} \times \frac{10^6\ \text{pg}}{1\ \text{µg}} \times \frac{1\ \text{pmol}}{660\ \text{pg}} \times \frac{1}{105} = 14.4\ \text{pmol/µg DNA}$$

$$Y\ \text{µL adaptor needed} = 1\ \text{µg DNA} \times \frac{14.4\ \text{pmol}}{1\ \text{µg DNA}} \times 30 \times \frac{1\ \text{µL adaptor needed}}{50\ \text{pmol}}$$

$$= 8.7\ \text{µL adaptor needed}$$

2. Combine and mix the following components (see Table 4).

Table 4 Ligation mix

Component	Volume (µL)
P1 Adaptor (ds), 50 pmol/µL	*Y*
P2 Adaptor (ds), 50 pmol/µL	*Y*
5X T4 Ligase Buffer	40
DNA	48 to 50
T4 Ligase, 5 U/µL	10
Nuclease-free water	Variable
Total	200

3. Incubate the mixture at room temperature for 15 minutes.

Purify the DNA with the PureLink™ PCR Purification Kit

1. Add 4 volumes (800 µL) of Binding Buffer (B2) with 40% isopropanol to the sample.
2. Apply about 700 µL of the ligated DNA in Binding Buffer (B2) to the column(s). The maximum yield of DNA can be achieved if the amount of DNA loaded to a PureLink™ column is ≤ 5 µg. Use more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 1 minute, then discard the flow-through.

5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the PureLink column(s) back into the same collection tube(s).

6. Add 650 µL of Wash Buffer (W2) to wash the column(s).

7. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.

8. Air-dry the column(s) for 2 minutes to evaporate residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).

9. Add 50 µL of Elution Buffer (E1) to the column(s) to elute the DNA, then let the column(s) stand for 2 minutes.

10. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 1 minute.

11. Add the eluate from step 10 back to the column(s), then let the column(s) stand for 2 minutes. Repeat step 10.

12. If necessary, pool the eluted DNA.

STOPPING POINT. Store the purified DNA in Elution Buffer (E1) at 4 °C, or proceed directly to "Size-select the DNA" on page 14.

Size-select the DNA

1. Remove an E-Gel® 2% SizeSelect™ gel from its package. Remove the combs from *top* sample-loading wells and *middle* collection wells. Set the E-Gel on the E-Gel iBase™ linked with the E-Gel Safe Imager™.

2. Load the E-Gel as follows:

   a. Load 20 µL of the ligated, purified DNA into each well of the *top row* of wells. If the sample volume < 20 µL, add nuclease-free water to the well for a total volume of 20 µL. Skip the center well (smaller well in the top center of the gel for the ladder); and skip a single well to the right and left of the center top well. Skip the two outermost wells (to avoid edge effects). Do not load more than 1 µg of DNA per lane.

   b. Load 2 µL of 50-bp ladder at 0.1 µg/µL to the center top well. Add 15 µL of water to fill the well.

   c. Fill empty wells in the top row with 20 µL of nuclease-free water.

   d. Fill each of the collection wells in the *middle* of the gel with 25 µL of nuclease-free water. Add 20 µL of nuclease-free water to the middle center well.

3. Run the gel:
   - iBase program: **Run E-Gel DC**
   - Run time: **11:40** (11 minutes and 40 seconds)

   Monitor the E-Gel in real-time with the E-Gel® Safe Imager.

4. If needed during the run, fill the middle collection wells with nuclease-free water.

5. When the 150-bp band from the marker lane clears the collection well and the 200-bp band is about to enter the collection well, stop the run if the run has not already stopped (see Figure 5 on page 15).

6. Collect the solution from the wells and pool according to samples.

7. Wash each collection well with 25 µL of nuclease-free water, then retrieve the wash solution with the solution collected in Step 6.

8. (Optional) Concentrate the DNA with a PureLink PCR purification column. No concentration of the DNA is needed, however, if the DNA will be nick-translated or amplified according to the procedures below.

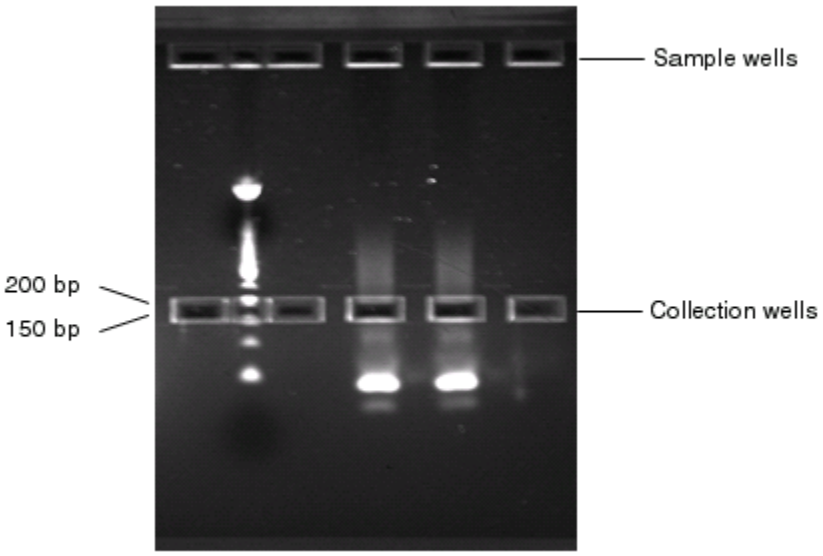


Figure 5 Elution of ~150- to 200-bp region from an E-Gel® 2% SizeSelect™ gel.

Nick-translate, then amplify the library

Nick-translate, then amplify the library

1. Prepare a master mix for the number of reactions needed based on the amount of starting input DNA, plus one additional reaction for the negative control (see Table 5).

Table 5 Suspend the gel eluate according to starting input DNA

If the gel eluate DNA is...	>100 µL	≤ 100 µL
Component	**Volume (µL)** **F = volume of eluate/100**	**Volume (µL)**
Platinum® PCR Amplification Mix	380 × F	380
Library PCR Primer 1, 50 µM	10 × F	10
Library PCR Primer 2, 50 µM	10 × F	10
Total	400 × F	400

2. If the volume of the eluate is
   - ≤ 100 µL, add 400 µL of master mix to the gel eluate, then distribute in 4 PCR reaction tubes
   - > 100 µL, add 400 µL of master mix to every 100 µL of eluate, then distribute in 125-µL aliquots to PCR reaction tubes.

3. Run the PCR (Table 6 on page 17).

IMPORTANT! Minimize the number of cycles to avoid overamplification and production of redundant molecules. Determine the number of cycles based on the amount of starting input DNA.

Table 6 PCR conditions to nick-translate and amplify the library

Stage	Step	Temp	Time
Holding	Nick translation	72 °C	20 min
Holding	Denature	95 °C	5 min
Cycling (2 to 10 cycles)‡	Denature	95 °C	15 sec
	Anneal	62 °C	15 sec
	Extend	70 °C	1 min
Holding	Extend	70 °C	5 min
Holding	—	4 °C	∞

‡ Starting amount of DNA: number of cycles:
10 ng to 100 ng: 10 cycles
100 ng to 1 µg: 6 to 8 cycles
1 µg to 2 µg: 4 to 6 cycles
2 µg to 20 µg: 2 to 3 cycles

4. Pool all of the PCR samples into a new 1.5-mL LoBind tube.

Purify the DNA with the PureLink™ PCR Purification Kit

1. Add 4 volumes of Binding Buffer (B2) with 40% isopropanol to the sample.
2. Apply about 700 µL of PCR product in the Binding Buffer (B2) to the column(s). The maximum yield of DNA can be achieved if the amount of DNA loaded to a PureLink™ column is ≤ 5 µg. Use more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the PureLink column(s) back into the same collection tube(s).
6. Add 650 µL of Wash Buffer (W2) to wash the column(s).
7. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 50 µL of Elution Buffer (E1) to the column(s) to elute the DNA, then let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 1 minute.

11. Add the eluate from step 10 back to the column(s), then let the column(s) stand for 2 minutes. Repeat step 10.

12. If necessary, pool the eluted DNA.

STOPPING POINT. Store the purified DNA in Elution Buffer (E1) at 4 °C, or proceed directly to "Quantitate the library by performing quantitative PCR (qPCR)" on page 18.

Quantitate the library by performing quantitative PCR (qPCR)

For accurate library quantitation, quantitative PCR is strongly recommended. For a TaqMan® or SYBR® qPCR protocol, see Appendix B, "SOLiD™ 3 System Library Quantitation" on page 155.

STOPPING POINT. Store the purified DNA in Elution Buffer (E1) at 4 °C, or proceed directly to emulsion PCR in the *Applied Biosystems SOLiD™ 3 System Templated Bead Preparation Guide* (PN 4407421).

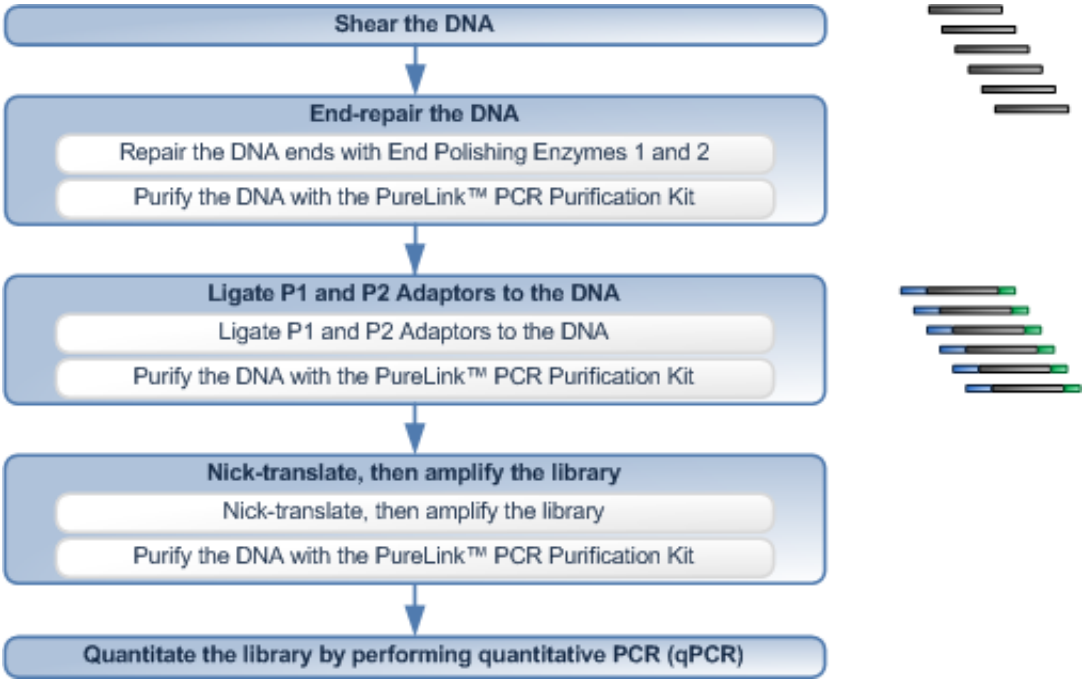
Section 2.2 Prepare an express fragment library

This protocol is designed for 10 ng to 20 µg of genomic DNA or ligated PCR product. You should modify the protocol with any change in the starting amount of DNA. If you are constructing a targeted, resequencing library with small-sized PCR products (≤ 500 bp), then you must perform a PCR-product ligation step. For a concatenation protocol, contact your field applications specialist.

Materials and equipment required

See Appendix A on page 127 for a list of equipment, kits, and consumables necessary for this procedure.

Workflow



Shear the DNA

This step involves sonicating the input DNA into small fragments with a mean size of 100 to 110 bp using the Covaris™ S2 System. The conditions have been tested for shearing 10 ng to 20 µg DNA in a total volume of 100 µL. For certain DNA samples, optimizing the shearing protocol may be necessary.

End-repair the DNA

End Polishing Enzyme 1 and End Polishing Enzyme 2 are used to convert DNA that has damaged or incompatible 5′-protruding and/or 3′-protruding ends to 5′-phosphorylated, blunt-ended DNA. The conversion to blunt-ended DNA results from 5′-to-3′ polymerase and the 3′-to-5′ exonuclease activities of End Polishing Enzyme 2. End Polishing Enzyme 1 and ATP are also included for phosphorylation of the 5′-ends of the blunt-ended DNA to allow for subsequent ligation.

Purify the DNA with the PureLink™ PCR Purification Kit

Sample purification is recommended with the PureLink™ PCR Purification columns supplied in the PureLink™ PCR Purification Kit. PureLink columns have a 40-µg capacity, but it may be necessary to use multiple columns during a purification step for higher yields.

Ligate P1 and P2 Adaptors to the DNA

P1 and P2 Adaptors are ligated to the ends of the end-repaired DNA. The P1 and P2 Adaptors are included in double-stranded form in the SOLiD™ Fragment Library Oligos Kit.

Nick-translate, then amplify the library

The adaptor-ligated, purified DNA undergoes nick translation, then amplification using Library PCR Primer 1 and Library PCR Primer 2 and Platinum® PCR Amplification Mix. After amplification, the PCR samples are purified with the PureLink PCR Purification Kit.

Quantitate the library by performing quantitative PCR (qPCR)

Quantitate the library by either the TaqMan® or SYBR® quantitative PCR (qPCR) method described in Appendix B, "SOLiD™ 3 System Library Quantitation" on page 155.

Tips

- Adjust microcentrifuge speeds and times according to the g-forces specified in the protocols. Applied Biosystems recommends the Eppendorf 5417R tabletop microcentrifuge.
- Perform all steps requiring 0.5-mL and 1.5-mL tubes with Eppendorf LoBind tubes.
- Thaw reagents on ice before use.

Shear the DNA

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. TRIS (Tris (hydroxymethyl)aminomethane).

Shear the DNA using the Covaris™ S2 System

1. Dilute the desired amount of DNA in 100 µL in 1✕ Low TE Buffer in a LoBind tube (see Table 7).

Table 7 Dilute the DNA for shearing

Component	Amount
DNA	10 ng to 20 µg
1✕ Low TE Buffer	Variable
Total	100 µL

2. Place a Covaris™ microTube into the loading station. Keep the cap on the tube and use a tapered pipette tip to slowly transfer the 100 µL of DNA sample through the pre-split septa. Be careful not to introduce a bubble into the bottom of the tube.

3. Shear the DNA using the following Covaris S2 System conditions:
   - Number of Cycles: **6**
   - Bath Temperature: **5** °C
   - Bath Temperature Limit: **30** °C
   - Mode: **Frequency sweeping**
   - Water Quality Testing Function: **Off**
   - Duty cycle: **20%**
   - Intensity: **5**
   - Cycles/burst: **200**
   - Time: **60 seconds**

IMPORTANT! Make sure that the water in the Covaris tank is filled with fresh deionized water to fill line level 12 on the graduated fill line label. The water should cover the visible glass part of the tube. Set the chiller temperature to between 2 to 5 °C to ensure that the temperature reading in the water bath displays 5 °C. The circulated water chiller should be supplemented with 20% ethylene glycol.

4. Place the Covaris microTube into the loading station. While keeping the snap-cap on, insert a pipette tip through the pre-split septa, then slowly remove the sheared DNA. Transfer the sheared DNA into a new 1.5-mL LoBind tube.

End-repair the DNA

Repair the DNA ends with End Polishing Enzyme 1 and End Polishing Enzyme 2

1. Combine and mix the following components in a 1.5-mL LoBind tube (see Table 8):

Table 8 Mix for end-repair of DNA

Component	Volume (µL)
Sheared DNA	100
5× End-Polishing Buffer	40
dNTP Mix, 10 mM	8
End Polishing Enzyme 1, 10 U/µL	2
End Polishing Enzyme 2, 5 U/µL	16
Nuclease-free water	34
Total	200

2. Incubate the mixture at room temperature for 30 minutes.

Purify the DNA with PureLink™ PCR Purification Kit

1. Add 4 volumes of Binding Buffer (B2) with 55% isopropanol to the end-repaired DNA.
2. Apply about 700 µL of end-repaired DNA in the Binding Buffer (B2) to the column(s). The maximum yield of DNA can be achieved if the amount of DNA loaded to a PureLink™ column is ≤ 5 µg. Use more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the PureLink column(s) back into the same collection tube(s).
6. Add 650 µL of Wash Buffer (W2) to wash the column(s).
7. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 50 µL of Elution Buffer (E1) to the column(s) to elute the DNA, then let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 1 minute.
11. Add the eluate from step 10 back to the column(s), then let the column(s) stand for 2 minutes. Repeat step 10.

12. If necessary, pool the eluted DNA.

13. If the starting DNA input amount is ≥ 500 ng, quantitate the purified DNA by using 2 µL of the sample on the NanoDrop® ND-1000 Spectrophotometer (see Appendix C, "Supplemental Procedures" on page 187). If the starting DNA input amount is < 500 ng, assume 70% recovery of input material after shearing.

STOPPING POINT. Store the purified DNA in Elution Buffer (E1) at 4 ºC, or proceed directly to "Ligate P1 and P2 Adaptors to the DNA" on page 25.

Ligate P1 and P2 Adaptors to the DNA

Ligate P1 and P2 Adaptors to the DNA

1. Calculate the amount of adaptor needed, *Y*, for the reaction based on the amount of DNA from the last purification step (for calculation details, see "Ligation of P1 and P2 Adaptors" on page 212). If DNA fragments were sheared using the standard protocol for fragment library preparation, the average insert size should be approximately 105 bp.

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{\text{Average insert size}}$$

$$Y \text{ µL adaptor needed} = \#\text{ µg DNA} \times \frac{X \text{ pmol}}{1 \text{ µg DNA}} \times 30 \times \frac{1 \text{ µL adaptor needed}}{50 \text{ pmol}}$$

Example:

For 1 µg of purified end-repaired DNA with an average insert size of 105 bp

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{105} = 14.4 \text{ pmol/µg DNA}$$

$$Y \text{ µL adaptor needed} = 1 \text{ µg DNA} \times \frac{14.4 \text{ pmol}}{1 \text{ µg DNA}} \times 30 \times \frac{1 \text{ µL adaptor needed}}{50 \text{ pmol}}$$

$$= 8.7 \text{ µL adaptor needed}$$

2. Combine (see Table 9):

Table 9 Ligation mix

Component	Volume (µL)
P1 Adaptor (ds), 50 pmol/µL	*Y*
P2 Adaptor (ds), 50 pmol/µL	*Y*
5× T4 Ligase Buffer	40
DNA	48 to 50
T4 Ligase, 5 U/µL	10
Nuclease-free water	Variable
Total	200

3. Incubate at room temperature for 10 minutes.

Purify the DNA with the PureLink™ PCR Purification Kit

1. Add 4 volumes (800 µL) of Binding Buffer (B2) with 40% isopropanol to the sample.
2. Apply about 700 µL of the ligated DNA in Binding Buffer (B2) to the column(s). The maximum yield of DNA can be achieved if the amount of DNA loaded to a PureLink™ column is ≤ 5 µg. Use more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 1 minute, then discard the flow-through.

5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the PureLink column(s) back into the same collection tube(s).

6. Add 650 µL of Wash Buffer (W2) to wash the column(s).

7. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.

8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).

9. Add 50 µL of Elution Buffer (E1) to the column(s) to elute the DNA, then let the column(s) stand for 2 minutes.

10. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 1 minute.

11. Add the eluate from step 10 back to the column(s), then let the column stand for 2 minutes. Repeat step 10.

12. If necessary, pool the eluted DNA.

STOPPING POINT. Store the purified DNA in Elution Buffer (E1) at 4 °C, or proceed directly to "Nick-translate, then amplify the library" on page 27.

Nick-translate, then amplify the library

Nick-translate, then amplify the library

1. Prepare a PCR reaction mix (see Table 10).

Table 10 PCR reaction mix: a mix for nick translation and amplification of the library

Component	Volume (µL)
Platinum® PCR Amplification Mix	400
Library PCR Primer 1, 50 µM	10
Library PCR Primer 2, 50 µM	10
Adaptor-ligated, purified DNA	48 to 50
Nuclease-free water	Variable
Total	500

2. Pipette 125 µL of the PCR reaction mix into each of four PCR tubes.

3. Run the PCR (Table 11).

IMPORTANT! Minimize the number of cycles to avoid overamplification and production of redundant molecules. Determine the number of cycles based on the amount of starting input DNA.

Table 11 PCR conditions to nick-translate and amplify the library

Stage	Step	Temp	Time
Holding	Nick translation	72 °C	20 min
Holding	Denature	95 °C	5 min
Cycling (2 to 10 cycles)‡	Denature	95 °C	15 sec
	Anneal	62 °C	15 sec
	Extend	70 °C	1 min
Holding	Extend	70 °C	5 min
Holding	—	4 °C	∞

‡ Starting amount of DNA: number of cycles:
10 ng to 100 ng: 10 cycles
100 ng to 1 µg: 6 to 8 cycles
1 µg to 2 µg: 4 to 6 cycles
2 µg to 20 µg: 2 to 3 cycles

4. Pool all of the PCR samples into a new 1.5-mL LoBind tube.

Purify the DNA with the PureLink™ PCR Purification Kit

1. Add 4 volumes of Binding Buffer (B2) with 40% isopropanol to the sample.
2. Apply about 700 µL of PCR product in the Binding Buffer (B2) to the column(s). The maximum yield of DNA can be achieved if the amount of DNA loaded to a PureLink™ column is ≤ 5 µg. Use more columns if necessary. Keep for now.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the PureLink column(s) back into the same collection tube(s).
6. Add 650 µL of Wash Buffer (W2) to wash the column(s).
7. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 50 µL of Elution Buffer (E1) to the column(s) to elute the DNA, then let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥ 10,000 × g (13,000 rpm) for 1 minute.
11. Add the eluate from step 10 back to the column(s), then let the column(s) stand for 2 minutes. Repeat step 10.
12. If necessary, pool the eluted DNA.

STOPPING POINT. Store the purified DNA in Elution Buffer (E1) at 4 °C, or proceed directly to "Quantitate the library by performing quantitative PCR (qPCR)".

Quantitate the library by performing quantitative PCR (qPCR)

Quantitate your library by quantitative PCR. For a TaqMan® or SYBR® qPCR protocol, see Appendix B, "SOLiD™ 3 System Library Quantitation" on page 155.

STOPPING POINT. Store the DNA in Elution Buffer (E1) at 4 °C, or proceed directly to emulsion PCR in the *Applied Biosystems SOLiD™ 3 System Templated Bead Preparation Guide* (PN 4407421).

3 Mate-Paired Library Preparation

This chapter covers:

Overview

This chapter describes the method to make a mate-paired library with insert sizes ranging from 600 bp to 6 kb. A mate-paired library consists of pairs of DNA fragments that are "mates" because they originated from the two ends of the same genomic DNA fragment. CAP adaptors connect the DNA mate pair together through an internal adaptor.

For 2 × 50 bp mate-paired libraries, size-selected genomic DNA fragments are ligated to LMP CAP Adaptors and circularized with internal adaptors (see Figure 6). The resulting DNA circle has one nick in each strand because the LMP CAP Adaptor does not have the 5′ phosphate in one of its oligonucleotides. Nick translation using *E. coli* DNA polymerase I "pushes" the nick into the genomic DNA region in 5′ to 3′ direction. The length of nick-translated DNA can be controlled by adjusting reaction temperature and time. T7 exonuclease and S1 nuclease digestion cuts the DNA at the position opposite to the nick and releases the DNA mate pair. P1 and P2 Adaptors are then ligated to the ends of the mate-paired library for subsequent amplification by PCR (see Figure 8 on page 33).

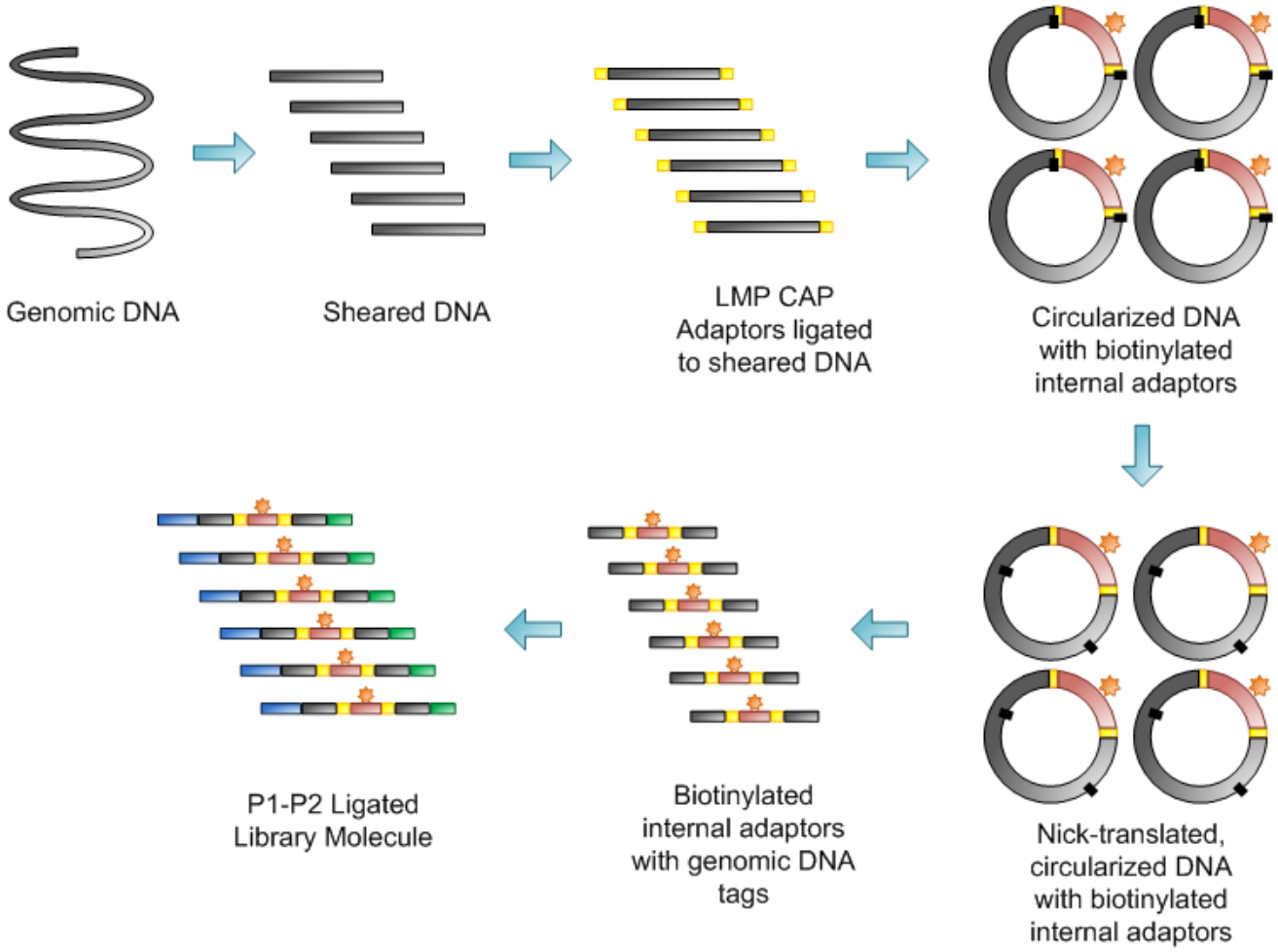


Figure 6 Basic 2 × 50 bp mate-paired library preparation workflow.

For 2 × 25 bp mate-paired libraries, EcoP15I CAP Adaptors are ligated to sheared, methylated DNA (see Figure 7). The EcoP15I restriction enzyme sites in the genomic DNA are methylated prior to EcoP15I CAP Adaptor ligation to ensure that only the unmethylated enzyme recognition sites in the CAP adaptor are recognized by EcoP15I during the restriction enzyme step. As a result, EcoP15I cleaves 25 to 27 bp away from the unmethylated enzyme recognition sites in the CAP linker, yielding mate-paired genomic DNA attached to either side of the internal adaptor. P1 and P2 Adaptors are then ligated to the ends of the mate-paired library for subsequent amplification by PCR. (see Figure 8 on page 33).

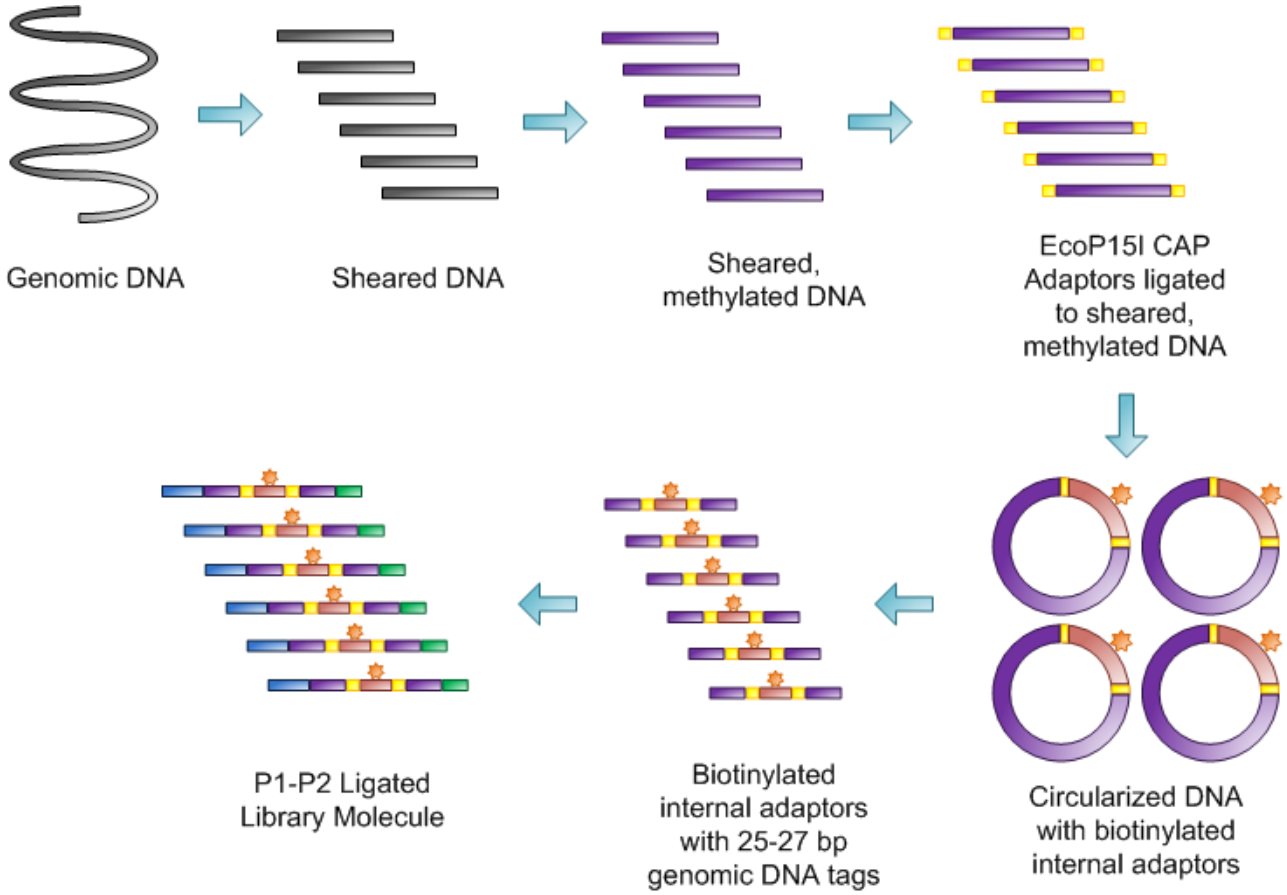


Figure 7 Basic 2 × 25 bp mate-paired library preparation workflow.

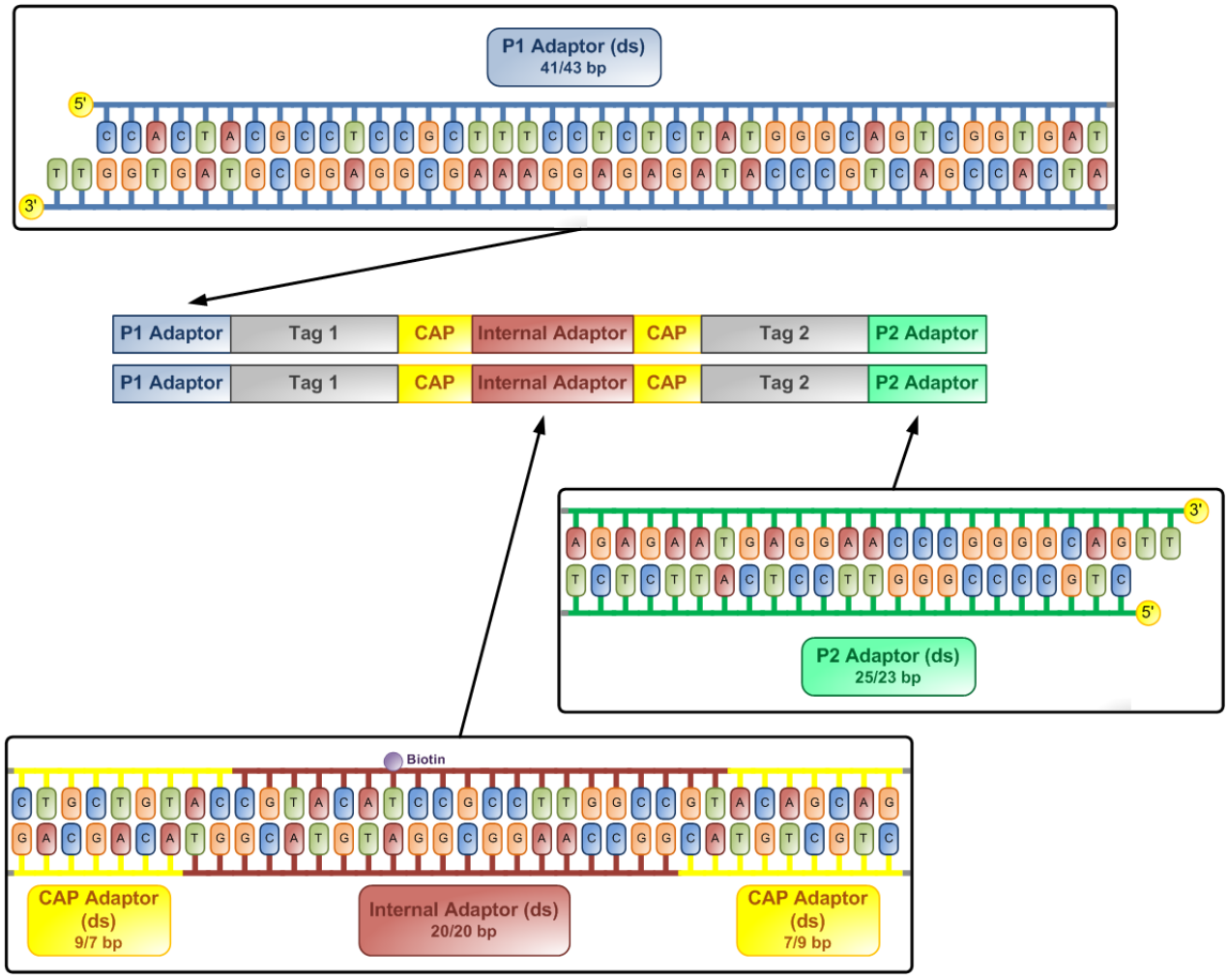


Figure 8 Mate-paired library design.

After P1 and P2 Adaptors are ligated to the sheared DNA, the library is amplified using primers specific to the P1 and P2 Adaptors (see Figure 9 on page 34). Library PCR Primer 1 is a 3′-truncated version of the 5′-strand sequence of P1, while Library PCR Primer 2 is a 3′-truncated version of the 5′-strand sequence of P2. These primers can be used only for library amplification and not for alternative or modified library construction adaptor design, because they do not have 3′ sequences compatible with the sequencing primers.

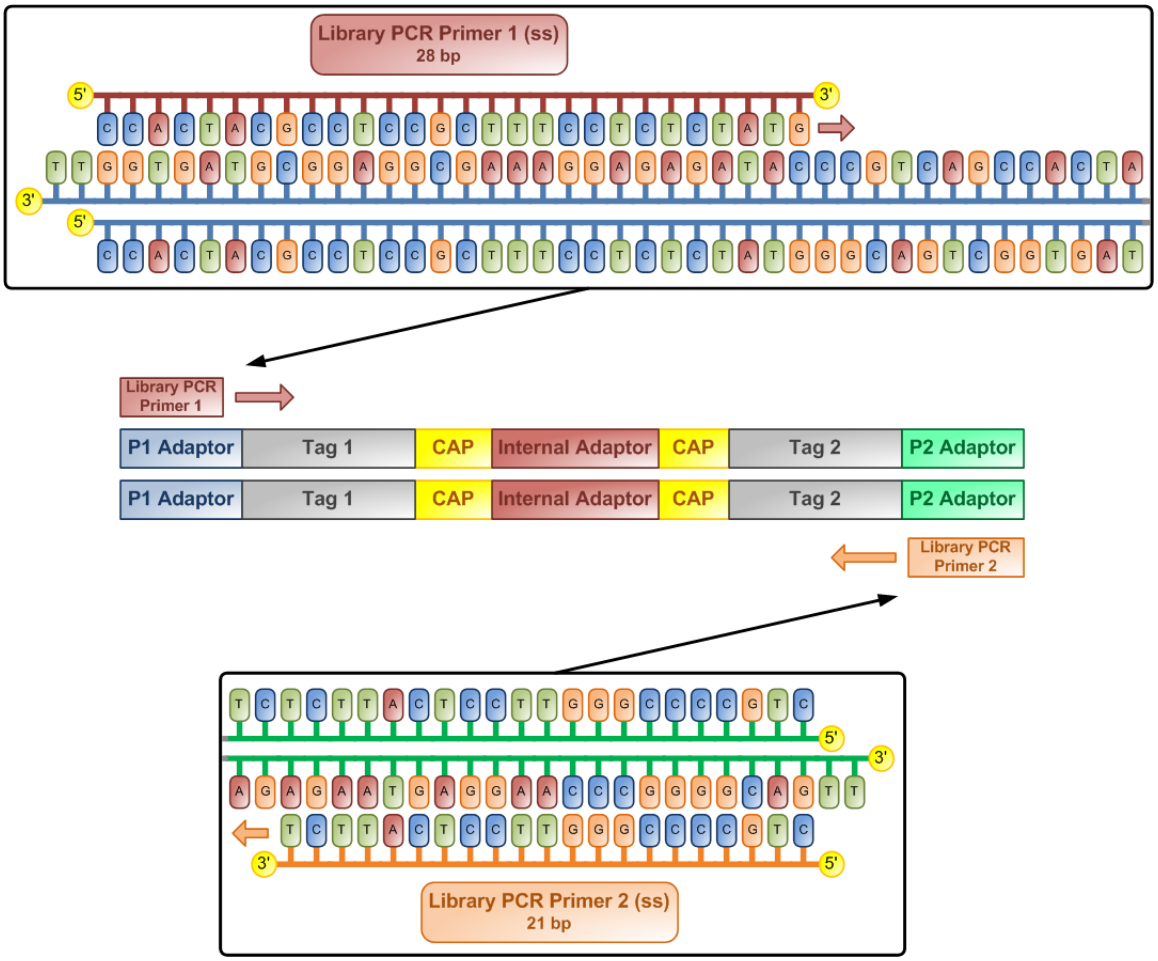


Figure 9 Mate-paired library amplification design.

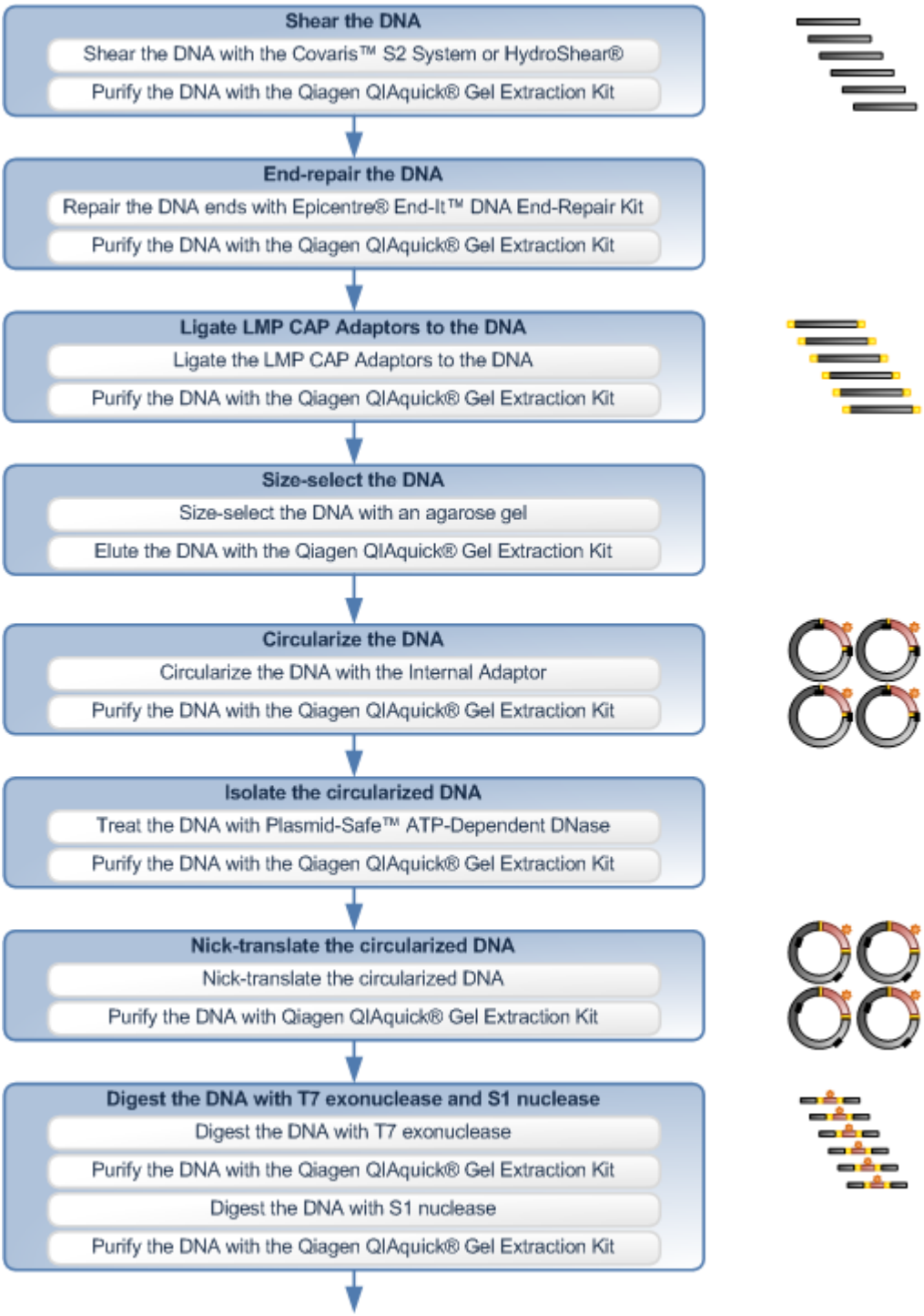
This chapter is organized into two sections. Section 3.1 describes how to generate a 2 × 50 bp mate-paired library. Section 3.2 describes how to generate a 2 × 25 bp mate-paired library.

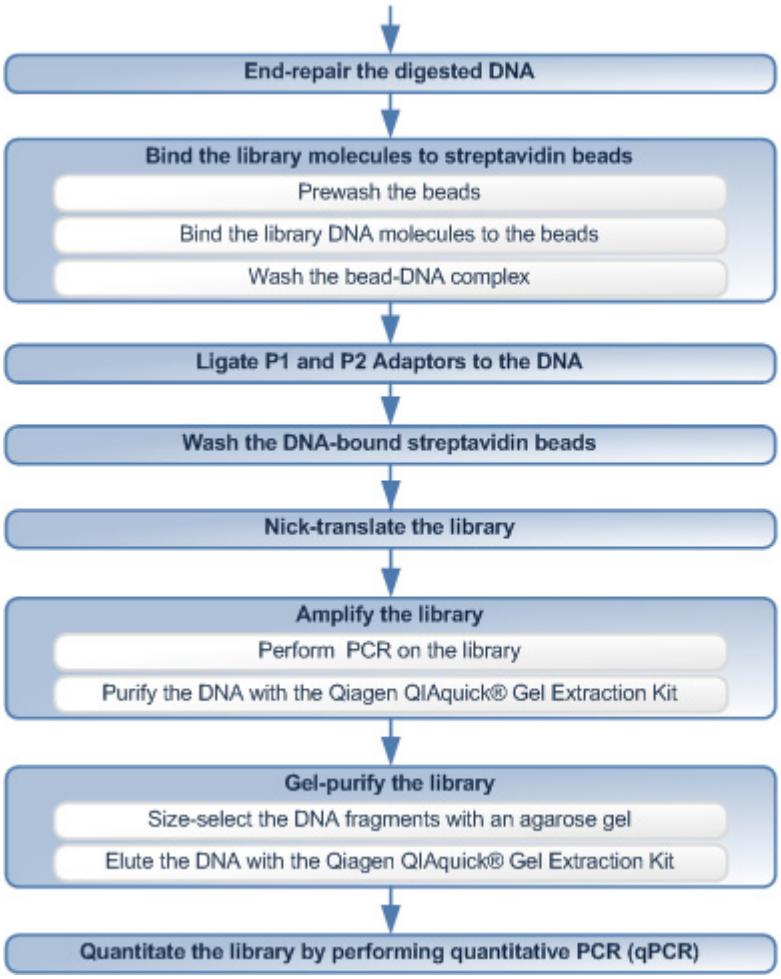
Section 3.1 Prepare a 2 × 50 bp mate-paired library

Materials and equipment required

See Appendix A on page 127 for a list of equipment, kits, and consumables necessary for this procedure.

Workflow


Shear the DNA
Shear the DNA with the Covaris™ S2 System or HydroShear®
Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit
End-repair the DNA
Repair the DNA ends with Epicentre® End-It™ DNA End-Repair Kit
Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit
Ligate LMP CAP Adaptors to the DNA
Ligate the LMP CAP Adaptors to the DNA
Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit
Size-select the DNA
Size-select the DNA with an agarose gel
Elute the DNA with the Qiagen QIAquick® Gel Extraction Kit
Circularize the DNA
Circularize the DNA with the Internal Adaptor
Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit
Isolate the circularized DNA
Treat the DNA with Plasmid-Safe™ ATP-Dependent DNase
Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit
Nick-translate the circularized DNA
Nick-translate the circularized DNA
Purify the DNA with Qiagen QIAquick® Gel Extraction Kit
Digest the DNA with T7 exonuclease and S1 nuclease
Digest the DNA with T7 exonuclease
Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit
Digest the DNA with S1 nuclease
Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit



Shear the DNA

The genomic DNA is sheared to yield 600 bp to 6 kb fragments. To shear for a mate-paired library with insert sizes between 600 bp and 1 kb, the Covaris™ S2 system is recommended. To shear for a mate-paired library with insert sizes between 1 kb and 6 kb, the HydroShear is recommended. HydroShear uses hydrodynamic shearing forces to fragment DNA strands. The DNA in solution flows through a tube with an abrupt contraction. As it approaches the contraction, the fluid accelerates to maintain the volumetric flow rate through the smaller area of the contraction. During this acceleration, drag forces stretch the DNA until it snaps and until the pieces are too short for the shearing forces to break the chemical bonds. The flow rate of the fluid and the size of the contraction determine the final DNA fragment sizes. While basic guidelines are given for shearing DNA using a HydroShear, every HydroShear® instrument may need an initial standard run, and speed codes may need adjusting for DNA from different organisms. A calibration run to assess the shearing efficacy of your device prior to starting your first library preparation is highly recommended.

Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit

Sample purification is recommended with Qiagen QIAquick® columns supplied in the QIAquick® Gel Extraction Kit. Qiagen QIAquick columns have a 10-µg capacity, so it may be necessary to use multiple columns during a purification step. For more detailed information on purification of DNA with Qiagen QIAquick columns, see the

manufacturer's instructions. If you have larger amounts of DNA for library construction, you can substitute this step with phenol-chloroform-isoamyl alcohol extraction and isopropyl alcohol precipitation (see Appendix C, "Supplemental Procedures" on page 187).

End-repair the DNA

The Epicentre® End-It™ DNA End-Repair Kit is used to convert DNA with damaged or incompatible 5′-protruding and/or 3′-protruding ends to 5′-phosphorylated, blunt-ended DNA for fast and efficient blunt-end ligation. The conversion to blunt-end DNA is accomplished by exploiting the 5′-to-3′ polymerase and the 3′-to-5′ exonuclease activities of T4 DNA Polymerase. T4 polynucleotide kinase and ATP are also included for phosphorylation of the 5′-ends of the blunt-ended DNA for subsequent ligation.

Ligate LMP CAP Adaptors to the DNA

LMP CAP ligation adds the LMP CAP Adaptors to the sheared, end-repaired DNA. The LMP CAP Adaptor is missing a 5′ phosphate from one of its oligonucleotides, which results in a nick on each strand when the DNA is circularized in a later step. The LMP CAP Adaptors are included in double-stranded form in the SOLiD™ Mate-Paired Library Oligos Plus Kit or SOLiD™ Mate-Paired Library Oligos Kit.

Size-select the DNA

Depending on the desired insert-size range, the ligated, purified DNA is run on a 0.8% or 1% agarose gel. The correctly sized ligation products are excised and purified using the Qiagen QIAquick® Gel Extraction Kit.

Size-selection after CAP adaptor ligation is needed to remove unbound CAP adaptors and should not be skipped under any circumstances. Contamination of unbound CAP adaptors can compromise the circularization reaction in the next step.

Circularize the DNA

Sheared DNA ligated to LMP CAP Adaptors is circularized with a biotinylated internal adaptor. To increase the chances that ligation will occur between two ends of one DNA molecule versus two different DNA molecules, a very dilute reaction is used. The circularization reaction products are purified using the QIAquick Gel Extraction Kit. The Internal Adaptor is included in double-stranded form in the SOLiD™ Mate-Paired Library Oligos Plus Kit or SOLiD™ Mate-Paired Library Oligos Kit.

Treat the DNA with Plasmid-Safe™ ATP-Dependent DNase

Epicentre® Plasmid-Safe™ ATP-Dependent DNase is used to eliminate uncircularized DNA. After the Plasmid-Safe DNase-treated DNA is purified using the QIAquick Gel Extraction Kit, the amount of circularized product is quantified. A minimum of 200 ng of circularized product is recommended to proceed with library construction. For more complex genomes, 600 ng to 1 µg circularized DNA is recommended for a high-complexity library.

Nick-translate the circularized DNA

Nick translation using *E. coli* DNA polymerase I translates the nick into the genomic DNA region. The size of the mate-paired tags to be produced can be controlled by adjusting the reaction temperature and time.

Digest the DNA with T7 exonuclease and S1 nuclease	T7 exonuclease recognizes the nicks within the circularized DNA. With its 5′-to-3′ exonuclease activity, T7 exonuclease digests the unligated strand away from the tags creating a gap in the sequence. This gap creates an unexposed single-stranded region that is more easily recognized by S1 nuclease, so the library molecule can be cleaved from the circularized template.
Bind the library molecules to the streptavidin beads	Streptavidin beads specifically bind to the biotin-labeled internal adaptor in the library molecules to purify the library from side products.
Ligate P1 and P2 Adaptors to the DNA	P1 and P2 adaptors are ligated to the ends of the end-repaired DNA. The P1 and P2 Adaptors are included in double-stranded form in the SOLiD™ Mate-Paired Library Oligos Plus Kit or SOLiD™ Mate-Paired Library Oligos Kit.
Wash the DNA-bound streptavidin beads	Library molecules bound to streptavidin beads are washed and purified from ligation side products.
Nick-translate the library	The ligated, purified DNA undergoes nick translation with DNA polymerase I.
Amplify the library	The library is amplified using Library PCR Primers 1 and 2 with the SOLiD™ Library PCR Master Mix. It is important to reduce the number of cycles as much as possible and use the entire nick-translated product for amplification to get maximum representation of the library and avoid PCR-related biases due to differential amplification of library molecules.
Gel-purify the library	The library is run on a 4% agarose gel and the correctly sized band (275 to 300 bp) is excised and eluted using the Qiagen QIAquick Gel Extraction Kit.
Quantitate the library by performing quantitative (qPCR)	Quantitate the library by either the TaqMan® or SYBR® quantitative PCR (qPCR) method described in Appendix B, "SOLiD™ 3 System Library Quantitation" on page 155.

Tips

- Adjust microcentrifuge speeds and times according to the g-forces specified in the protocols. Applied Biosystems recommends the Eppendorf 5417R tabletop microcentrifuge.
- Perform all steps requiring 0.5-mL and 1.5-mL tubes with Eppendorf LoBind tubes.
- Thaw reagents on ice before use.

Shear the DNA

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. 3 M Sodium acetate.

Prepare for shearing

1. Choose the appropriate shearing method based on the desired insert size of the mate-paired library (see Table 12).

   Note: These conditions are only guidelines, and a shearing trial prior to large-scale shearing is recommended if additional DNA is available.

Table 12 Recommended shearing conditions for mate-paired library insert sizes.

Insert size	Shearing method	Shearing conditions
600 to 800 bp	Covaris™ Shearing in 20% glycerol (13 mm × 65 mm borosilicate tube)	• Number of Cycles: **75** • Bath Temperature: **5** °C • Bath Temperature Limit: **12** °C • Mode: **Frequency sweeping** • Water Quality Testing Function: **Off** • Duty cycle: **2%** • Intensity: **7** • Cycles/burst: **200** • Time: **10 seconds**
800 to 1000 bp	Covaris™ Shearing in 20% glycerol (13 mm × 65 mm borosilicate tube)	• Number of Cycles: **30** • Bath Temperature: **5** °C • Bath Temperature Limit: **12** °C • Mode: **Frequency sweeping** • Water Quality Testing Function: **Off** • Duty cycle: **2%** • Intensity: **5** • Cycles/burst: **200** • Time: **10 seconds**
1 to 2 kb	HydroShear® Standard Shearing Assembly	• SC5‡ • 20 cycles
2 to 3 kb	HydroShear® Standard Shearing Assembly	• SC9 • 20 cycles
3 to 4 kb	HydroShear® Standard Shearing Assembly	• SC13 • 20 cycles
4 to 5 kb	HydroShear® Standard Shearing Assembly	• SC15 • 5 cycles
5 to 6 kb	HydroShear® Standard Shearing Assembly	• SC16 • 25 cycles

‡ Speed code (SC): 5.

IMPORTANT! Set the chiller temperature to between 2 to 5 °C to ensure that the temperature reading in the water bath displays 5 °C. The circulated water chiller should be supplemented with 20% ethylene glycol.

2. If the DNA source is not limiting, ensure that the shearing conditions result in the desired insert sizes. Shear 5 µg DNA and run 150 ng sheared DNA on a 0.8% E-Gel® according to the manufacturer's specifications.

Shear the DNA using the Covaris™ S2 System

1. In a round-bottomed 13 mm × 65 mm borosilicate tube, dilute 5 to 20 µg DNA in 500 µL so that the final volume contains 20% glycerol in nuclease-free water. (see Table 13).

Table 13 Dilute the DNA for shearing

Component	Amount
99% glycerol	100 µL
DNA	5 to 20 µg
Nuclease-free water	Variable
Total	500 µL

2. Shear the DNA using the Covaris™ S2 System shearing program described in Table 12 on page 41.
3. Transfer 500 µL of sheared DNA into a clean 1.5-mL LoBind tube.
4. Wash the borosilicate tube with 100 µL of nuclease-free water and transfer the wash to the 1.5-mL LoBind tube. Mix by vortexing and then proceed to "Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit" on page 43.

Shear the DNA using the HydroShear®

1. Divide the DNA equally into two aliquots in 1.5-mL LoBind tubes, then adjust the volume of each aliquot to 125 µL using nuclease-free water. If you are starting with an input < 15 µg, shear all of the DNA in one 125-µL aliquot.
2. On the Edit Wash Scheme tab, specify the solution and cycles as follows:
   - 2 cycles WS1 (0.2 N HCl)
   - 2 cycles WS2 (0.2 N NaOH)
   - 3 cycles nuclease-free water
3. Run the wash scheme on the HydroShear.
4. Adjust the speed code and number of cycles according to Table 12 on page 41 and adjust the volume setting to **150** µL.
5. Begin shearing. Repeat the shearing for the other aliquot of DNA. It is not necessary to run the wash cycle if both tubes contain the same DNA.
6. Run the wash scheme after DNA shearing of both aliquots is complete.
7. If needed, pool the aliquots of sheared DNA.

Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit

1. Add 3 volumes of Buffer QG and 1 volume of isopropyl alcohol to the sheared DNA. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.
2. Apply 750 µL of sheared DNA in Buffer QG to the column(s). The maximum amount of DNA that can be applied to a QIAquick® column is 10 µg. Use more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute, then discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).
6. Add 750 µL of Buffer PE to wash the column(s).
7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. Repeat steps 9 and 10.
12. If necessary, pool the eluted DNA.
13. Quantitate the purified DNA by using 2 µL of the sample on the NanoDrop® ND-1000 Spectrophotometer (see Appendix C, "Supplemental Procedures" on page 187).

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "End-repair the sheared DNA" on page 44.

End-repair the sheared DNA

Repair the DNA ends with the Epicentre® End-It™ DNA End-Repair Kit

1. Combine and mix the following components in a 1.5-mL LoBind tube, where *X* is the number of micrograms of sheared DNA (see Table 14):

Table 14 Combine components for end-repair of DNA

Component	Amount
Sheared DNA	*X* µg
10X End-repair Buffer‡	*X* µL
ATP, 10 mM‡	*X* µL
dNTPs, 2.5 mM each‡	*X* µL
End-Repair Enzyme Mix‡	(*X* ÷ 3) µL
Nuclease-free water	Variable
Total	(*X* × 10) µL

‡ From Epicentre® End-It™ Kit.

Example

For 15 µg sheared DNA:

Component	Amount
Sheared DNA	15 µg
10X End-Repair Buffer‡	15 µL
ATP, 10 mM‡	15 µL
dNTPs, 2.5 mM each‡	15 µL
End-Repair Enzyme Mix‡	5 µL
Nuclease-free water	Variable
Total	150 µL

‡ From Epicentre® End-It™ Kit.

2. Incubate the mixture at room temperature for 30 minutes.

Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit

1. Add 3 volumes of Buffer QG and 1 volume of isopropyl alcohol to the end-repaired DNA. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.

2. Apply 750 µL of end-repaired DNA in Buffer QG to the column(s). The maximum amount of DNA that can be applied to a QIAquick® column is 10 µg. Use more columns if necessary.

3. Let the column(s) stand for 2 minutes at room temperature.

4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute, then discard the flow-through.

5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the QIAquick® column(s) back into the same collection tube(s).

6. Add 750 µL of Buffer PE to wash the column(s).

7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.

8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).

9. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.

10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.

11. Repeat steps 9 and 10.

12. If necessary, pool the eluted DNA.

13. Quantitate the purified DNA by using 2 µL of the sample on the NanoDrop® ND-1000 Spectrophotometer (see Appendix C, "Supplemental Procedures" on page 187).

14. For structural variation studies where tighter size selection of fragments is required, perform one of two size selections (see "Size-select the DNA" on page 48), then see "Ligate LMP CAP Adaptors to the DNA" on page 46. If tight insert size distribution is not critical, see "Ligate LMP CAP Adaptors to the DNA" on page 46. If the starting DNA is less than 10 µg, do not perform the optional size-selection.

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "Ligate LMP CAP Adaptors to the DNA" on page 46.

Ligate LMP CAP Adaptors to the DNA

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. 3 M Sodium acetate.

Ligate the adaptors to the DNA

1. Calculate the amount of adaptor needed, *Y*, for the reaction based on the amount of DNA from the last purification step (see "Ligation of LMP CAP Adaptors" on page 212 for calculation details).

   Example:

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{\text{Average insert size}}$$

$$Y \text{ µL adaptor needed} = \#\text{ µg DNA} \times \frac{X \text{ pmol}}{1 \text{ µg DNA}} \times 100 \times \frac{1 \text{ µL adaptor needed}}{50 \text{ pmol}}$$

Example:

For 12 µg of purified end-repaired DNA with an average insert size of 1.5 kb

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{1500} = 1.0 \text{ pmol/µg DNA}$$

$$Y \text{ µL adaptor needed} = 12 \text{ µg DNA} \times \frac{1.0 \text{ pmol}}{1 \text{ µg DNA}} \times 100 \times \frac{1 \text{ µL adaptor needed}}{50 \text{ pmol}}$$

$$= 24 \text{ µL adaptor needed}$$

2. Combine and mix the components below (see Table 15). If a larger reaction volume is required to incorporate all of the DNA, scale up the Quick Ligase and Quick Ligase Buffer. Add 1 µL of Quick Ligase per 40 µL of reaction volume. Add 1 µL of 2× Quick Ligase Buffer per 2 µL of reaction volume.

   Table 15 Ligation mix

Component	Volume (µL)
LMP CAP Adaptor (ds), 50 µM	*Y*
2× Quick Ligase Buffer‡	150
Quick Ligase Enzyme‡	7.5
DNA	Variable
Nuclease-free water	Variable
Total	300

‡ From New England Biolabs (NEB).

3. Incubate the reaction mixture at room temperature for 10 minutes.

Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit

1. Add 3 volumes of Buffer QG and 1 volume of isopropyl alcohol to the ligated DNA. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.
2. Apply 750 µL of ligated DNA in Buffer QG to the column(s). The maximum amount of DNA that can be applied to a QIAquick column is 10 µg. Use more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).
6. Add 750 µL of Buffer PE to wash the column(s).
7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. If necessary, pool the eluted DNA.

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "Size-select the DNA" on page 48.

Size-select the DNA

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. 10× TAE, 3 M Sodium acetate, gel loading solution, ethidium bromide.

Size-select the DNA fragments with an agarose gel

1. Determine the appropriate percentage of agarose gel needed to size-select DNA (see Table 16).

Table 16 Percent agarose gel needed to size-select DNA

Desired insert size	Agarose gel needed (%)
600 to 3000 bp	1.0
3 to 6 kb	0.8

2. Prepare the appropriate percentage agarose gel in 1× TAE buffer with 10 µL of 10 mg/mL ethidium bromide per 100 to 150 mL gel volume. To prepare the 1% gels, use either Agarose-LE (Applied Biosystems, AM9040) or 1% Mini ReadyAgarose Gel (Bio-Rad, 161-3016).

3. Add 10× Gel Loading Solution to the purified ligated DNA (1 µL of 10× Gel Loading Solution for every 10 µL mate-paired library).

4. Load 1 µL of 1 kb DNA ladder. Load 11 µL of dye-mixed sample per well. There should be at least one lane in between the ladder well and the sample wells to avoid contamination of the sample with ladder.

5. Run the gel at 120 V until the marker is close to the edge of the gel.

6. Destain the gel in nuclease-free water twice for 2 minutes each time, then visualize the gel on a UV transilluminator with a ruler lying on top.

7. Using the ladder bands and the ruler for reference, excise the band of the gel corresponding to the insert size range of interest with a clean razor blade (see Figure 10 on page 49). If desired, a tighter size selection can be carried out at this stage by taking a tighter cut. If the gel piece is large, slice into smaller pieces.

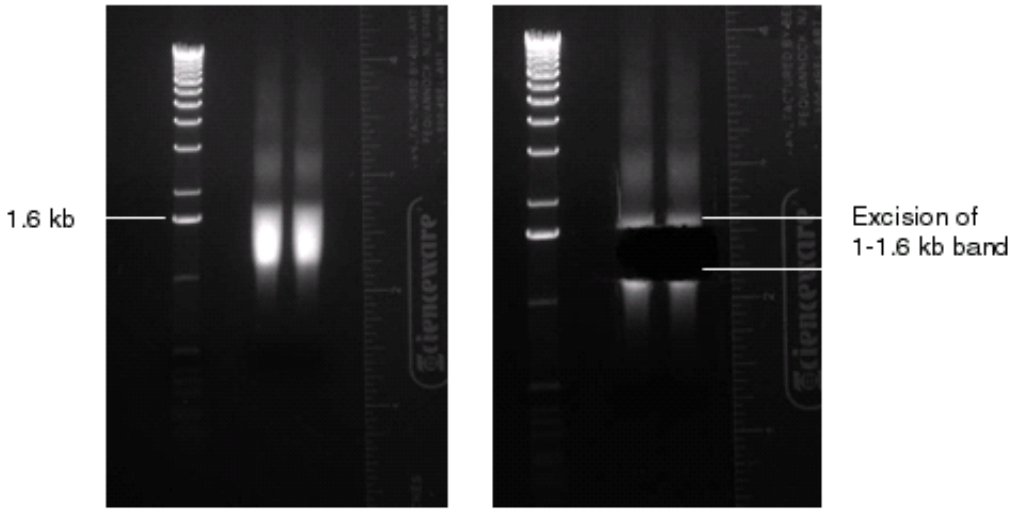


Figure 10 Excision of 1-1.6 kb range in a 1.0% agarose gel.

Elute the DNA using the Qiagen QIAquick® Gel Extraction Kit

1. Weigh the gel slice(s) in a 15-mL polypropylene conical colorless tube.
2. Add 3 volumes of Buffer QG to 1 volume of gel.
3. Dissolve the gel slice by vortexing at *room temperature* until the gel slice has dissolved completely (~5 minutes).
4. If the color of the mixture is yellow, proceed to step 5. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.
5. Add one *gel volume* of isopropyl alcohol to the sample and mix by inverting the tube several times.
6. Apply about 750 µL of sample to the column(s). The maximum amount of gel that can be applied to a QIAquick® column is 400 mg. Use more columns if necessary.
7. Let the column(s) stand for 2 minutes at room temperature.
8. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
9. Repeat steps 6 and 8 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).
10. Add 500 µL of Buffer QG to the column(s).
11. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute, then discard the flow-through.
12. Add 750 µL of Buffer PE to wash the column(s).

13. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.

14. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).

15. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.

16. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.

17. Repeat steps 15 and 16.

18. If necessary, pool the eluted DNA in a 1.5-mL LoBind tube.

19. Quantitate the purified DNA by using 2 µL of the sample on the NanoDrop® ND-1000 Spectrophotometer (see Appendix C, "Supplemental Procedures" on page 187).

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "Circularize the DNA" on page 51.

Circularize the DNA

Circularize the DNA

1. Prepare a circularization reaction by mixing the following components (in order) based on the desired insert size, where *X* is the number of micrograms of DNA to be circularized (see Table 17). If a larger reaction volume is required, scale up the Quick Ligase and Quick Ligase Buffer. Add 1 µL of Quick Ligase per 40 µL of reaction volume. Add 1 µL of 2✕ Quick Ligase Buffer per 2 µL of reaction volume (see "DNA circularization" on page 213 for calculation details).

Table 17 Mix for DNA circularization by insert size

Components	600 to 800 bp	800 to 1000 bp	1 to 2 kb	2 to 3 kb	3 to 4 kb	4 to 5 kb	5 to 6 kb
Nuclease-free water	Variable	Variable	Variable	Variable	Variable	Variable	Variable
DNA	*X* µg	*X* µg	*X* µg	*X* µg	*X* µg	*X* µg	*X* µg
2✕ Quick Ligase Buffer	(*X* × 117.5) µL	(*X* × 135) µL	(*X* × 182.5) µL	(*X* × 250) µL	(*X* × 280) µL	(*X* × 312.5) µL	(*X* × 360) µL
Internal Adaptor (ds), 2 µM	(*X* × 3.75) µL	(*X* × 2.84) µL	(*X* × 1.5) µL	(*X* × 0.9) µL	(*X* × 0.65) µL	(*X* × 0.5) µL	(*X* × 0.4) µL
Quick Ligase	(*X* × 6) µL	(*X* × 6.75) µL	(*X* × 9) µL	(*X* × 12.5) µL	(*X* × 14) µL	(*X* × 15.6) µL	(*X* × 18) µL
Total	(*X* × 235) µL	(*X* × 270) µL	(*X* × 365) µL	(*X* × 500) µL	(*X* × 560) µL	(*X* × 625) µL	(*X* × 720) µL

Example

For 10 µg of DNA in 1 to 2 kb size range to be circularized:

Components	Amount
Nuclease-free water	Variable
DNA	10 µg
Quick Ligase Buffer, 2✕	1825 µL
Internal Adaptor (ds), 2 µM	15 µL
Quick Ligase	90 µL
Total	3650 µL

2. Incubate the reaction at room temperature for 10 minutes.

Purify the DNA with Qiagen QIAquick® Gel Extraction Kit

1. Add 3 volumes of Buffer QG and 1 volume of isopropyl alcohol to the circularized DNA. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.

2. Apply 750 µL of circularized DNA in Buffer QG to the column(s). The maximum amount of DNA that can be applied to a QIAquick column is 10 µg. Use more columns if necessary.

3. Let the column(s) stand for 2 minutes at room temperature.

4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).
6. Add 750 µL of Buffer PE to wash the column(s).
7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. Repeat steps 9 and 10.
12. If necessary, pool the eluted DNA.

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "Isolate the circularized DNA" on page 53.

Isolate the circularized DNA

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. 3 M Sodium acetate.

Treat the DNA with Plasmid-Safe™ ATP-Dependent DNase

1. Combine and mix the following components, where *X* is the volume in µL of DNA and *Y* is the number of micrograms of DNA used in the circularization reaction (see Table 18).

Table 18 Mix for DNase treatment of DNA

Component	Volume (µL)
ATP, 25 mM	5
10× Plasmid-Safe™ Buffer	10
Plasmid-Safe™ DNase, 10 U/µL	(*Y* ÷ 3)
DNA	*X*
Nuclease-free water	Variable
Total	100

If *X* exceeds 85 µL, adjust the total reaction volume, accordingly. The volume of ATP and Plasmid-Safe™ Buffer, 10× should be proportional to the total reaction volume.

Example

For 10 µg DNA used in the circularization reaction:

Component	Volume (µL)
ATP, 25 mM	5
10× Plasmid-Safe™ Buffer	10
Plasmid-Safe™ DNase, 10 U/µL	3.3
DNA	60
Nuclease-free water	21.7
Total	100

2. Incubate the reaction mixture at 37 °C for 40 minutes.

Purify the DNA with Qiagen QIAquick® Gel Extraction Kit

1. Add 3 volumes of Buffer QG and 1 volume of isopropyl alcohol to the Plasmid-Safe™ DNase-treated DNA. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.

2. Apply 750 µL of Plasmid-Safe™ DNase-treated DNA in Buffer QG to the column(s). The maximum amount of DNA that can be applied to a QIAquick column is 10 µg. Use more columns if necessary.

3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).
6. Add 750 µL of Buffer PE to wash the column(s).
7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. Repeat steps 9 and 10.
12. If needed, pool the eluted DNA.
13. Quantitate the purified DNA by using 2 µL of the sample on the NanoDrop® ND-1000 Spectrophotometer (see Appendix C, "Supplemental Procedures" on page 187).

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "Nick-translate the circularized DNA" on page 55.

Nick-translate the circularized DNA

Nick-translate the circularized DNA

1. Combine and mix the following components on ice, where *X* is the number of nanograms of circularized DNA. Mix all components except the enzyme and chill on ice, then add the enzyme and quickly vortex (see Table 19).

   IMPORTANT! Before adding enzyme, chill the reaction on ice for 5 minutes. DNA polymerase I is very sensitive to slight changes in temperature.

   Table 19 Mix for nick translation of DNA

Component	Amount
dNTP Mix, 100 mM, 25 mM each	(*X* ÷ 200) µL
10× NEBuffer 2	(*X* ÷ 20) µL
DNA	*X* ng
Nuclease-free water	Variable
DNA Polymerase I, 10 U/µL	(*X* ÷ 100) µL
Total	(*X* ÷ 2) µL

   Example

   For 1 µg of circularized DNA:

Component	Amount
dNTP Mix, 100 mM, 25 mM each	5 µL
10× NEBuffer 2	50 µL
DNA	1000 ng
Nuclease-free water	Variable
DNA Polymerase I, 10 U/µL	10 µL
Total	500 µL

2. Incubate the reaction at 0 °C in an ice-water bath for 12 to 14 minutes.

3. Stop the reaction immediately by proceeding to "Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit" on page 55.

Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit

1. Add 3 volumes of Buffer QG and 1 volume of isopropyl alcohol to the nick-translated DNA. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.

2. Apply 750 µL of nick-translated DNA in Buffer QG to the column(s). The maximum amount of DNA that can be applied to a QIAquick column is 10 µg. Use more columns if necessary.

3. Let the column(s) stand for 2 minutes at room temperature.

4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute, then discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).
6. Add 750 µL of Buffer PE to wash the column(s).
7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. Repeat steps 9 and 10.
12. If needed, pool the eluted DNA.

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "Digest the DNA with T7 exonuclease and S1 nuclease" on page 57.

Digest the DNA with T7 exonuclease and S1 nuclease

Digest the DNA with T7 exonuclease

1. Combine (see Table 20):

Table 20 T7 exonuclease reaction mix from *X* ng circularized DNA

Component	Amount
DNA	From *X* ng circularized DNA
10× NEBuffer 4	(X ÷ 20) µL
T7 exonuclease, 10 U/µL	(*X* ÷ 50) U
Nuclease-free water	Variable
Total	(X ÷ 2) µL

Example

For 1 µg of circularized DNA:

Component	Amount
DNA	From 1000 ng circularized DNA
10× NEBuffer 4	50 µL
T7 exonuclease, 10 U/µL	20 µL
Nuclease-free water	Variable
Total	500 µL

2. Incubate the reaction mixture at 37 °C for 30 minutes.

Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit

1. Add 3 volumes of Buffer QG and 1 volume of isopropyl alcohol to the digested DNA. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.
2. Apply 750 µL of T7 exonuclease-digested DNA in Buffer QG to the column(s). The maximum amount of DNA that can be applied to a QIAquick column is 10 µg. Use more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).
6. Add 750 µL of Buffer PE to wash the column(s).
7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.

8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. Repeat steps 9 and 10.
12. If necessary, pool the eluted DNA.

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "Digest the circularized DNA with S1 nuclease".

Digest the circularized DNA with S1 nuclease

1. Freshly dilute Invitrogen™ S1 Nuclease to 1 U/µL with S1 Dilution Buffer.
2. Combine (see Table 21):

Table 21 S1 nuclease reaction mix

Component	Amount
T7 exonuclease-digested DNA	From *X* ng circularized DNA
10× S1 Nuclease Buffer	(X ÷ 20) µL
Sodium chloride, 3 M	(*X* ÷ 40) µL
Magnesium chloride, 100 mM	(*X* ÷ 20) µL
S1 nuclease, 1 U/µL	(*X* ÷ 50) µL
Nuclease-free water	Variable
Total	(X ÷ 2) µL

Example

For T7 exonuclease-digested DNA from 1000 ng of circularized DNA:

Component	Amount
T7 exonuclease-digested DNA	From 1000 ng circularized DNA
10× S1 Nuclease Buffer	50 µL
Sodium chloride, 3 M	25 µL
Magnesium chloride, 100 mM	50 µL
S1 nuclease, 1 U/µL	20 µL
Nuclease-free water	Variable
Total	500 µL

3. Incubate the reaction mixture at 37 °C for 30 minutes. *Immediately* proceed to the next step, "Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit" on page 59.

Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit

1. Add 3 volumes of Buffer QG and 1 volume of isopropyl alcohol to the digested DNA. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.
2. Apply 750 µL of digested DNA in Buffer QG to the column(s). The maximum amount of DNA that can be applied to a QIAquick column is 10 µg. Use more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).
6. Add 750 µL of Buffer PE to wash the column(s).
7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. Repeat steps 9 and 10.
12. If necessary, pool the eluted DNA.

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "End-repair the digested DNA" on page 60.

End-repair the digested DNA

Repair the digested DNA ends with the Epicentre® End-It™ DNA End-Repair Kit

1. Combine (see Table 22):

Table 22 Streptavidin Binding Buffer

Component	Volume (µL)
Tris-HCl, pH 7.5, 500 mM	10
Sodium chloride, 5 M	200
EDTA, 500 mM	1
Nuclease-free water	289
Total	500

2. Combine (see Table 23):

Table 23 Reaction mix

Component	Amount
S1-digested DNA	*X* ng
10× End-repair buffer‡	10 µL
ATP, 10 mM‡	10 µL
dNTPs, 2.5 mM each‡	10 µL
End-Repair Enzyme Mix‡	2 µL
Nuclease-free water	Variable
Total	100 µL

‡ From the Epicentre® End-It™ DNA End-Repair Kit.

3. Incubate the reaction mix at room temperature for 30 minutes.

4. Stop the reaction by combining and mixing (see Table 24):

Table 24 Stop end-repair mix

Component	Volume (µL)
End-repaired DNA	100
500 mM EDTA	5
Streptavidin Binding Buffer	200
Nuclease-free water	95
Total	400

Bind the library molecules to streptavidin beads

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. 1× Bead Wash Buffer.

CAUTION! CHEMICAL HAZARD. 1× Bind & Wash Buffer, 1× Bead Wash Buffer, 1× Low Salt Binding Buffer.

Pre-wash the beads

1. Combine (see Table 25):

Table 25 1× BSA mix

Component	Volume (µL)
100× BSA	5
Nuclease-free water	495
Total	500

2. Vortex the bottle of Dynal® MyOne C1 streptavidin beads and transfer 90 µL into a 1.5-mL LoBind Tube.
3. Add 500 µL of 1× Bead Wash Buffer and vortex for 15 seconds, then pulse-spin.
4. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
5. Add 500 µL of 1× BSA and vortex for 15 seconds, then pulse-spin.
6. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
7. Add 500 µL of 1× Bind & Wash Buffer and vortex for 15 seconds, then pulse-spin.
8. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.

Bind the library DNA molecules to the beads

1. Add the entire 400 µL solution of library DNA in Streptavidin Binding Buffer to the pre-washed beads and vortex.
2. Mix by rotation at room temperature for 30 minutes, then pulse-spin.

Wash the bead-DNA complex

1. Combine (see Table 26):

Table 26 1× Quick Ligase Buffer

Component	Volume (µL)
2× Quick Ligase Buffer	300
Nuclease-free water	300
Total	600

2. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
3. Resuspend the beads in 500 µL of 1× Bead Wash Buffer and transfer beads to a new 1.5-mL LoBind tube. Vortex for 15 seconds, then pulse-spin.
4. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
5. Resuspend the beads in 500 µL of 1× Bind & Wash Buffer. Vortex for 15 seconds, then pulse-spin.
6. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
7. Resuspend the beads in 500 µL of 1× Bind & Wash Buffer and transfer beads to a new 1.5-mL LoBind tube. Vortex for 15 seconds, then pulse-spin.
8. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
9. Resuspend the beads in 500 µL of 1× Quick Ligase Buffer. Vortex for 15 seconds, then pulse-spin.
10. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
11. Resuspend the beads in 97.5 µL of 1× Quick Ligase Buffer.

Ligate P1 and P2 Adaptors to the DNA

Ligate the P1 and P2 Adaptors to the end-repaired DNA

1. Calculate the amount of P1 and P2 Adaptors needed for the ligation reaction based on the amount of circularized DNA from "Isolate the circularized DNA" on page 53 and the calculation below. For calculation details, "Ligation of P1 and P2 Adaptors" on page 214.

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{\text{Circularized DNA size}}$$

$$Y \text{ µL adaptor needed} = \#\text{ µg DNA} \times \frac{X \text{ pmol}}{1 \text{ µg DNA}} \times 30 \times \frac{1 \text{ µL adaptor needed}}{50 \text{ pmol}}$$

Example:
For 1 µg of purified circularized DNA with an average size of 1536 (1500 bp insert + 36 bp internal adaptor)

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{1536} = 1 \text{ pmol/µg DNA}$$

$$Y \text{ µL adaptor needed} = 1 \text{ µg DNA} \times \frac{1 \text{ pmol}}{1 \text{ µg DNA}} \times 30 \times \frac{1 \text{ µL adaptor needed}}{50 \text{ pmol}}$$

$$= 0.6 \text{ µL adaptor needed}$$

2. Combine (see Table 27):

Table 27 Mix for ligation of end-repaired DNA to P1 and P2 Adaptors

Component	Volume (µL)
DNA-bead complex	97.5
P1 Adaptor (ds), 50 µM	*Y*
P1 Adaptor (ds), 50 µM	*Y*
Quick Ligase	2.5
Total	Variable

3. Incubate the reaction mixture at room temperature for 15 minutes.

Wash the DNA-bound streptavidin beads

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. 1× Bead Wash Buffer.

CAUTION! CHEMICAL HAZARD. 1× Bind & Wash Buffer, 1× Low Salt Binding Buffer.

Wash the bead-DNA complex

1. Combine (see Table 28):

Table 28 Prepare 1× NEBuffer 2

Components	Volume (µL)
10× NEBuffer 2	60
Nuclease-free water	540
Total	600

2. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
3. Resuspend the beads in 500 µL of 1× Bead Wash Buffer and transfer beads to a new 1.5-mL LoBind tube. Vortex for 15 seconds, then pulse-spin.
4. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
5. Resuspend the beads in 500 µL of 1× Bind & Wash Buffer. Vortex for 15 seconds, then pulse-spin.
6. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
7. Resuspend the beads in 500 µL of 1× Bind & Wash Buffer and transfer beads to a new 1.5-mL LoBind tube. Vortex for 15 seconds, then pulse-spin.
8. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
9. Resuspend the beads in 500 µL of 1× NEBuffer 2. Vortex for 15 seconds, then pulse-spin.
10. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
11. Resuspend the beads in 96 µL of 1× NEBuffer 2.

Nick-translate the DNA

Nick-translate the DNA

1. Combine (see Table 29):

Table 29 Mix for nick translation

Component	Volume (µL)
DNA-bead complex	96
100 mM dNTP mix, 25 mM each	2
DNA Polymerase I, 10 U/µL	2
Total	100

2. Incubate the reaction mixture at 16 °C for 30 minutes.
3. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
4. Resuspend the beads in 500 µL of Buffer EB (Qiagen).
5. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
6. Resuspend the beads in 30 µL of Buffer EB.
7. (Optional) Save 3 µL of nick-translated library DNA for troubleshooting.

STOPPING POINT. Store the DNA-Bead complexes in Buffer EB at 4 °C, or go to "Amplify the library" on page 66.

Amplify the library

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. 3 M Sodium acetate.

Perform PCR on the library

1. Prepare a master mix for four 100-µL PCR reactions (see Table 30):

Table 30 PCR master mix for amplification of the library

Component	Volume (µL)
SOLiD™ Library PCR Master Mix	200
Library PCR Primer 1, 50 µM	8
Library PCR Primer 2, 50 µM	8
Cloned Pfu, 2.5 U/µL	1
Nuclease-free water	143
Total	360

2. For the negative control, aliquot 90 µL of PCR master mix to a PCR tube. Add 10 µL of nuclease-free water to the tube.

3. Add 27 or 30 µL of DNA-bead complex solution to the remaining 270 µL of PCR master mix. Vortex to mix, then divide evenly among three PCR tubes.

4. Run (see Table 31):

Table 31 PCR conditions to amplify the library

Stage	Step	Temp	Time
Holding	Denature	95 °C	10 min
Cycling (5 cycles)	Denature	95 °C	15 sec
	Anneal	62 °C	15 sec
	Extend	60 °C	4 min
Holding	—	4 °C	∞

Confirm library amplification with a Lonza FlashGel®

1. Add 1 µL of 5× FlashGel® Loading Dye to 4 µL of sample from the 100 µL of PCR reaction, then load the mixture on a 2.2% Lonza FlashGel®. Load FlashGel DNA Marker (50 bp to 1.5 kb or 100 bp to 4 kb) in an adjacent well for reference.

2. Run the FlashGel for 6 minutes at 275 V (see Figure 11 on page 67).

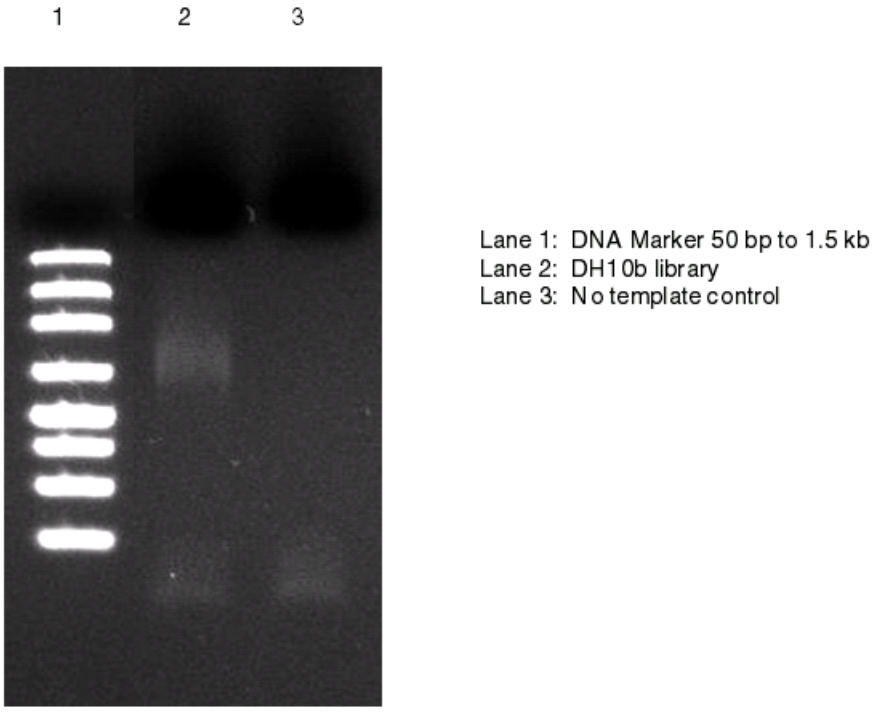


Figure 11 Mate-paired library amplification sample run on a Lonza FlashGel.

3. If fairly robust amplification products are visible, proceed to step 7; otherwise, return the tubes to the thermal cycler and run the PCR cycling program (see Table 32.

Table 32 PCR conditions to amplify the trial library

Stage	Step	Temp	Time
Cycling (2 cycles)	Denature	95 °C	15 sec
	Anneal	62 °C	15 sec
	Extend	60 °C	4 min
Holding	—	4 °C	∞

4. Add 1 µL of 5✕ FlashGel® Loading Dye to 4 µL of sample from the 100 µL of PCR reaction, then load the mixture on a 2.2% Lonza FlashGel®. Load FlashGel DNA Marker (50 bp to 1.5 kb or 100 bp to 4 kb) in an adjacent well for reference.

5. Run the FlashGel for 6 minutes at 275 V to ensure amplification after a minimal number of cycles.

6. If amplification is still not observed, repeat steps 3 to 5 until amplification is observed. Be careful not to overamplify the sample.

7. Pool all of the PCR samples into a 2.0-mL LoBind tube.

8. Place the tube of beads in a magnetic rack and transfer the supernatant to a fresh 2.0-mL LoBind tube. Discard the tube containing the beads.

Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit

1. Add 3 volumes of Buffer QG and 1 volume of isopropyl alcohol to each PCR product aliquot. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.
2. Apply 750 µL of PCR product in Buffer QG to the column(s). The maximum amount of DNA that can be applied to a QIAquick column is 10 µg. Use more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).
6. Add 750 µL of Buffer PE to wash the column(s).
7. Centrifuge the columns at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the columns for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 30 µL of Buffer EB to the column(s) to elute the DNA, then let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. Pool the eluted DNA.

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "Gel-purify the library" on page 69.

Gel-purify the library

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. 10× TAE, 10× TBE, 3 M Sodium acetate, gel loading solution, ethidium bromide.

Size-select the DNA fragments with an agarose gel

1. Prepare a 4% agarose gel in 1× TAE or 1× TBE. To prepare the 4% gel, use either Agarose-LE (Applied Biosystems, AM9040) or Reliant® Precast 4% NuSieve® 3:1 Plus Agarose Gel (Lonza, 54927). Do not use a FlashGel® or 4% E-Gel for size-selection.

   Note: Size-selection may also be performed with 6% PAGE gels (Invitrogen, EC6365BOX). To elute the DNA from the PAGE gel, see "PAGE gel DNA elution" on page 198.

2. Add 10× Gel Loading Solution to the mate-paired library: 1 µL of 10× Gel Loading Solution for every 10 µL of mate-paired library.

3. Load 2 µL of TrackIt™ 25-bp ladder. Load 11 µL of dye-mixed sample per well. There should be at least one lane in between the ladder well and the sample wells to avoid contamination of the sample with ladder.

4. Run the gel at 120 V until the marker is close to the edge of the gel.

5. If needed, stain the gel in 50 to 100 mL of 1× TAE or 1× TBE Buffer with 8 µL of ethidium bromide (10 mg/mL) for 5 minutes.

6. Destain the gel in nuclease-free water twice for 2 minutes each time and visualize the gel on a UV transilluminator.

7. Excise the entire band that has an average size ranging from 275 to 300 bp using a clean razor blade (see Figure 12 on page 70).

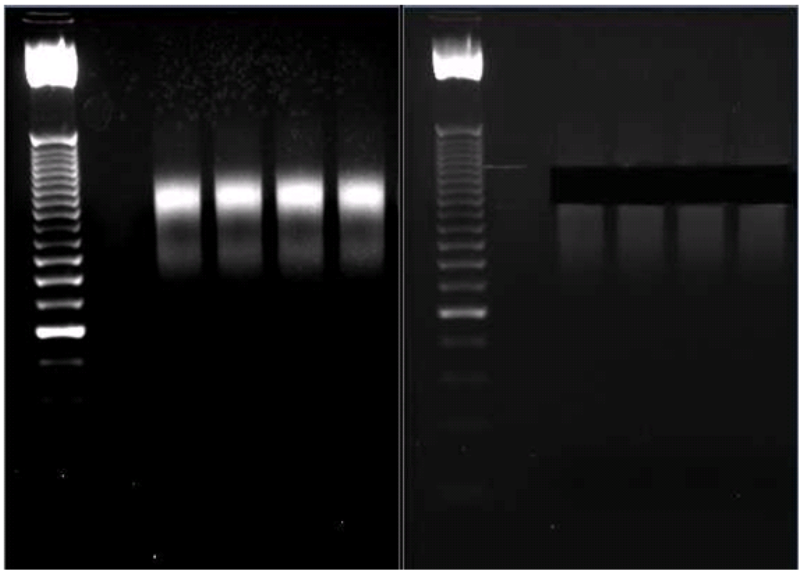


Figure 12 Excision of 275-300 bp library.

Elute the DNA with the Qiagen QIAquick® Gel Extraction Kit

1. Weigh the gel slice(s) in a 15-mL polypropylene conical colorless tube.
2. Add 6 volumes of Buffer QG to 1 volume of gel.
3. Dissolve the gel slice by vortexing it at *room temperature* until the gel slice has dissolved completely (~5 minutes).

   IMPORTANT! Do not dissolve the gel slice by heating. Although Qiagen recommends dissolving the gel slice at 50 °C for 10 minutes or until the gel slice has completely dissolved, these conditions affect the fragment library deleteriously, resulting in denaturation of the fragments and lead to formation of heteroduplexes.

4. If the color of the mixture is yellow, proceed to step 5. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.
5. Add one *gel volume* of isopropyl alcohol to the sample and mix by inverting the tube several times.
6. Apply about 750 µL of sample to the column(s). The maximum amount of gel that can be applied to a QIAquick® column is 400 mg. Use more columns if necessary.
7. Let the column(s) stand for 2 minutes at room temperature.
8. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.

9. Repeat steps 6 and 8 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).

10. Add 500 µL of Buffer QG to the column(s).

11. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute, then discard the flow-through.

12. Add 750 µL of Buffer PE to wash the column(s).

13. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.

14. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).

15. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.

16. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.

17. If necessary, pool the eluted DNA in a 1.5-mL LoBind tube.

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "Quantitate the library by performing quantitative PCR (qPCR)" on page 72.

Quantitate the library by performing quantitative PCR (qPCR)

For accurate library quantitation, quantitative PCR is strongly recommended. For a TaqMan® or SYBR® qPCR protocol, see Appendix B, "SOLiD™ 3 System Library Quantitation" on page 155.

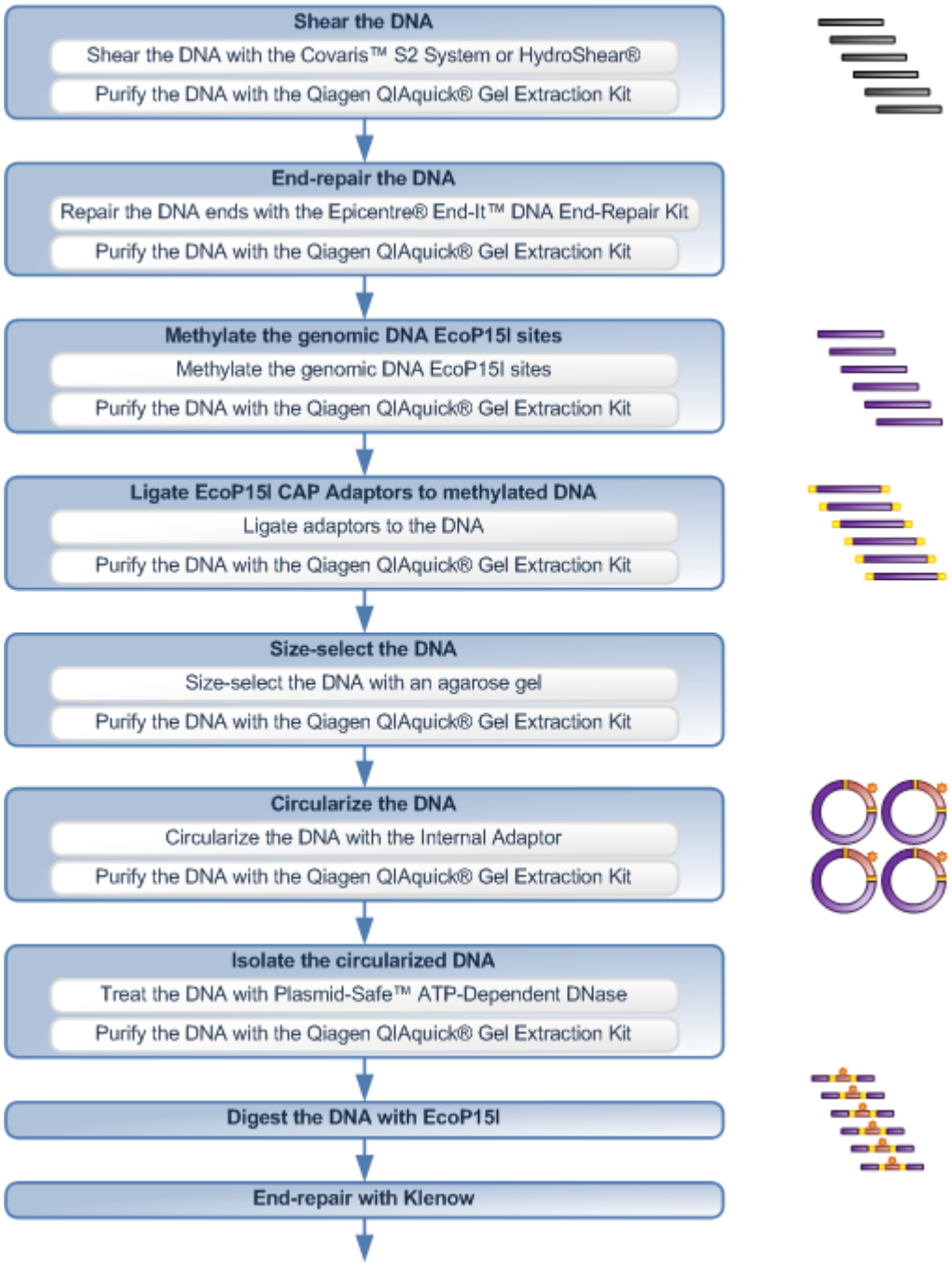
STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to emulsion PCR in the *Applied Biosystems SOLiD™ 3 System Templated Bead Preparation Guide* (PN 4407421).

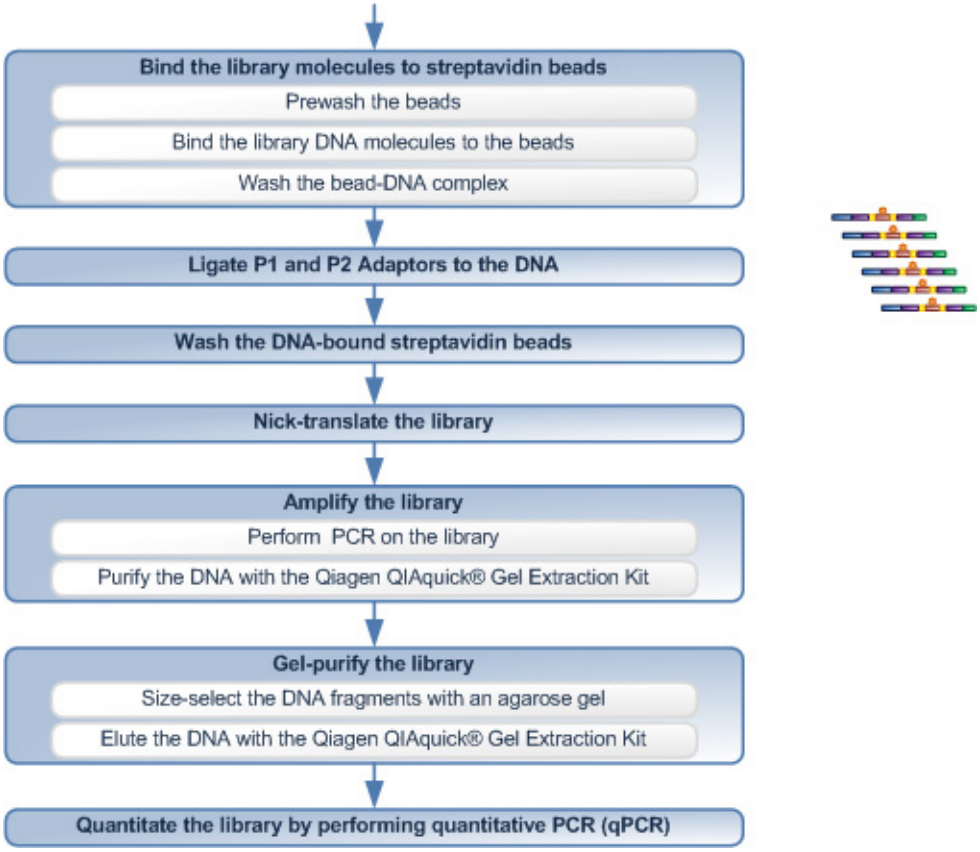
Section 3.2 Prepare a 2 × 25 bp mate-paired library

Materials and equipment required

See Appendix A on page 127 for a list of equipment, kits, and consumables necessary for this procedure.

Workflow


Shear the DNA
Shear the DNA with the Covaris™ S2 System or HydroShear®
Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit
End-repair the DNA
Repair the DNA ends with the Epicentre® End-It™ DNA End-Repair Kit
Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit
Methylate the genomic DNA EcoP15I sites
Methylate the genomic DNA EcoP15I sites
Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit
Ligate EcoP15I CAP Adaptors to methylated DNA
Ligate adaptors to the DNA
Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit
Size-select the DNA
Size-select the DNA with an agarose gel
Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit
Circularize the DNA
Circularize the DNA with the Internal Adaptor
Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit
Isolate the circularized DNA
Treat the DNA with Plasmid-Safe™ ATP-Dependent DNase
Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit
Digest the DNA with EcoP15I
End-repair with Klenow



Shear the DNA

The genomic DNA is sheared to yield 600 bp to 6 kb fragments. To shear for a mate-paired library with insert sizes between 600 bp and 1 kb, the Covaris™ S2 system is recommended. To shear for a mate-paired library with insert sizes between 1 kb and 6 kb, the HydroShear is recommended. HydroShear uses hydrodynamic shearing forces to fragment DNA strands. The DNA in solution flows through a tube with an abrupt contraction. As it approaches the contraction, the fluid accelerates to maintain the volumetric flow rate through the smaller area of the contraction. During this acceleration, drag forces stretch the DNA until it snaps and until the pieces are too short for the shearing forces to break the chemical bonds. The flow rate of the fluid and the size of the contraction determine the final DNA fragment sizes. While basic guidelines are given for shearing DNA using a HydroShear, every HydroShear® instrument may need an initial standard run, and speed codes may need adjusting for DNA from different organisms. A calibration run to assess the shearing efficacy of your device prior to starting your first library preparation is highly recommended.

Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit

Sample purification is recommended with Qiagen QIAquick® columns supplied in the QIAquick® Gel Extraction Kit. Qiagen QIAquick columns have a 10-µg capacity, so it may be necessary to use multiple columns during a purification step. For more detailed information on purification of DNA with Qiagen QIAquick columns, see the manufacturer's instructions. If you have larger amounts of DNA for library construction, you can substitute this step with phenol-chloroform-isoamyl alcohol extraction and isopropyl alcohol precipitation (see Appendix C, “Supplemental Procedures” on page 187).

End-repair the DNA

The Epicentre® End-It™ DNA End-Repair Kit is used to convert DNA with damaged or incompatible 5′-protruding and/or 3′-protruding ends to 5′-phosphorylated, blunt-ended DNA for fast and efficient blunt-end ligation. The conversion to blunt-end DNA is accomplished by exploiting the 5′-to-3′ polymerase and the 3′-to-5′ exonuclease activities of T4 DNA Polymerase. T4 polynucleotide kinase and ATP are also included for phosphorylation of the 5′-ends of the blunt-ended DNA for subsequent ligation.

Methylate the genomic DNA EcoP15I sites

Methylation of the EcoP15I sites in the genomic DNA prevents digestion at the EcoP15I sites. EcoP15I is a type III restriction enzyme that recognizes the nucleotide sequence CAGCAG. For effective cleavage of a DNA molecule, EcoP15I needs two unmethylated, inversely-oriented EcoP15I recognition sites and cleaves the DNA 25/27 bp away from its binding site. The restriction activity requires ATP and in its absence, EcoP15I only methylates the fifth-base adenine in its binding site CAGC**A**G. This methylation is further boosted in the presence of exogenous S-adenosylmethionine, a methyl group donor. After methylation of genomic DNA EcoP15I sites, completion of methylation can be confirmed with a test digestion in the presence of ATP and its analysis on an agarose gel.

Ligate EcoP15I CAP Adaptors to the methylated DNA

EcoP15I CAP ligation adds the EcoP15I CAP Adaptors to the sheared, methylated DNA. The adaptors contain the EcoP15I restriction site that ultimately is utilized to make 25 to 27 bp genomic DNA mate-paired tags. The EcoP15I CAP Adaptors are included in double-stranded form in the SOLiD™ Mate-Paired Library Oligos Plus Kit or SOLiD™ Mate-Paired Library Oligos Kit.

Size-select the DNA

Depending on the desired insert-size range, the ligated, purified DNA is run on a 0.8% or 1% agarose gel. The correctly sized ligation products are excised and purified using the Qiagen QIAquick Gel Extraction Kit.

Size-selection after CAP adaptor ligation is needed to remove unbound CAP adaptors and should not be skipped under any circumstances. Contamination of unbound CAP adaptors can compromise the circularization reaction in the next step.

Circularize the DNA

Sheared, methylated DNA ligated to EcoP15I CAP Adaptors is circularized with a biotinylated internal adaptor. To increase the chances that ligation will occur between two ends of one DNA molecule versus two different DNA molecules, a very dilute reaction is used. The circularization reaction products are purified using the QIAquick Gel Extraction Kit. The Internal Adaptor is included in double-stranded form in the SOLiD™ Mate-Paired Library Oligos Plus Kit or SOLiD™ Mate-Paired Library Oligos Kit.

Treat the DNA with Plasmid-Safe™ ATP-Dependent DNase

Epicentre® Plasmid-Safe™ ATP-Dependent DNase is used to eliminate uncircularized DNA. After the Plasmid-Safe DNase-treated DNA is purified using the QIAquick Gel Extraction Kit, the amount of circularized product is quantified. A minimum of 200 ng of circularized product is recommended to proceed with library construction. For more complex genomes, 600 ng to 1 µg circularized DNA is recommended for a high-complexity library.

Digest the DNA with EcoP15I

In the presence of sinefungin, EcoP15I digests the circularized DNA 25 to 27 bp away from the CAGCAG recognition site. The digestion creates two genomic DNA tags 25 to 27 bp long connected with an internal adaptor in-between.

End-repair with Klenow

The Klenow fragment is used to convert 5′-protruding and/or 3′-protruding ends to 5′-phosphorylated, blunt-ended DNA for blunt-end ligation.

Bind the DNA molecules to the streptavidin beads

Streptavidin beads specifically bind to the biotin-labeled Internal Adaptor in the library molecules to purify the library from side products.

Ligate P1 and P2 Adaptors to the DNA

P1 and P2 adaptors are ligated to the ends of the end-repaired DNA. The P1 and P2 Adaptors are included in double-stranded form in the SOLiD™ Mate-Paired Library Oligos Plus Kit or SOLiD™ Mate-Paired Library Oligos Kit.

Wash the DNA-bound streptavidin beads

Library molecules bound to streptavidin beads are washed and purified from ligation side products.

Nick-translate the library

The ligated, purified DNA undergoes nick translation with DNA polymerase I.

Amplify the library

The library is amplified using Library PCR Primers 1 and 2 with the SOLiD™ Library PCR Master Mix. It is important to reduce the number of cycles as much as possible and use the entire nick-translated product for amplification to get maximum representation of the library and avoid PCR-related biases due to differential amplification of library molecules.

Gel-purify the library

The library is run on a 4% agarose gel and the library band (154 to 156 bp) is excised and eluted using the Qiagen QIAquick® Gel Extraction Kit.

Quantitate the library by performing quantitative (qPCR)

Quantitate the library by either the TaqMan® or SYBR® quantitative PCR (qPCR) method described in Appendix B, “SOLiD™ 3 System Library Quantitation” on page 155.

Tips

- Adjust microcentrifuge speeds and times according to the g-forces specified in the protocols. Applied Biosystems recommends the Eppendorf 5417R tabletop microcentrifuge.
- Perform all steps requiring 0.5-mL and 1.5-mL tubes with Eppendorf LoBind tubes.
- Thaw reagents on ice before use.

Shear the DNA

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. 3 M Sodium acetate and glycerol.

Prepare for shearing

1. Choose the appropriate shearing method based on the desired insert size of the mate-paired library (see Table 33 on page 80).

   Note: These conditions are only guidelines, and a shearing trial prior to large-scale shearing is recommended if additional DNA is available.

Table 33 Recommended shearing conditions for mate-paired library insert sizes.

Insert size	Shearing method	Shearing conditions
600 to 800 bp	Covaris™ Shearing in 20% glycerol (13 mm × 65 mm borosilicate tube)	• Number of Cycles: **75** • Bath Temperature: **5** °C • Bath Temperature Limit: **12** °C • Mode: **Frequency sweeping** • Water Quality Testing Function: **Off** • Duty cycle: **2%** • Intensity: **7** • Cycles/burst: **200** • Time: **10 seconds**
800 to 1000 bp	Covaris™ Shearing in 20% glycerol (13 mm × 65 mm borosilicate tube)	• Number of Cycles: **30** • Bath Temperature: **5** °C • Bath Temperature Limit: **12** °C • Mode: **Frequency sweeping** • Water Quality Testing Function: **Off** • Duty cycle: **2%** • Intensity: **5** • Cycles/burst: **200** • Time: **10 seconds**
1 to 2 kb	HydroShear® Standard Shearing Assembly	• SC5 • 20 cycles
2 to 3 kb	HydroShear® Standard Shearing Assembly	• SC9 • 20 cycles
3 to 4 kb	HydroShear® Standard Shearing Assembly	• SC13 • 20 cycles
4 to 5 kb	HydroShear® Standard Shearing Assembly	• SC15 • 5 cycles
5 to 6 kb	HydroShear® Standard Shearing Assembly	• SC16 • 25 cycles

IMPORTANT! If you are using the Covaris™ S2 System, set the chiller temperature to between 2 to 5 °C to ensure that the temperature reading in the water bath displays 5 °C. The circulated water chiller should be supplemented with 20% ethylene glycol.

2. If the DNA source is not limiting, ensure that the shearing conditions result in the desired insert sizes. Shear 5 µg DNA and run 150 ng sheared DNA on a 0.8% E-Gel® according to the manufacturer's specifications.

Shear the DNA with the Covaris™ S2 System

1. In a round bottom 13 mm × 65 mm borosilicate tube, dilute 5 to 20 µg DNA in 500 µL so that the final volume contains 20% glycerol in nuclease-free water (see Table 34).

Table 34 Dilute the DNA for shearing

Component	Amount
99% glycerol	100 µL
DNA	5 to 20 µg
Nuclease-free water	Variable
Total	500 µL

2. Shear the DNA using the Covaris™ S2 System shearing program described Table 33 on page 80.
3. Transfer 500 µL of sheared DNA into a clean 1.5-mL LoBind tube.
4. Wash the borosilicate tube with 100 µL of nuclease-free water and transfer the wash to the 1.5-mL LoBind tube. Mix by vortexing and then proceed to "Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit".

Shear the DNA with the HydroShear®

1. Divide the DNA equally into two aliquots in 1.5-mL LoBind tubes, then adjust the volume of each aliquot to 125 µL using nuclease-free water. If you are starting with an input < 15 µg, shear all of the DNA in one 125-µL aliquot.
2. On the Edit Wash Scheme tab, specify the solution and cycles:
   - 2 cycles WS1 (0.2 N HCl)
   - 2 cycles WS2 (0.2 N NaOH)
   - 3 cycles nuclease-free water
3. Run the wash scheme on the HydroShear.
4. Adjust the speed code and number of cycles according to Table 33 on page 80 and adjust the volume setting to 150 µL.
5. Begin shearing. Repeat the shearing for the other aliquot of DNA. It is not necessary to run the wash cycle if both tubes contain the same DNA.
6. Run the wash scheme after DNA shearing of both aliquots is complete.
7. Pool the aliquots of sheared DNA.

Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit

1. Add 3 volumes of Buffer QG and 1 volume of isopropyl alcohol to the sheared DNA. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.

2. Apply 750 µL of sheared DNA in Buffer QG to the column(s). The maximum amount of DNA that can be applied to a QIAquick column is 10 µg. Use more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).
6. Add 750 µL of Buffer PE to wash the column(s).
7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. Repeat steps 9 and 10.
12. If necessary, pool the eluted DNA.
13. Quantitate the purified DNA by using 2 µL of the sample on the NanoDrop® ND-1000 Spectrophotometer (see Appendix C, "Supplemental Procedures" on page 187).

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "End-repair the sheared DNA" on page 83.

End-repair the sheared DNA

Repair the DNA ends with the Epicentre® End-It™ DNA End-Repair Kit

1. Combine and mix the following components in a LoBind tube, where *X* is the number of micrograms of sheared DNA (see Table 35):

Table 35 Combine components for end-repair of DNA

Component	Amount
Sheared DNA	*X* µg
10× End-Repair Buffer‡	*X* µL
ATP, 10 mM‡	*X* µL
dNTPs, 2.5 mM each‡	*X* µL
End-repair enzyme mix‡	(*X* ÷ 3) µL
Nuclease-free water	Variable
Total	(*X* × 10) µL

‡ From Epicentre® End-It™ Kit.

Example

For 15 µg sheared DNA:

Combine components for end-repair of DNA

Component	Amount
Sheared DNA	15 µg
10× End-Repair Buffer‡	15 µL
ATP, 10 mM‡	15 µL
dNTPs, 2.5 mM each‡	15 µL
End-repair enzyme mix‡	5 µL
Nuclease-free water	Variable
Total	150 µL

‡ From Epicentre® End-It™ Kit.

2. Incubate the mixture at room temperature for 30 minutes.

Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit

1. Add 3 volumes of Buffer QG and 1 volume of isopropyl alcohol to the end-repaired DNA. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.

2. Apply 750 µL of end-repaired DNA in Buffer QG to the column(s). The maximum amount of DNA that can be applied to a QIAquick column is 10 µg. Use more columns if necessary.

3. Let the column(s) stand for 2 minutes at room temperature.

4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.

5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).

6. Add 750 µL of Buffer PE to wash the column(s).

7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.

8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).

9. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.

10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.

11. Repeat steps 9 and 10.

12. If necessary, pool the eluted DNA.

13. Quantitate the purified DNA using 2 µL of the sample on the NanoDrop® ND-1000 Spectrophotometer (see Appendix C, "Supplemental Procedures" on page 187).

14. For structural variation studies where tighter size selection of fragments is required, perform one of two size selections (see "Size-select the DNA" on page 89, then see "Methylate the genomic DNA EcoP15I sites" on page 85. If tight insert size distribution is not as critical, proceed directly to "Methylate the genomic DNA EcoP15I sites" on page 85.

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "Methylate the genomic DNA EcoP15I sites" on page 85.

Methylate the genomic DNA EcoP15I sites

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. 3 M Sodium acetate.

Methylate the genomic DNA EcoP15I sites

1. Combine and mix the following components, where *X* indicates the number of micrograms of end-repaired DNA. Use a final concentration of at least 360 µM S-adenosylmethionine and 10 U of EcoP15I enzyme per 1 µg of end-repaired DNA (see Table 36).

Table 36 Combine components for methylation of DNA

Component	Amount
Sheared, end-repaired DNA	*X* µg
10× NEBuffer 3	*X* µL
100× BSA	(X ÷ 10) µL
EcoP15I, 10 U/µL	*X* µL
S-adenosylmethionine, 32 mM	(X × 3 ÷ 25) µL
Nuclease-free water	Variable
Total	(X × 10) µL

Example

For 12.5 µg of sheared, end-repaired DNA:

Combine components for methylation of DNA

Component	Amount
Sheared, end-repaired DNA	12.5 µg
10× NEBuffer 3	12.5 µL
100× BSA	1.25 µL
EcoP15I, 10 U/µL	12.5 µL
S-adenosylmethionine, 32 mM	1.5 µL
Nuclease-free water	Variable
Total	125 µL

IMPORTANT! S-adenosylmethionine is an extremely labile molecule and is sensitive to repeated freeze-thaw cycles. It should not be used beyond its expiry date.

2. Incubate the methylation reaction mixture at 37 °C for 2 hours or overnight.

Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit

1. Add 3 volumes of Buffer QG and 1 volume of isopropyl alcohol to the methylated DNA. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.
2. Apply 750 µL of methylated DNA in Buffer QG to the column(s). The maximum amount of DNA that can be applied to a QIAquick column is 10 µg. Use more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).
6. Add 750 µL of Buffer PE to wash the column(s).
7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. Repeat steps 9 and 10.
12. If necessary, pool the eluted DNA.
13. Quantitate the purified DNA by using 2 µL of the sample on the NanoDrop® ND-1000 Spectrophotometer (see Appendix C, "Supplemental Procedures" on page 187).
14. (Optional) To confirm DNA methylation, follow the "Confirm complete methylation of DNA fragments" on page 202.

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "Ligate EcoP15I CAP Adaptors to the methylated DNA" on page 87.

Ligate EcoP15I CAP Adaptors to the methylated DNA

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. 3 M Sodium acetate.

Ligate the adaptors to the methylated DNA

1. Calculate the amount of adaptor, *Y*, needed for the reaction based on the amount of DNA from the last purification step (see "Ligation of EcoP15I CAP Adaptors" on page 215 for calculation details).

$$\textbf{\textit{X}}\ \text{pmol/µg DNA} = 1\ \text{µg DNA} \times \frac{10^6\ \text{pg}}{1\ \text{µg}} \times \frac{1\ \text{pmol}}{660\ \text{pg}} \times \frac{1}{\text{Average insert size}}$$

$$\textbf{\textit{Y}}\ \text{µL adaptor needed} = \#\ \text{µg DNA} \times \frac{\textbf{\textit{X}}\ \text{pmol}}{1\ \text{µg DNA}} \times 100 \times \frac{1\ \text{µL adaptor needed}}{50\ \text{pmol}}$$

Example:

For 12 µg of purified end-repaired DNA with an average insert size of 1.5 kb

$$\textbf{\textit{X}}\ \text{pmol/µg DNA} = 1\ \text{µg DNA} \times \frac{10^6\ \text{pg}}{1\ \text{µg}} \times \frac{1\ \text{pmol}}{660\ \text{pg}} \times \frac{1}{1500} = 1.0\ \text{pmol/µg DNA}$$

$$\textbf{\textit{Y}}\ \text{µL adaptor needed} = 12\ \text{µg DNA} \times \frac{1.0\ \text{pmol}}{1\ \text{µg DNA}} \times 100 \times \frac{1\ \text{µL adaptor needed}}{50\ \text{pmol}}$$

$$= 24\ \text{µL adaptor needed}$$

2. Combine and mix the following components (see Table 37). If a larger reaction volume is required to incorporate all of the methylated DNA, scale up the Quick Ligase and Quick Ligase Buffer. Add 1 µL of Quick Ligase per 40 µL of reaction volume. Add 1 µL of 2× Quick Ligase Buffer per 2 µL of reaction volume.

Table 37 Ligation mix

Component	Volume (µL)
EcoP15I CAP Adaptor (ds), 50 pmol/µL	*Y*
2× Quick Ligase Buffer‡	150
Quick Ligase Enzyme‡	7.5
DNA	Variable
Nuclease-free water	Variable
Total	300

‡ From NEB.

3. Incubate at room temperature for 10 minutes.

Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit

1. Add 3 volumes of Buffer QG and 1 volume of isopropyl alcohol to the ligated DNA. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.
2. Apply 750 µL of ligated DNA in Buffer QG to the column(s). The maximum amount of DNA that can be applied to a QIAquick column is 10 µg. Use more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).
6. Add 750 µL of Buffer PE to wash the column(s).
7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. If necessary, pool the eluted DNA.

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "Size-select the DNA" on page 89.

Size-select the DNA

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. 10× TAE, 3 M Sodium acetate, gel loading solution, ethidium bromide.

Size-select the DNA fragments with an agarose gel

1. Determine the appropriate percentage of agarose gel needed to size-select DNA (see Table 38).

   Table 38 Percent agarose gel needed to size-select DNA

Desired insert size	Agarose gel needed (%)
600 to 3000 bp	1.0
3 to 6 kb	0.8

2. Prepare the appropriate percentage agarose gel in 1× TAE buffer with 10 µL of 10 mg/mL ethidium bromide per 100 to 150 mL gel volume. To prepare the 1% gels, use either Agarose-LE (Applied Biosystems, AM9040) or 1% Mini ReadyAgarose Gel (Bio-Rad, 161-3016).

3. Add 10× Gel Loading Solution to the purified ligated DNA (1 µL of 10× Gel Loading Solution for every 10 µL of mate-paired library).

4. Load 1 µL of 1 kb DNA ladder. Load 11 µL of dye-mixed sample per well. There should be at least one lane in between the ladder well and the sample wells to avoid contamination of the sample with ladder.

5. Run the gel at 120 V until the marker is close to the edge of the gel.

6. Destain the gel in nuclease-free water twice for 2 minutes each time and visualize the gel on a UV transilluminator with a ruler lying on top.

7. Using the ladder bands and the ruler for reference, excise the band of the gel corresponding to the insert size range of interest with a clean razor blade (see Figure 13 on page 90). If desired, a tighter size selection can be carried out at this stage by taking a tighter cut. If the gel piece is large, slice into smaller pieces.

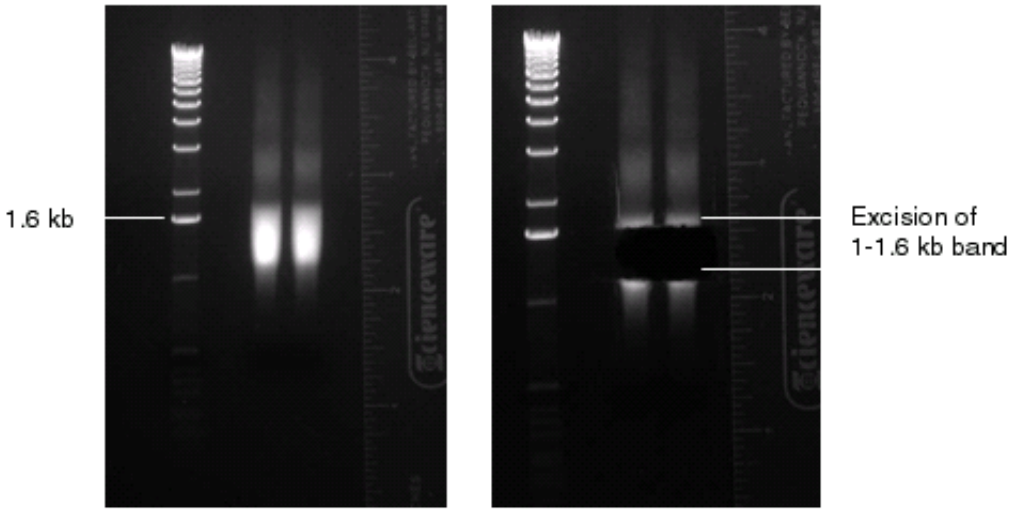


Figure 13 Excision of 1-1.6 kb range in a 1.0% agarose gel.

Elute the DNA using the Qiagen QIAquick® Gel Extraction Kit

1. Weigh the gel slice in a 15-mL polypropylene conical colorless tube.
2. Add 3 volumes of Buffer QG to 1 volume of gel.
3. Dissolve the gel slice by vortexing at *room temperature* until the gel slice has dissolved completely (~5 minutes).
4. If the color of the mixture is yellow, proceed to step 5. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.
5. Add one *gel volume* of isopropyl alcohol to the sample and mix by inverting the tube several times.
6. Apply about 750 µL of sample to the column(s). The maximum amount of gel that can be applied to a QIAquick® column is 400 mg. Use more columns if necessary.
7. Let the column(s) stand for 2 minutes at room temperature.
8. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
9. Repeat steps 6 and 8 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).
10. Add 500 µL of Buffer QG to the column(s).
11. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute, then discard the flow-through.
12. Add 750 µL of Buffer PE to wash the column(s).

13. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.

14. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).

15. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.

16. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.

17. Repeat steps 15 and 16.

18. If necessary, pool the eluted DNA in a 1.5-mL LoBind tube.

19. Quantitate the purified DNA by using 2 µL of the sample on the NanoDrop® ND-1000 Spectrophotometer (see Appendix B)

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "Circularize the DNA" on page 92.

Circularize the DNA

Circularize the DNA

1. Prepare a circularization reaction by mixing the following components listed (in order) based on the desired insert size, where *X* is the number of micrograms of DNA to be circularized (see Table 39). If a larger reaction volume is required, scale up the Quick Ligase and Quick Ligase Buffer. Add 1 µL of Quick Ligase per 40 µL of reaction volume. Add 1 µL of 2✕ Quick Ligase Buffer per 2 µL of reaction volume (see "DNA circularization" on page 215 for calculation details).

Table 39 Mix for DNA circularization by insert size

Components	600 to 800 bp	800 to 1000 bp	1 to 2 kb	2 to 3 kb	3 to 4 kb	4 to 5 kb	5 to 6 kb
Nuclease-free water	Variable	Variable	Variable	Variable	Variable	Variable	Variable
DNA	*X* µg	*X* µg	*X* µg	*X* µg	*X* µg	*X* µg	*X* µg
Quick Ligase Buffer, 2✕	(*X* × 117.5) µL	(*X* × 135) µL	(*X* × 182.5) µL	(*X* × 250) µL	(*X* × 280) µL	(*X* × 312.5) µL	(*X* × 360) µL
Internal Adaptor (ds), 2 µM	(*X* × 3.75) µL	(*X* × 2.84) µL	(*X* × 1.5) µL	(*X* × 0.9) µL	(*X* × 0.65) µL	(*X* × 0.5) µL	(*X* × 0.4) µL
Quick Ligase	(*X* × 6) µL	(*X* × 6.75) µL	(*X* × 9) µL	(*X* × 12.5) µL	(*X* × 14) µL	(*X* × 15.6) µL	(*X* × 18) µL
Total	(*X* × 235) µL	(*X* × 270) µL	(*X* × 365) µL	(*X* × 500) µL	(*X* × 560) µL	(*X* × 625) µL	(*X* × 720) µL

Example

For 10 µg of DNA in 1 to 2 kb size range to be circularized:

Mix for DNA circularization by insert size

Components	Amount
Nuclease-free water	Variable
DNA	10 µg
2✕ Quick Ligase Buffer	1825 µL
Internal Adaptor (ds), 2 µM	15 µL
Quick Ligase	90 µL
Total	3650 µL

2. Incubate at room temperature for 10 minutes.

Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit

1. Add 3 volumes of Buffer QG and 1 volume of isopropyl alcohol to the circularized DNA. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.
2. Apply 750 µL of circularized DNA in Buffer QG to the column(s). The maximum amount of DNA that can be applied to a QIAquick column is 10 µg. Use more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).
6. Add 750 µL of Buffer PE to wash the column(s).
7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. Repeat steps 9 and 10.
12. If necessary, pool the eluted DNA.

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "Isolate the circularized DNA" on page 94.

Isolate the circularized DNA

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. 3 M Sodium acetate.

Treat the DNA with Plasmid-Safe™ ATP-Dependent DNase

1. Combine and mix the following components, where *X* is the volume in µL of DNA and *Y* is the number of micrograms of DNA used in the circularization reaction (see Table 40).

Table 40 Mix for DNase treatment of DNA

Component	Volume (µL)
ATP, 25 mM	5
Plasmid-Safe™ Buffer, 10×	10
Plasmid-Safe™ DNase, 10 U/µL	(*Y* ÷ 3)
DNA	*X*
Nuclease-free water	Variable
Total	100

If *X* exceeds 85 µL, adjust the total reaction volume, accordingly. The volume of ATP and Plasmid-Safe™ Buffer, 10× should be proportional to the total reaction volume.

Example

For 10 µg DNA used in the circularization reaction:

Mix for DNase treatment of DNA

Component	Volume (µL)
ATP, 25 mM	5
Plasmid-Safe™ Buffer, 10×	10
Plasmid-Safe™ DNase, 10 U/µL	3.3
DNA	60
Nuclease-free water	21.7
Total	100

2. Incubate the reaction mixture at 37 °C for 40 minutes.

Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit

1. Add 3 volumes of Buffer QG and 1 volume of isopropyl alcohol to the Plasmid-Safe™ DNase-treated DNA. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.

2. Apply 750 µL of Plasmid-Safe™ DNase-treated DNA in Buffer QG to the column(s). The maximum amount of DNA that can be applied to a QIAquick column is 10 µg. Use more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).
6. Add 750 µL of Buffer PE to wash the column(s).
7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 40 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. If necessary, pool the eluted DNA.

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "Digest the circularized DNA with EcoP15I" on page 96.

Digest the circularized DNA with EcoP15I

Digest the circularized DNA with EcoP15I

1. Calculate the amount of EcoP15I enzyme, *X*, needed to digest the circularized DNA:

For insert sizes in 600 bp to 2 kb range:

$$X\ \mu L\ EcoP15I = \#\ ng\ DNA \times \frac{10\ U}{100\ ng\ DNA} \times \frac{1\ \mu L}{10\ U}$$

For insert sizes in 2 kb to 6 kb range:

$$X\ \mu L\ EcoP15I = \#\ ng\ DNA \times \frac{5\ U}{100\ ng\ DNA} \times \frac{1\ \mu L}{10\ U}$$

Example:

For a 1-2 kb insert

$$X\ \mu L\ EcoP15I = 1000\ ng\ DNA \times \frac{10\ U}{100\ ng\ DNA} \times \frac{1\ \mu L}{10\ U}$$

$$= 10\ \mu L\ EcoP15I$$

2. Combine in a LoBind tube (see Table 41):

Table 41 Mix to digest circularized DNA with EcoP15I

Component	Volume (µL)
Circularized DNA	38
10X NEBuffer 3	10
100X BSA	1
Sinefungin, 10 mM	1
10X ATP	20
EcoP15I, 10 U/µL	*X*
Nuclease-free water	Variable
Total	100

3. Incubate the reaction mixtures at 37 °C overnight.

4. Combine (see Table 42):

Table 42 Mix to digest circularized DNA with EcoP15I

Component	Volume (µL)
EcoP15I-digested DNA	100
10 mM Sinefungin	1
10× ATP	2
EcoP15I Enzyme, 10 U/µL	0.5
Total	103.5

5. Incubate the reaction mixture at 37 °C for 1 hour.

6. Denature the enzyme at 65 °C for 20 minutes and chill on ice for 5 minutes.

End-repair with Klenow

Repair the DNA ends with Klenow large-fragment DNA polymerase

1. Combine (see Table 43):

Table 43 Mix to digest circularized DNA with EcoP15I

Component	Volume (µL)
EcoP15I-digested DNA	103.5
dNTP Mix, 100 mM, 25 mM each	1.5
DNA polymerase, Klenow large fragment	1
Total	106

2. Incubate the reaction mixture at room temperature for 30 minutes.
3. Denature the enzyme at 65 °C for 20 minutes and chill on ice for 5 minutes.
4. Prepare Streptavidin Binding Buffer (see Table 44):

Table 44 Streptavidin Binding Buffer

Component	Volume (µL)
Tris-HCl, pH 7.5, 500 mM	10
Sodium chloride, 5 M	200
EDTA, 500 mM	1
Nuclease-free water	289
Total	500

5. Combine (see Table 45):

Table 45 Stop end-repair mix

Component	Volume (µL)
End-repaired DNA	106
Streptavidin Binding Buffer	200
Nuclease-free water	94
Total	400

Bind the library molecules to streptavidin beads

Pre-wash the beads

1. Combine (see Table 46):

Table 46 Mix for 1× BSA solution

Component	Volume (µL)
100× BSA	5
Nuclease-free water	495
Total	500

2. Vortex the bottle of Dynal® MyOne C1 streptavidin beads and transfer 90 µL into a 1.5-mL LoBind Tube.
3. Add 500 µL of 1× Bead Wash Buffer and vortex for 15 seconds, then pulse-spin.
4. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
5. Add 500 µL of 1× BSA and vortex for 15 seconds, then pulse-spin.
6. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
7. Add 500 µL of 1× Bind & Wash Buffer and vortex for 15 seconds, then pulse-spin.
8. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.

Bind the library DNA molecules to the beads

1. Add the entire 400 µL solution of library DNA in Streptavidin Binding Buffer to the pre-washed beads and vortex.
2. Mix by rotation at room temperature for 30 minutes, then pulse-spin.

Wash the bead-DNA complex

1. Combine (see Table 47):

Table 47 1× Quick Ligase Buffer

Component	Volume (µL)
2× Quick Ligase Buffer	300
Nuclease-free water	300
Total	600

2. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
3. Resuspend the beads in 500 µL of 1× Bead Wash Buffer and transfer beads to a new 1.5-mL LoBind tube. Vortex for 15 seconds, then pulse-spin.

4. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.

5. Resuspend the beads in 500 µL of 1× Bind & Wash Buffer. Vortex for 15 seconds, then pulse-spin.

6. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.

7. Resuspend the beads in 500 µL of 1× Bind & Wash Buffer and transfer beads to a new 1.5-mL LoBind tube. Vortex for 15 seconds, then pulse-spin.

8. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.

9. Resuspend the beads in 500 µL of 1× Quick Ligase Buffer. Vortex for 15 seconds, then pulse-spin.

10. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.

11. Resuspend the beads in 97.5 µL of 1× Quick Ligase Buffer.

Ligate P1 and P2 Adaptors to the DNA

Ligate P1 and P2 Adaptors to the DNA

1. Calculate the amount of P1 and P2 Adaptors needed for the ligation reaction based on the amount of circularized DNA from "Isolate the circularized DNA" on page 94 and the calculation below. For calculation details, see "Ligation of P1 and P2 Adaptors" on page 214.

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{\text{Circularized DNA size}}$$

$$Y \text{ µL adaptor needed} = \#\text{ µg DNA} \times \frac{X \text{ pmol}}{1 \text{ µg DNA}} \times 30 \times \frac{1 \text{ µL adaptor needed}}{50 \text{ pmol}}$$

Example:
For 1 µg of purified circularized DNA with an average size of 1536 (1500 bp insert + 36 bp internal adaptor)

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{1536} = 1 \text{ pmol/µg DNA}$$

$$Y \text{ µL adaptor needed} = 1 \text{ µg DNA} \times \frac{1 \text{ pmol}}{1 \text{ µg DNA}} \times 30 \times \frac{1 \text{ µL adaptor needed}}{50 \text{ pmol}}$$

$$= 0.6 \text{ µL adaptor needed}$$

2. Combine (see Table 48):

Table 48 Mix for ligation of end-repaired DNA to P1 and P2 Adaptors

Component	Volume (µL)
DNA-bead complex	97.5
P1 Adaptor (ds), 50 µM	*Y*
P1 Adaptor (ds), 50 µM	*Y*
Quick Ligase	2.5
Total	Variable

3. Incubate the reaction mixture at room temperature for 15 minutes.

Wash the DNA-bound streptavidin beads

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. 1× Bead Wash Buffer.

CAUTION! CHEMICAL HAZARD. 1× Bind & Wash Buffer, 1× Low Salt Binding Buffer.

Wash the bead-DNA complex

1. Combine (see Table 49):

Table 49 Prepare 1× NEBuffer 2

Components	Volume (µL)
10× NEBuffer 2	60
Nuclease-free water	540
Total	600

2. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
3. Resuspend the beads in 500 µL of 1× Bead Wash Buffer and transfer beads to a new 1.5-mL LoBind tube. Vortex for 15 seconds, then pulse-spin.
4. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
5. Resuspend the beads in 500 µL of 1× Bind & Wash Buffer. Vortex for 15 seconds, then pulse-spin.
6. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
7. Resuspend the beads in 500 µL of 1× Bind & Wash Buffer and transfer beads to a new 1.5-mL LoBind tube. Vortex for 15 seconds, then pulse-spin.
8. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
9. Resuspend the beads in 500 µL of 1× NEBuffer 2. Vortex for 15 seconds, then pulse-spin.
10. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
11. Resuspend the beads in 96 µL of 1× NEBuffer 2.

Nick-translate the DNA

Nick-translate the library

1. Combine (see Table 50):

Table 50 Mix for nick translation

Component	Volume (µL)
DNA-bead complex	96
100 mM dNTP mix, 25 mM each	2
DNA Polymerase I, 10 U/µL	2
Total	100

2. Incubate the reaction mixture at 16 °C for 30 minutes.
3. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
4. Resuspend the beads in 500 µL of Buffer EB (Qiagen).
5. Place the tube in a magnetic rack for at least 1 minute. After the solution clears, remove and discard the supernatant.
6. Resuspend the beads in 30 µL of Buffer EB.
7. (Optional) Save 3 µL of nick-translated library DNA for troubleshooting.

STOPPING POINT. Store the DNA-Bead complexes in Buffer EB at 4 ºC, or proceed directly to "Amplify the library" on page 104.

Amplify the library

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. 3 M Sodium acetate.

Perform PCR on the library

1. Prepare a master mix for four 100-µL PCR reactions (see Table 51):

Table 51 PCR master mix for amplification of the library

Component	Volume (µL)
SOLiD™ Library PCR Master Mix	200
Library PCR Primer 1, 50 µM	8
Library PCR Primer 2, 50 µM	8
Cloned Pfu, 2.5 U/µL	1
Nuclease-free water	143
Total	360

2. For the negative control, aliquot 90 µL of PCR master mix to a PCR tube. Add 10 µL of nuclease-free water to the tube.

3. Add 27 or 30 µL of DNA-bead complex solution to the remaining 270 µL of PCR master mix. Vortex to mix, then divide evenly among three PCR tubes.

4. Run (see Table 52):

Table 52 PCR conditions to amplify the trial library

Stage	Step	Temp	Time
Holding	Denature	95 °C	10 min
Cycling (5 cycles)	Denature	95 °C	15 sec
	Anneal	62 °C	15 sec
	Extend	60 °C	4 min
Holding	—	4 °C	∞

Confirm library amplification with a Lonza FlashGel®

1. Add 1 µL of 5✕ FlashGel® Loading Dye to 4 µL of sample from the 100 µL of PCR reaction, then load on a 2.2% Lonza FlashGel®. Load FlashGel DNA Marker (50 bp to 1.5 kb or 100 bp to 4 kb) in an adjacent well for reference.

2. Run the FlashGel for 6 minutes at 275 V (see Figure 14 on page 105).

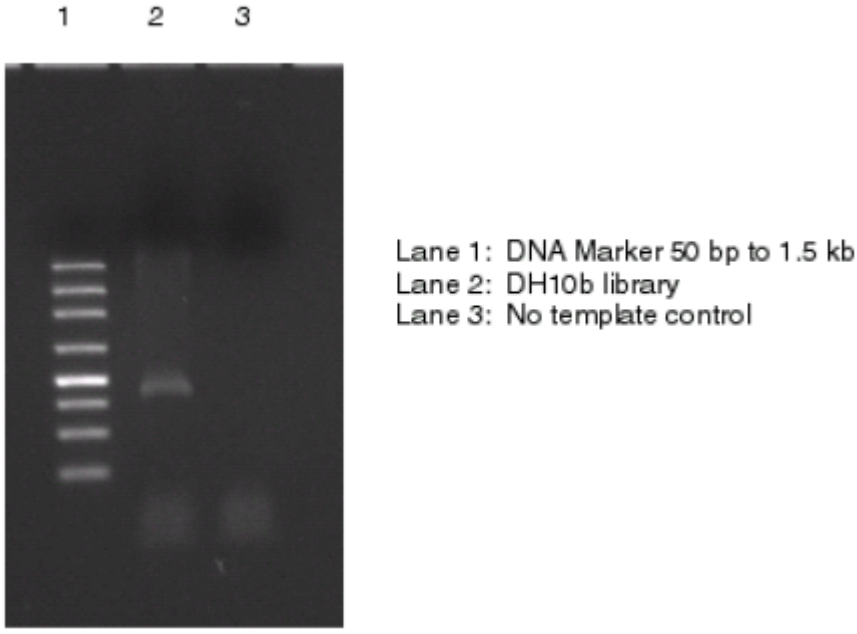


Figure 14 Mate-paired library amplification sample run on a Lonza FlashGel.

3. If fairly robust amplification products are visible, proceed to step 5. If robust amplification products are *not* visible, return the tubes to the thermal cycler and run the PCR cycling program (see Table 53), then repeat steps 1 and 2.

Table 53 PCR conditions to amplify the trial library

Stage	Step	Temp	Time
Cycling (2 cycles)	Denature	95 °C	15 sec
	Anneal	62 °C	15 sec
	Extend	60 °C	4 min
Holding	—	4 °C	∞

4. If amplification is still not observed, repeat steps 1 through 3 until amplification is observed. Be careful not to overamplify the sample.

5. Pool all of the PCR samples into a 2.0-mL LoBind tube.

6. Place the tube of beads in a magnetic rack and transfer the supernatant to a fresh 2.0-mL LoBind tube. Discard the tube containing the beads.

Purify the DNA with the Qiagen QIAquick® Gel Extraction Kit

1. Add 3 volumes of Buffer QG and 1 volume of isopropyl alcohol to each PCR product aliquot. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.

2. Apply 750 µL of PCR product in Buffer QG to the column(s). The maximum amount of DNA that can be applied to a QIAquick column is 10 µg. se more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).
6. Add 750 µL of Buffer PE to wash the column(s).
7. Centrifuge the columns at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the columns for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. If necessary, pool the eluted DNA.

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "Gel-purify the library" on page 107.

Gel-purify the library

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. 10× TAE, 10× TBE, 3 M Sodium acetate, gel loading solution, ethidium bromide.

Size-select the DNA fragments with an agarose gel

1. Prepare a 4% agarose gel in 1× TAE or 1× TBE. To prepare the 4% gel, use either Agarose-LE (Applied Biosystems, AM9040) or Reliant® Precast 4% NuSieve® 3:1 Plus Agarose Gel (Lonza, 54927). Do not use a FlashGel® or 4% E-Gel for size-selection.

   Note: Size-selection may also be performed with 6% PAGE gels (Invitrogen, EC6365BOX). To elute the DNA from the PAGE gel, see "PAGE gel DNA elution" on page 198.

2. Add 10× Gel Loading Solution to the mate-paired library: 1 µL of 10× Gel Loading Solution for every 10 µL of mate-paired library.

3. Load 2 µL of TrackIt™ 25-bp ladder. Load 11 µL of dye-mixed sample per well. There should be at least one lane in between the ladder well and the sample wells to avoid contamination of the sample with ladder.

4. Run the gel at 120 V until the marker is close to the edge of the gel.

5. If needed, stain the gel in 50 to 100 mL of 1× TAE or 1× TBE Buffer with 8 µL of ethidium bromide (10 mg/mL) for 5 minutes.

6. Destain the gel in nuclease-free water twice for 2 minutes each time and visualize the gel on a UV transilluminator.

7. Excise the band between 154 to 156 bp using a clean razor blade (see Figure 15 on page 108).

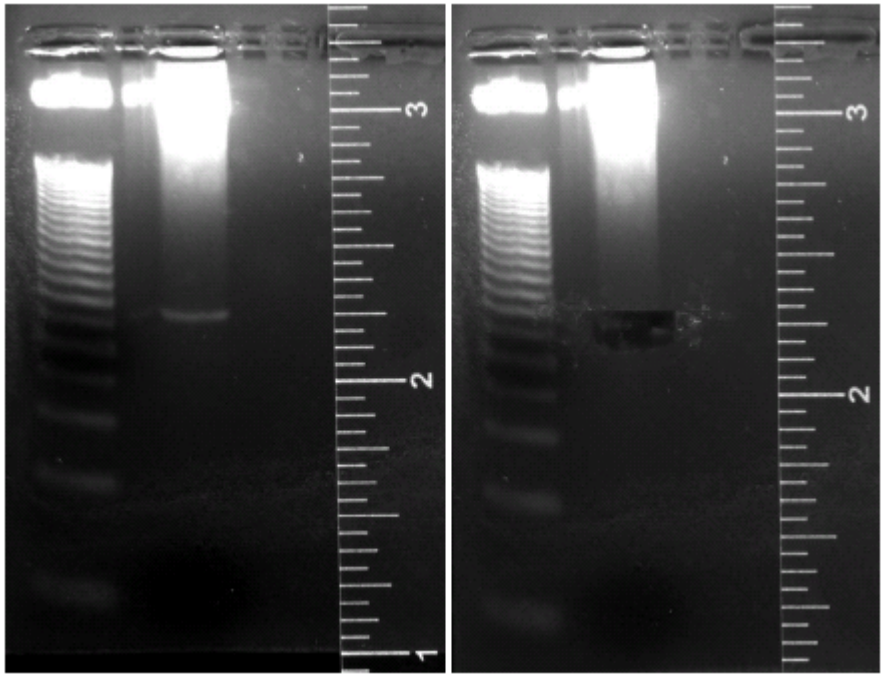


Figure 15 Excision of 154 to 156 bp library band.

Elute the DNA with the Qiagen QIAquick® Gel Extraction Kit

1. Weigh the gel slice(s) in a 15-mL polypropylene conical colorless tube.
2. Add 6 volumes of Buffer QG to 1 volume of gel.
3. Dissolve the gel slice by vortexing at *room temperature* until the gel slice has dissolved completely (~5 minutes).

   IMPORTANT! Do not dissolve the gel slice by heating. Although Qiagen recommends dissolving the gel slice at 50 °C for 10 minutes or until the gel slice has completely dissolved, these conditions affect the fragment library deleteriously, resulting in denaturation of the fragments and lead to formation of heteroduplexes.

4. If the color of the mixture is yellow, proceed to step 5. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.
5. Add one *gel volume* of isopropyl alcohol to the sample and mix by inverting the tube several times.
6. Apply about 750 µL of sample to the column(s). The maximum amount of gel that can be applied to a QIAquick column is 400 mg. Use more columns if necessary.
7. Let the column(s) stand for 2 minutes at room temperature.
8. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.

9. Repeat steps 6 and 8 until the entire sample has been loaded onto the column(s). Place the QIAquick column(s) back into the same collection tube(s).

10. Add 500 µL of Buffer QG to the column(s).

11. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute, then discard the flow-through.

12. Add 750 µL of Buffer PE to wash the column(s).

13. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.

14. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).

15. Add 30 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.

16. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.

17. If necessary, pool the eluted DNA in a 1.5-mL LoBind tube.

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to "Quantitate the library by performing quantitative PCR (qPCR)" on page 110.

Quantitate the library by performing quantitative PCR (qPCR)

For accurate library quantitation, quantitative PCR is strongly recommended. For a TaqMan® or SYBR® qPCR protocol, see Appendix B, "SOLiD™ 3 System Library Quantitation" on page 155.

STOPPING POINT. Store the purified DNA in Buffer EB at 4 °C, or proceed directly to emulsion PCR in the *Applied Biosystems SOLiD™ 3 System Templated Bead Preparation Guide* (PN 4407421).

4 Barcoded Fragment Library Preparation

This chapter covers:

Overview

This chapter describes the method to generate a small-insert library (100 to 110 bp) tagged with a unique sequence identifier, or barcode, to enable multiplexed sequencing analysis. This method involves shearing DNA into small fragments and ligating Multiplex P1 and P2 Adaptors specific for barcoded library preparation (see Figure 16).

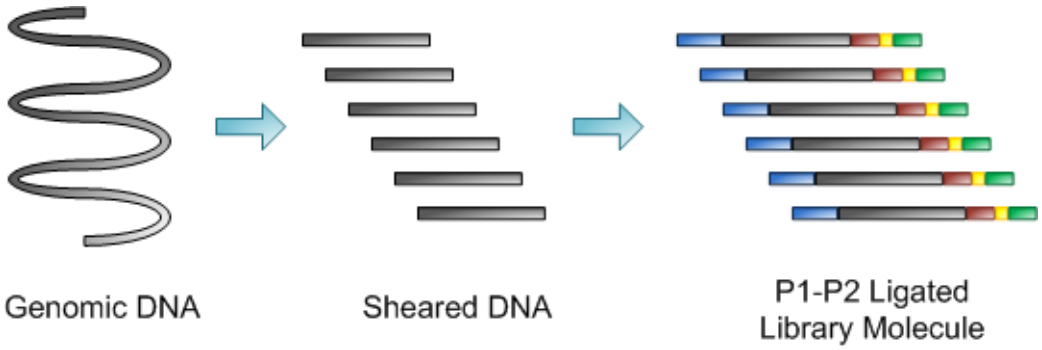


Figure 16 Basic barcoded fragment library preparation workflow overview.

The Multiplex P2 Adaptor consists of 3 segments of sequence:

1. Internal adaptor sequence, which is necessary for sequencing the barcode
2. Barcode sequence
3. P2 adaptor sequence, which is used for library amplification and emulsion PCR

The Multiplex P1 Adaptor is a truncated version of the standard P1 Adaptor. The Multiplex P1 Adaptor is shorter to make up for the increased length of the Multiplex P2 Adaptor. Different libraries to be multiplexed in the same sequencing run are ligated to Multiplex P2 Adaptors with different barcode sequences. Twenty barcode sequences are provided to tag different libraries (see Figure 17 on page 113).

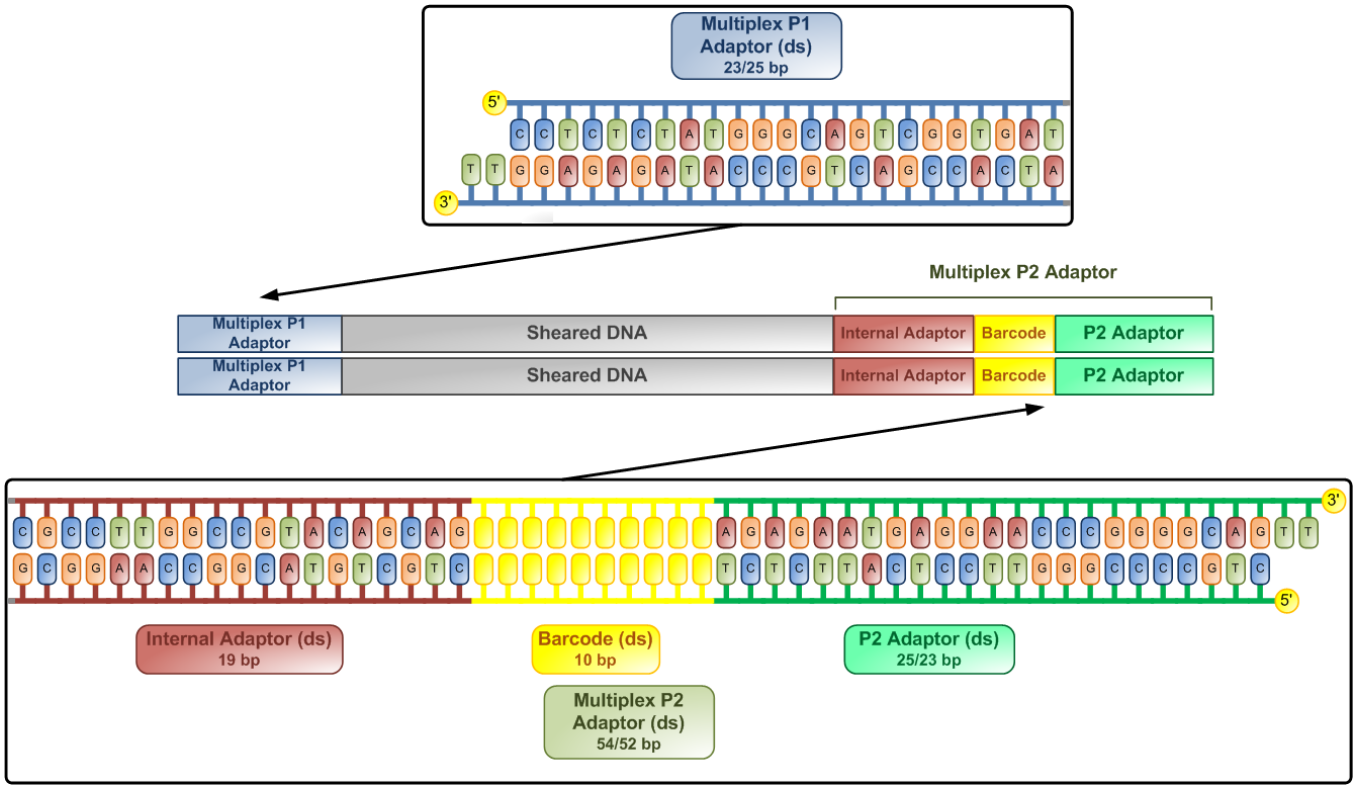


Figure 17 Barcoded fragment library design.

After Multiplex P1 and P2 Adaptors are ligated to the sheared DNA, the library is amplified using primers specific to the Multiplex P1 and P2 Adaptors (see Figure 18 on page 114). Multiplex Library PCR Primer 1 is a 3′-truncated version of the 5′-strand sequence of Multiplex P1, while Library PCR Primer 2 is a 3′-truncated version of the 5′-strand sequence of standard P2. Amplification with Multiplex Library PCR Primer 1 adds back the P1 sequence that was truncated in the Multiplex P1 Adaptor. These primers can be used only for library amplification and not for alternative or modified library construction adaptor design, because they do not have 3′sequences compatible with the sequencing primers.

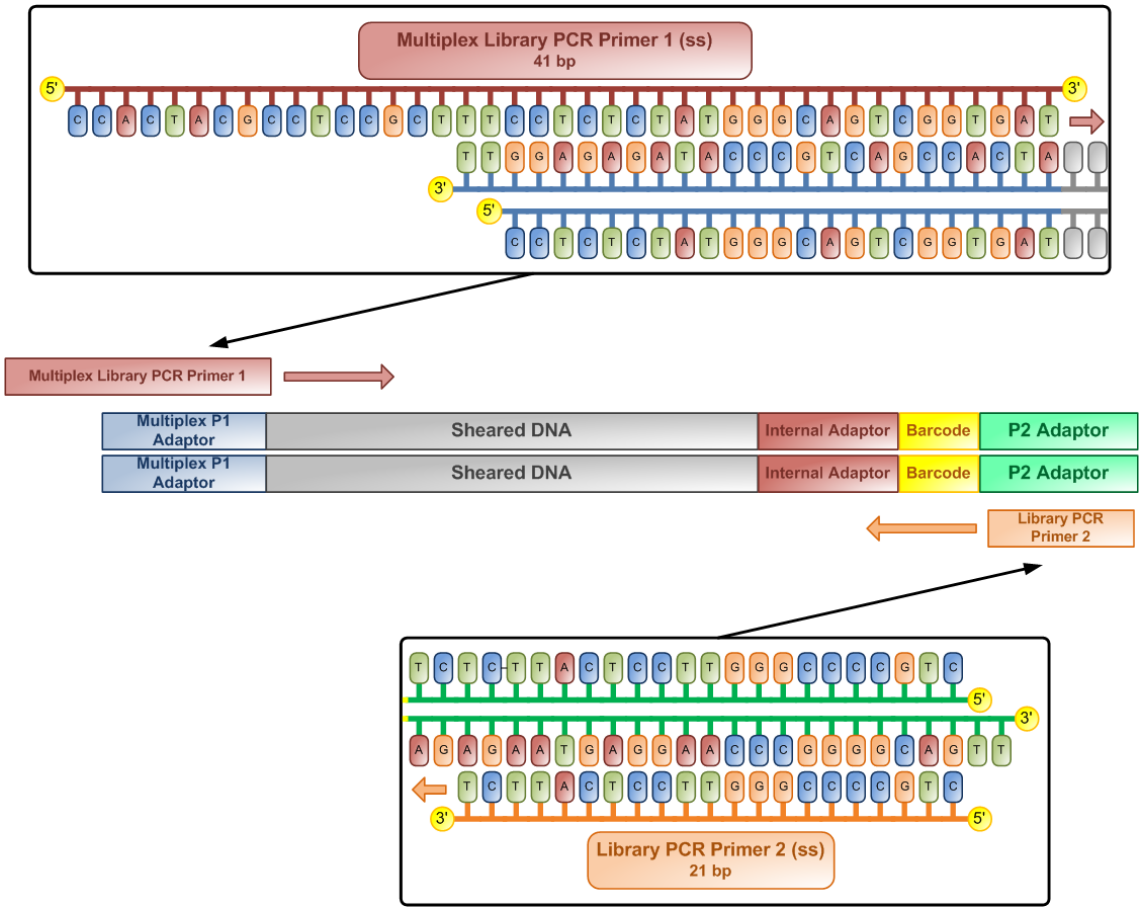


Figure 18 Barcoded fragment library amplification design.

This chapter describes how to generate a barcoded small-insert DNA library. An alternative method to generate barcoded fragment libraries not described in this chapter involves use of the SOLiD™ Small RNA Expression Kit (PN 4397682).

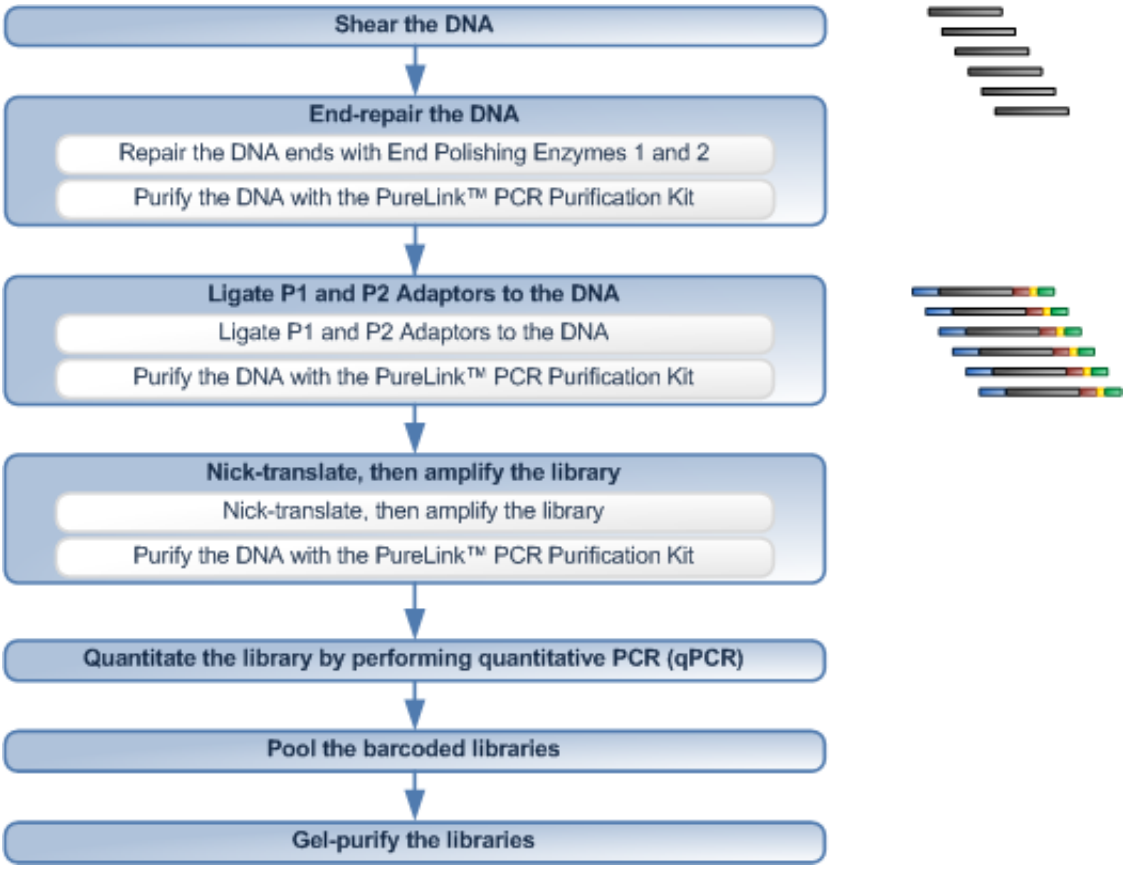
Prepare a barcoded fragment library

The protocol is designed for 10 ng to 20 µg of genomic DNA or ligated PCR product. You should modify the protocol with any change in the starting amount of DNA. If you are trying to construct a targeted, resequencing library with small-sized PCR products (≤ 500 bp), then you must first perform a PCR-product ligation step. For a concatenation protocol, contact your field applications specialist.

Materials and equipment required

See Appendix A on page 127 for a list of equipment, kits, and consumables necessary for this procedure.

Workflow



Shear the DNA

This step involves sonicating the input DNA into small fragments with a mean size of 100 to 110 bp using the Covaris™ S2 System. The conditions have been tested for shearing 10 ng to 20 µg DNA in a total volume of 100 µL. For certain DNA samples, optimizing the shearing protocol may be necessary.

End-repair the DNA

End Polishing Enzyme 1 and End Polishing Enzyme 2 are used to convert DNA that has damaged or incompatible 5′-protruding and/or 3′-protruding ends to 5′-phosphorylated, blunt-ended DNA. The conversion to blunt-ended DNA results from 5′-to-3′ polymerase and the 3′-to-5′ exonuclease activities of End Polishing Enzyme 2. End Polishing Enzyme 1 and ATP are also included for phosphorylation of the 5′-ends of the blunt-ended DNA to allow for subsequent ligation.

Purify the DNA with the PureLink™ PCR Purification Kit

Sample purification is recommended with the PureLink™ PCR Purification columns supplied in the PureLink™ PCR Purification Kit. PureLink columns have a 40-µg capacity, but it may be necessary to use multiple columns during a purification step for higher yields.

Ligate P1 and P2 Adaptors to the DNA

Multiplex P1 and P2 Adaptors are ligated to the ends of the end-repaired DNA. The Multiplex P1 and P2 Adaptors are not included in the SOLiD™ Fragment Library Oligos Kit. Custom order them from an oligonucleotide vendor (for sequences, see Appendix D, "Oligonucleotide Sequences" on page 205). Anneal the custom-ordered single-stranded oligonucleotides to make double-stranded adaptors (see "Hybridization of oligonucleotides" on page 188). You can design experiments to use as few as 4 barcodes, as long as at least one of the following full sets of four barcodes are used: Barcodes 1–4, 5–8, 9–12, 13–16, or 17 –20. If fewer than four libraries are to be prepared for sequencing, multiple barcodes should be used per sample in equal ratios.

Nick-translate, then amplify the library

The adaptor-ligated, purified DNA undergoes nick translation, then amplification using Multiplex Library PCR Primer 1 and Library Primer 2 and Platinum® PCR Amplification Mix. After amplification, the PCR samples are purified with the PureLink PCR Purification Kit.

Quantitate the library by performing quantitative PCR (qPCR)

Quantitate the library by either the TaqMan® or SYBR® quantitative PCR (qPCR) method described in Appendix B, "SOLiD™ 3 System Library Quantitation" on page 155.

Pool the barcoded libraries

Equal molar amounts of each barcoded library are mixed together. This sample of combined barcoded libraries can be processed together through templated bead preparation [see the *Applied Biosystems SOLiD™ 3 System Templated Bead Preparation Guide* (PN 4407421)]. If accurate quantitation of each barcoded library is not crucial, barcoded libraries can be pooled just prior to nick translation and amplification.

Gel-purify the libraries

The library is run on an E-Gel® 2% SizeSelect™ gel. The correctly sized ligation products (150 to 200 bp) are electrophoresed to the collection wells of the SizeSelect™ gel. If needed, the eluate can be concentrated using the PureLink™ PCR Purification Kit

Tips

- Adjust microcentrifuge speeds and times according to the g-forces specified in the protocols. Applied Biosystems recommends the Eppendorf 5417R tabletop microcentrifuge.
- Perform all steps requiring 0.5-mL and 1.5-mL tubes with Eppendorf LoBind tubes.
- Thaw reagents on ice before use.

Shear the DNA

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. TRIS (Tris (hydroxymethyl)aminomethane).

Shear the DNA using the Covaris™ S2 System

1. Dilute the desired amount of DNA in 100 µL in 1✕ Low TE Buffer in a LoBind tube (see Table 54).

Table 54 Dilute the DNA for shearing

Component	Amount
DNA	10 ng to 20 µg
1✕ Low TE Buffer	Variable
Total	100 µL

2. Place a Covaris™ microTube into the loading station. Keep the cap on the tube and use a tapered pipette tip to slowly transfer the 100 µL of DNA sample through the pre-split septa. Be careful not to introduce a bubble into the bottom of the tube.

3. Shear the DNA using the following Covaris S2 System conditions:
   - Number of Cycles: **6**
   - Bath Temperature: **5** °C
   - Bath Temperature Limit: **30** °C
   - Mode: **Frequency sweeping**
   - Water Quality Testing Function: **Off**
   - Duty cycle: **20%**
   - Intensity: **5**
   - Cycles/burst: **200**
   - Time: **60 seconds**

IMPORTANT! Make sure that the water in the Covaris tank is filled with fresh deionized water to fill line level 12 on the graduated fill line label. The water should cover the visible glass part of the tube. Set the chiller temperature to between 2 to 5 °C to ensure that the temperature reading in the water bath displays 5 °C. The circulated water chiller should be supplemented with 20% ethylene glycol.

4. Place the Covaris microTube into the loading station. While keeping the snap-cap on, insert a pipette tip through the pre-split septa, then slowly remove the sheared DNA. Transfer the sheared DNA into a new 1.5-mL LoBind tube.

End-repair the DNA

Repair the DNA ends with End Polishing Enzyme 1 and End Polishing Enzyme 2

1. Combine and mix the following components in a 1.5-mL LoBind tube (see Table 55):

Table 55 Mix for end-repair of DNA

Component	Volume (µL)
Sheared DNA	100
5× End-Polishing Buffer	40
dNTP Mix, 10 mM	8
End Polishing Enzyme 1, 10 U/µL	2
End Polishing Enzyme 2, 5 U/µL	16
Nuclease-free water	34
Total	200

2. Incubate the mixture at room temperature for 30 minutes.

Purify the DNA with PureLink™ PCR Purification Kit

1. Add 4 volumes of Binding Buffer (B2) with 55% isopropanol to the end-repaired DNA.
2. Apply about 700 µL of end-repaired DNA in the Binding Buffer (B2) to the column(s). The maximum yield of DNA can be achieved if the amount of DNA loaded to a PureLink™ column is ≤ 5 µg. Use more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the PureLink column(s) back into the same collection tube(s).
6. Add 650 µL of Wash Buffer (W2) to wash the column(s).
7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 50 µL of Elution Buffer (E1) to the column(s) to elute the DNA, then let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. Add the eluate from step 10 back to the column(s), then let the column(s) stand for 2 minutes. Repeat step 10.

12. If necessary, pool the eluted DNA.

13. If the starting DNA input amount is ≥500 ng, quantitate the purified DNA by using 2 µL of the sample on the NanoDrop® ND-1000 Spectrophotometer (see Appendix C, "Supplemental Procedures" on page 187). If the starting DNA input amount is < 500 ng, assume 70% recovery of input material after shearing.

STOPPING POINT. Store the purified DNA in Elution Buffer (E1) at 4 ºC, or proceed directly to "Ligate P1 and P2 Adaptors to the DNA" on page 121.

Ligate P1 and P2 Adaptors to the DNA

Ligate P1 and P2 Adaptors to the DNA

1. Generate double-stranded Multiplex P1 and P2 Adaptors (see "Hybridization of oligonucleotides" on page 188).

2. Calculate the amount of adaptor needed, *Y*, for the reaction based on the amount of DNA from the last purification step (for calculation details, see "Ligation of P1 and P2 Adaptors" on page 212). If DNA fragments were sheared using the standard protocol for fragment library preparation, the average insert size should be approximately 105 bp.

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{\text{Average insert size}}$$

$$Y \text{ µL adaptor needed} = \#\text{ µg DNA} \times \frac{X \text{ pmol}}{1 \text{ µg DNA}} \times 30 \times \frac{1 \text{ µL adaptor needed}}{50 \text{ pmol}}$$

Example:

For 1 µg of purified end-repaired DNA with an average insert size of 105 bp

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{105} = 14.4 \text{ pmol/µg DNA}$$

$$Y \text{ µL adaptor needed} = 1 \text{ µg DNA} \times \frac{14.4 \text{ pmol}}{1 \text{ µg DNA}} \times 30 \times \frac{1 \text{ µL adaptor needed}}{50 \text{ pmol}}$$

$$= 8.7 \text{ µL adaptor needed}$$

IMPORTANT! For each multiplexed sequencing run, use at least one of the following full sets of four barcodes: Barcodes 1–4, 5–8, 9–12, 13–16, or 17 –20. Use only one of the barcoded Multiplex P2 Adaptors for each ligation reaction, unless fewer than four libraries are being barcoded. If fewer than four samples are to be prepared for sequencing, use multiple barcodes per sample in equal ratios (see next step).

3. Combine (see Table 56):

Table 56 Ligation mix

Component	Volume (µL)
Multiplex P1 Adaptor (ds), 50 pmol/µL	*Y*
Multiplex P2 Adaptor (ds), 50 pmol/µL	*Y*
5X T4 Ligase Buffer	40
DNA	48 to 50
T4 Ligase, 5 U/µL	10
Nuclease-free water	Variable
Total	200

4. Incubate at room temperature for 10 minutes.

Purify the DNA with the PureLink™ PCR Purification Kit

1. Add 4 volumes (800 µL) of Binding Buffer (B2) with 40% isopropanol to the sample.
2. Apply about 700 µL of the ligated DNA in Binding Buffer (B2) to the column(s). The maximum yield of DNA can be achieved when the amount of DNA loaded to a PureLink™ column is ≤ 5 µg. Use more columns if necessary.
3. Let the column stand for 2 minutes at room temperature.
4. Centrifuge the column at ≥10,000 × g (13,000 rpm) for 1 minute, then discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the PureLink column(s) back into the same collection tube(s).
6. Add 650 µL of Wash Buffer (W2) to wash the column(s).
7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 50 µL of Elution Buffer (E1) to the column(s) to elute the DNA, then let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. Add the eluate from step 10 back to the column(s), then let the column(s) stand for 2 minutes. Repeat step 10.
12. If necessary, pool the eluted DNA.

STOPPING POINT. Store the purified DNA in Elution Buffer (E1) at 4 °C, or proceed directly to "Nick-translate, then amplify the library" on page 123.

Nick-translate, then amplify the library

Nick-translate, then amplify the library

1. Prepare a PCR reaction mix (see Table 57).

Table 57 PCR reaction mix: a mix for nick translation and amplification of the library

Component	Volume (µL)
Platinum® PCR Amplification Mix	400
Multiplex Library PCR Primer 1, 50 µM	10
Library PCR Primer 2, 50 µM	10
Adaptor-ligated, purified DNA	48 to 50
Nuclease-free water	Variable
Total	500

2. Pipette 125 µL of the PCR reaction mix into each of four PCR tubes.

3. Run the PCR (Table 58).

IMPORTANT! The number of cycles should be minimized and determined based on the amount of starting input DNA. Minimal cycling is desirable to avoid overamplification and production of redundant molecules.

Table 58 PCR conditions to nick-translate and amplify the library

Stage	Step	Temp	Time
Holding	Nick translation	72 °C	20 min
Holding	Denature	95 °C	5 min
Cycling (2 to 10 cycles)‡	Denature	95 °C	15 sec
	Anneal	62 °C	15 sec
	Extend	70 °C	1 min
Holding	Extend	70 °C	5 min
Holding	—	4 °C	∞

‡ Starting amount of DNA: number of cycles:
10 ng to 100 ng: 10 cycles
100 ng to 1 µg: 6 to 8 cycles
1 µg to 2 µg: 4 to 6 cycles
2 µg to 20 µg: 2 to 3 cycles

4. Pool all of the PCR samples into a new 1.5-mL LoBind tube.

Purify the DNA with the PureLink™ PCR Purification Kit

1. Add 4 volumes of Binding Buffer (B2) with 40% isopropanol to the sample.
2. Apply about 700 µL of PCR product in the Binding Buffer (B2) to the column(s). The maximum yield of DNA can be achieved if the amount of DNA loaded to a PureLink™ column is ≤ 5 µg. Use more columns if necessary.
3. Let the column(s) stand for 2 minutes at room temperature.
4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the PureLink column(s) back into the same collection tube(s).
6. Add 650 µL of Wash Buffer (W2) to wash the column(s).
7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 50 µL of Elution Buffer (E1) to the column(s) to elute the DNA, then let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. Add the eluate from step 10 back to the column(s), then let the column(s) stand for 2 minutes. Repeat step 10.
12. If necessary, pool the eluted DNA.

STOPPING POINT. Store the purified DNA in Elution Buffer (E1) at 4 °C, or proceed directly to "Quantitate the library by performing quantitative PCR (qPCR)".

Quantitate the library by performing quantitative PCR (qPCR)

Quantitate your library by quantitative PCR. For a TaqMan® or SYBR® qPCR protocol, see Appendix B, "SOLiD™ 3 System Library Quantitation" on page 155.

STOPPING POINT. Store the DNA in Elution Buffer (E1) at 4 °C, or proceed directly to "Pool the barcoded libraries" on page 125.

Pool the barcoded libraries

To multiplex, simply mix equal molar amounts of each barcoded library together in a single tube. Vortex the tube.

STOPPING POINT. Store the library DNA in Elution Buffer (E1) at 4 °C, or proceed directly to "Gel-purify the libraries" on page 125.

Gel-purify the libraries

Size-select the DNA fragments with an E-Gel® 2% SizeSelect™ gel

1. Remove an E-Gel® 2% SizeSelect™ gel from its package. Remove the combs from *top* sample-loading wells and *middle* collection wells. Set the E-Gel on the E-Gel iBase™ linked with the E-Gel Safe Imager™.
2. Load the E-Gel as follows:
   a. Load 20 µL of the pooled library DNA into each well of the *top row* of wells. If the sample volume < 20 µL, add nuclease-free water to the well for a total volume of 20 µL. Skip the center well (smaller well in the top center of the gel for the ladder); and skip a single well to the right and left of the center top well. Skip the two outermost wells (to avoid edge effects). Do not load more than 1 µg of DNA per lane.
   b. Load 2 µL of 50-bp ladder at 0.1 µg/µL to the center top well. Add 15 µL of water to fill the well.
   c. Fill the empty wells in the top row with 20 µL of nuclease-free water.
   d. Fill each of the collection wells in the *middle* of the gel with 25 µL of nuclease-free water. Add 20 µL of nuclease-free water to the middle center well.
3. Run the gel:
   - iBase program: **Run E-Gel DC**
   - Run time: **11:40** (11 minutes and 40 seconds)

   Monitor the E-Gel in real-time with the E-Gel® Safe Imager.
4. If needed during the run, fill the middle collection wells with nuclease-free water.
5. When the 150-bp band from the marker lane clears the collection well and the 200-bp band is about to enter the collection well, stop the run if the run has not already stopped (see Figure 19 on page 126).
6. Collect the solution from the wells and pool according to samples.
7. Wash each collection well with 25 µL with nuclease-free water, then retrieve the wash solution with the solution collected in Step 6.

8. (Optional) Concentrate the DNA with a PureLink PCR purification column.

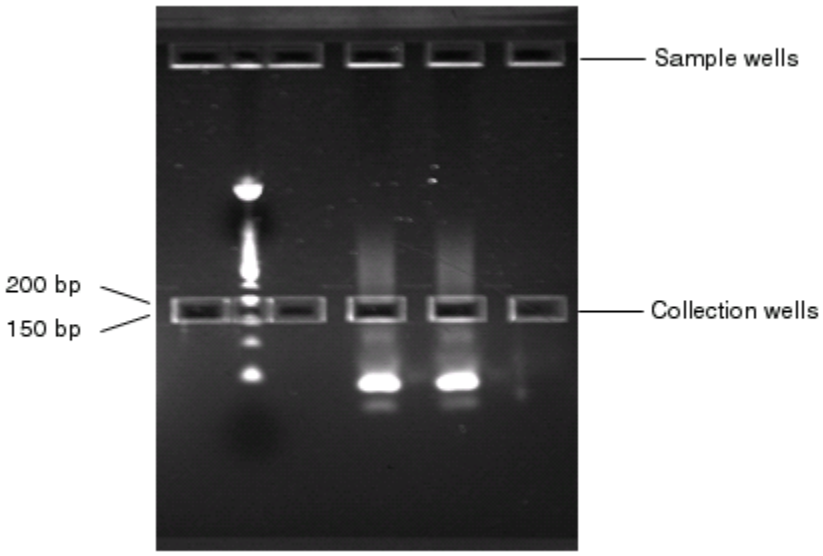


Figure 19 Elution of ~150- to 200-bp region from an E-Gel® 2% SizeSelect™ gel.

STOPPING POINT. Store the purified DNA in Elution Buffer (E1) at 4 °C, or proceed directly to the *Applied Biosystems SOLiD™ 3 System Templated Bead Preparation Guide* (PN 4407421).

A Required Materials

This appendix covers:

Prepare a standard fragment library

Required Applied Biosystems reagent kits

Item (part number)‡	Components	Kit components used in…
SOLiD™ Fragment Library Oligos Kit (4401151)	SOLiD™ Library Oligos Kit 1 – P1 Adaptor (ds)	Ligation of adaptors
	SOLiD™ Library Oligos Kit 1 – P2 Adaptor (ds)	
	SOLiD™ Library Oligos Kit 1 – Library PCR Primer 1	Library amplification
	SOLiD™ Library Oligos Kit 1 – Library PCR Primer 2	

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Required Invitrogen reagent kits

Item (Invitrogen part number)‡§	Components	Kit components used in…
SOLiD™ Fragment Library Construction Kit with SizeSelect™ Gels (S3100101)#	SOLiD™ Fragment Library Enzyme/Core Kit: • 5X End Polishing Buffer • dNTP, 10 mM • End Polishing Enzyme 1 • End Polishing Enzyme 2	DNA end repair
	• 5X Ligase Buffer • T4 DNA Ligase	Ligation of P1 and P2 Adaptors
	Platinum® PCR Amplification Mix	Nick translation/library amplification
	PureLink™ PCR Purification Kit	DNA end repair, ligation of P1 and P2 Adaptors, and nick translation/library amplification
	E-Gel® SizeSelect™ 2%, 10 gels	Size selection

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ Invitrogen products can be ordered at **www.invitrogen.com**.
# For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Optional Invitrogen reagent kits

Item (part number)‡	Components	Kit components used in…
SOLiD™ Fragment Library Construction Kit (S3100102)§	SOLiD™ Fragment Library Enzyme/Core Kit: • 5× End Polishing Buffer • dNTP, 10 mM • End Polishing Enzyme 1 • End Polishing Enzyme 2	DNA end repair
	• 5× Ligase Buffer • T4 DNA Ligase	Ligation of P1 and P2 Adaptors
	Platinum® PCR Amplification Mix	Nick translation/library amplification
	PureLink™ PCR Purification Kit	DNA end repair, ligation of P1 and P2 Adaptors, and nick translation/library amplification
SOLiD™ Fragment Library Construction Kit Reagents (S3100105)	SOLiD™ Fragment Library Enzyme/Core Kit: • 5× End Polishing Buffer • dNTP, 10 mM • End Polishing Enzyme 1 • End Polishing Enzyme 2	DNA end repair
	• 5× Ligase Buffer • T4 DNA Ligase	Ligation of P1 and P2 Adaptors
	– Platinum® PCR Amplification Mix	Nick translation/library amplification

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

§ For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Required equipment

Item‡	Source
Covaris™ S2 System (110 V for U.S. customers) (220 V for international customers) The system includes: • Covaris™ S2 sonicator • Latitude™ laptop from Dell® Inc. • MultiTemp III Thermostatic Circulator • Covaris-2 series Machine Holder for (one) 1.5-mL microcentrifuge tube • Covaris-2 series Machine Holder for (one) 0.65-mL microcentrifuge tube • Covaris-2 series Machine Holder for (one) 13 mm × 65 mm tube For system materials summary, see "Covaris™ S2 System Materials Summary," *SOLiD™ 3 System Site Preparation Guide*.	• Applied Biosystems 4387833 (110 V) • Applied Biosystems 4392718 (220 V) or Covaris™ Inc.
Covaris™ microTube loading and unloading station	Covaris 500142
Covaris™-2 series Machine Holder for (one) microTube	Covaris 500114
Covaris™ S2 System Pump Kit, with water fill level label	Covaris 500165
Microcentrifuge 5417R, refrigerated, without rotor	• Eppendorf§ 022621807 (120 V/60 Hz) • Eppendorf‡ 022621840 (230 V/50 Hz)
FA-45-24-11, fixed-angle rotor, 24 × 1.5/2 mL, including aluminum lid, aerosol-tight	Eppendorf‡ 022636006
96-well GeneAmp® PCR System 9700 (thermal cycler)	• Applied Biosystems N8050200 (Base) • Applied Biosystems 4314443 (Block)‡
NanoDrop® ND-1000 Spectrophotometer (computer required)	Thermo Scientific ND-1000
6-Tube Magnetic Stand	Applied Biosystems AM10055
E-Gel® iBase™ and E-Gel® Safe Imager™ Combo Kit	Invitrogen G6465
Vortexer	MLS
Picofuge	MLS
Pipettors, 2 µL	MLS

Item‡	Source
Pipettors, 20 µL	MLS
Pipettors, 200 µL	MLS
Pipettors, 1000 µL	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ Or equivalent but validation of the equipment for library preparation is required.

Optional equipment

Item‡	Source
2100 Bioanalyzer	• Agilent Technologies G2938C
Qubit™ Quantitation Starter Kit	Invitrogen Q32860

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Required consumables

Item‡	Source
1× Low TE Buffer	Applied Biosystems 4389764
Nuclease-free Water, 1 L	Applied Biosystems AM9932
Covaris microTubes	Covaris 520045
Isopropyl alcohol	Sigma-Aldrich I9516
Ethylene glycol	American Bioanalytical AB00455-01000
50-bp ladder	Invitrogen 10416-014
0.5-mL LoBind Tubes	Eppendorf 022431005
1.5-mL LoBind Tubes	Eppendorf 022431021
CF-1 Calibration Fluid Kit	Thermo Scientific CF-1
PR-1 Conditioning Kit§	Thermo Scientific PR-1

Item‡	Source
Filtered pipettor tips	MLS#
PCR strip tubes	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

§ The NanoDrop® Conditioning Kit is useful for "reconditioning" the sample measurement pedestals to a hydrophobic state if they become "unconditioned" (see NanoDrop user's manual for more information). The PR-1 kit consists of a container of specially formulated polishing compound and a supply of convenient applicators.

For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Optional consumables

Item‡	Source
Agilent DNA 1000 Kit§	Agilent Technologies 5067-1504

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

§ For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Prepare an express fragment library

Required Applied Biosystems reagent kits

Item (Part number)‡	Components	Kit components used in…
SOLiD™ Fragment Library Oligos Kit (4401151)	SOLiD™ Library Oligos Kit 1 – P1 Adaptor (ds)	Ligation of adaptors
	SOLiD™ Library Oligos Kit 1 – P2 Adaptor (ds)	
	SOLiD™ Library Oligos Kit 1 – Library PCR Primer 1	Library amplification
	SOLiD™ Library Oligos Kit 1 – Library PCR Primer 2	

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Required Invitrogen reagent kits

Item (part number)‡	Components	Kit components used in…
SOLiD™ Fragment Library Construction Kit (S3100102)§	SOLiD™ Fragment Library Enzyme/Core Kit: • 5X End Polishing Buffer • dNTP, 10 mM • End Polishing Enzyme 1 • End Polishing Enzyme 2	DNA end repair
	• – 5X Ligase Buffer • – T4 DNA Ligase	Ligation of P1 and P2 Adaptors
	Platinum® PCR Amplification Mix	Nick translation/library amplification
	PureLink™ PCR Purification Kit	DNA end repair, ligation of P1 and P2 Adaptors, and nick translation/library amplification

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

§ For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Optional Invitrogen reagent kits

Item (Invitrogen part number)‡§	Components	Kit components used in…
SOLiD™ Fragment Library Construction Kit Reagents (S3100105)	SOLiD™ Fragment Library Enzyme/Core Kit: • 5X End Polishing Buffer • dNTP, 10 mM • End Polishing Enzyme 1 • End Polishing Enzyme 2	DNA end repair
	• 5X Ligase Buffer • T4 DNA Ligase	Ligation of P1 and P2 Adaptors
	Platinum® PCR Amplification Mix	Nick translation/library amplification

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ Invitrogen products can be ordered at **www.invitrogen.com**.

Required equipment

Item‡	Source
Covaris™ S2 System (110 V for U.S. customers) (220 V for international customers) The system includes: • Covaris™ S2 sonicator • Latitude™ laptop from Dell® Inc. • MultiTemp III Thermostatic Circulator • Covaris-2 series Machine Holder for (one) 1.5-mL microcentrifuge tube • Covaris-2 series Machine Holder for (one) 0.65-mL microcentrifuge tube • Covaris-2 series Machine Holder for (one) 13 mm × 65 mm tube For system materials summary, see "Covaris™ S2 System Materials Summary," *SOLiD™ 3 System Site Preparation Guide*.	• Applied Biosystems 4387833 (110 V) • Applied Biosystems 4392718 (220 V) or Covaris™ Inc.
Covaris™ microTUBE loading and unloading station	Covaris 500142
Covaris™-2 series Machine Holder for (one) microTube	Covaris 500114
Covaris™ S2 System Pump Kit, with water fill level label	Covaris 500165
Microcentrifuge 5417R, refrigerated, without rotor	• Eppendorf§ 022621807 (120 V/60 Hz) • Eppendorf‡ 022621840 (230 V/50 Hz)

Item‡	Source
FA-45-24-11, fixed-angle rotor, 24 × 1.5/2 mL, including aluminum lid, aerosol-tight	Eppendorf‡ 022636006
96-well GeneAmp® PCR System 9700 (thermal cycler)	• Applied Biosystems N8050200 (Base) • Applied Biosystems 4314443 (Block)‡
NanoDrop® ND-1000 Spectrophotometer (computer required)	Thermo Scientific ND-1000
6-Tube Magnetic Stand	Applied Biosystems AM10055
E-Gel® iBase™ and E-Gel® Safe Imager™ Combo Kit	Invitrogen 6465
Vortexer	MLS
Picofuge	MLS
Pipettors, 2 µL	MLS
Pipettors, 20 µL	MLS
Pipettors, 200 µL	MLS
Pipettors, 1000 µL	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ Or equivalent but validation of the equipment for library preparation is required.

Required consumables

Item‡	Source
1X Low TE Buffer	Applied Biosystems 4389764
Nuclease-free Water, 1 L	Applied Biosystems AM9932
Covaris™ microTUBEs	Covaris™ 520045
Isopropyl alcohol	Sigma-Aldrich I9516
Ethylene glycol	American Bioanalytical AB00455-01000
0.5-mL LoBind Tubes	Eppendorf 022431005
1.5-mL LoBind Tubes	Eppendorf 022431021
CF-1 Calibration Fluid Kit	Thermo Scientific CF-1

Item‡	Source
PR-1 Conditioning Kit§	Thermo Scientific PR-1
Filtered pipettor tips	MLS#
PCR strip tubes	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

§ The NanoDrop® Conditioning Kit is useful for "reconditioning" the sample measurement pedestals to a hydrophobic state if they become "unconditioned" (see NanoDrop user's manual for more information). The PR-1 kit consists of a container of specially formulated polishing compound and a supply of convenient applicators.

For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Prepare a 2 × 50 bp mate-paired library

Required Applied Biosystems reagent kits

Item (Part number)‡	Components	Kit components used in…
SOLiD™ Mate-Paired Library Oligos Plus Kit (4425772)	SOLiD™ Library Oligos Kit 1 – P1 Adaptor (ds)	Ligation of adaptors
	SOLiD™ Library Oligos Kit 1 – P2 Adaptor (ds)	
	SOLiD™ Library Oligos Kit 1 – Library PCR Primer 1	Library amplification
	SOLiD™ Library Oligos Kit 1– Library PCR Primer 2	
	SOLiD™ Library Oligos Kit 2 – LMP CAP Adaptor (ds)	Ligation of LMP CAP Adaptors to DNA
	SOLiD™ Library Oligos Kit 2 – Internal Adaptor (ds)	DNA circularization
	SOLiD™ Library Oligos Kit 2 – EcoP15I CAP Adaptor (ds)	2 × 25 bp Mate-Paired Library Preparation
	SOLiD™ Library PCR Master Mix	Library amplification
SOLiD™ Buffer Kit (4387918)	1X Bead Wash Buffer (4389758)	Binding of DNA to streptavidin beads
	1X Bind & Wash Buffer (4389760)	
	1X Low Salt Binding Buffer (4389762)	Templated bead preparation (see Applied Biosystems SOLiD™ 3 Templated Bead Preparation Guide)
	1X Low TE Buffer (4389764)	
	1X TEX Buffer (4389766)	
	2-Butanol§ (4389770)	

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ The tube is labeled as “butanol” in the kit.

Optional Applied Biosystems reagent kits

Item (Part number)‡	Components	Kit components used in…
SOLiD™ Mate-Paired Library Oligos Kit (4400468)	SOLiD™ Library Oligos Kit 1 – P1 Adaptor (ds)	Ligation of adaptors
	SOLiD™ Library Oligos Kit 1 – P2 Adaptor (ds)	
	SOLiD™ Library Oligos Kit 1 – Library PCR Primer 1	Library amplification
	SOLiD™ Library Oligos Kit 1– Library PCR Primer 2	
	SOLiD™ Library Oligos Kit 2 – LMP CAP Adaptor (ds)	Ligation of LMP CAP Adaptors to DNA
	SOLiD™ Library Oligos Kit 2 – Internal Adaptor (ds)	DNA circularization

Item (Part number)‡	Components	Kit components used in…
	SOLiD™ Library Oligos Kit 2 – EcoP15I CAP Adaptor (ds)	2 × 50 bp Mate-Paired Library Preparation

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Required equipment

Product name‡	Vendor
HydroShear® from Genomic Solutions®§	• Applied Biosystems 4392889 (115 V) • Applied Biosystems 4392890 (230 V)
Covaris™ S2 System (110 V for U.S. customers) (220 V for international customers) The system includes: • Covaris™ S2 sonicator • Latitude™ laptop from Dell® Inc. • MultiTemp III Thermostatic Circulator • Covaris-2 series Machine Holder for (one) 1.5-mL microcentrifuge tube • Covaris-2 series Machine Holder for (one) 0.65-mL microcentrifuge tube • Covaris-2 series Machine Holder for (one) 13 mm × 65 mm tube For system materials summary, see "Covaris™ S2 System Materials Summary," *SOLiD™ 3 System Site Preparation Guide*.	• Applied Biosystems 4387833 (110 V) • Applied Biosystems 4392718 (220 V) or Covaris™ Inc.
Microcentrifuge 5417R, refrigerated, without rotor	• Eppendorf# 022621807 (120 V/60 Hz) • Eppendorf§ 022621840 (230 V/50 Hz)
FA-45-24-11, fixed-angle rotor, 24 × 1.5/2 mL, including aluminum lid, aerosol-tight	Eppendorf§ 022636006
96-well GeneAmp® PCR System 9700 (thermal cycler)	• Applied Biosystems N8050200 (Base) • Applied Biosystems 4314443 (Block)‡
NanoDrop® ND-1000 Spectrophotometer (computer required)	Thermo Scientific ND-1000
Labquake Rotisserie Rotator, Barnstead/Thermolyne	VWR 56264-312
6-Tube Magnetic Stand	Applied Biosystems AM10055

Product name‡	Vendor
0.8% E-Gel Starter Pack	Invitrogen™ Corporation G5018-08
FlashGel® DNA starter pack	Lonza 57026
Gel Imaging System	MLS‡‡
Tabletop Centrifuge	MLS
Gel boxes and power supplies for agarose gels	MLS
Vortexer	MLS
Picofuge	MLS
Incubator, 16 °C	MLS
Incubator, 37 °C	MLS
Scale	MLS
Water bath	MLS
Thermometer	MLS
Pipettors, 2 µL	MLS
Pipettors, 20 µL	MLS
Pipettors, 200 µL	MLS
Pipettors, 1000 µL	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ For more information on HydroShear® materials, refer to the manufacturer's documentation.
# Or equivalent but validation of the equipment for library preparation is required.
‡‡ For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Optional equipment

Product name‡	Vendor
2100 Bioanalyzer	Agilent Technologies G2938C

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Required consumables

Product name‡	Vendor
GeneAmp® dNTP Blend, 100 mM	Applied Biosystems N8080261
3 M Sodium acetate, pH 5.5	Applied Biosystems AM9740
• 10× TAE, 1 L *and/or* • 10× TBE, 1 L	• Applied Biosystems AM9869 • Applied Biosystems AM9863

Product name‡	Vendor
Agarose-LE§	Applied Biosystems AM9040
Gel Loading Solution, All-purpose	Applied Biosystems AM8556
Nuclease-free Water, 1 L	Applied Biosystems AM9932
1 M Magnesium chloride	Applied Biosystems AM9530G
5 M Sodium chloride, 100 mL	Applied Biosystems AM9760G
500 mM EDTA	Applied Biosystems AM9261
Covaris Tubes and Caps, 125	Applied Biosystems 4399054
10× NEBuffer 2	New England BioLabs® Inc. B7002S
100× BSA	New England BioLabs® Inc. B9001S
T7 exonuclease	New England Biolabs® M0263L
DNA Polymerase I, *E. coli*, 10 U/µL	New England BioLabs® Inc. M0209L
Quick Ligation™ Kit	New England BioLabs® Inc. M2200L
Isopropyl alcohol	Sigma-Aldrich I9516
Glycerol, 99% pure	Sigma-Aldrich G5516-1L
Ethylene glycol	American Bioanalytical AB00455-01000
Ethidium bromide	American Bioanalytical AB00512-00010
End-It™ DNA End-Repair Kit	Epicentre® ER0720
Plasmid-Safe™ ATP-Dependent DNase	Epicentre® E3110K
TrackIt™ 25 bp Ladder	Invitrogen™ Corporation 10488-022
1 kb Ladder	Invitrogen™ Corporation 15615-016

Product name‡	Vendor
Dynabeads® MyOne™ Streptavidin C1	Invitrogen™ Corporation 650-02
0.8% E-Gel	Invitrogen™ Corporation G6000-08
S1 Nuclease	Invitrogen™ Corporation 18001-016
Cloned Pfu polymerase, 2.5 U/µL	Stratagene 600153
QIAquick® Gel Extraction Kit	Qiagen 28706
FlashGel® DNA Cassette, 2.2%, 12 + 1 wells, single tier, 9 pk	Lonza 57031
FlashGel® DNA Marker • 50 bp to 1.5 kb, 500 µL *or* • 100 bp to 4 kb, 500 µL	• Lonza 57033 • Lonza 50473
FlashGel® Loading Dye, 5 × 1 mL	Lonza 50462
0.5-mL LoBind Tubes	Eppendorf 022431005
1.5-mL LoBind Tubes	Eppendorf 022431021
2.0-mL LoBind Tubes	Eppendorf 022431048
Hydrochloric Acid, 0.20 N	VWR VW8888-0
Sodium Hydroxide, 0.20 N	VWR VW8889-0
CF-1 Calibration Fluid Kit	Thermo Scientific CF-1
PR-1 Conditioning Kit#	Thermo Scientific PR-1

Product name‡	Vendor
500 mM Tris-HCl, pH 7.5	MLS‡‡
Filtered pipettor tips	MLS
Razor blades	MLS
PCR strip tubes	MLS
15-mL conical polypropylene tubes	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

§ For library gel purification, Reliant® Precast 4% NuSieve® 3:1 Plus Agarose Gel (Lonza, 54927) or 3% Mini ReadyAgarose Gel (Bio-Rad, 161-3018) may be substituted. For size selection using 1% agarose gels, 1% Mini ReadyAgarose Gel (Bio-Rad, 161-3016) may be substituted.

The NanoDrop® Conditioning Kit is useful for "reconditioning" the sample measurement pedestals to a hydrophobic state if they become "unconditioned" (see NanoDrop user's manual for more information). The PR-1 kit consists of a container of specially formulated polishing compound and a supply of convenient applicators.

‡‡ For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Optional consumables

Product name‡	Vendor
Agilent DNA 1000 Kit	Agilent Technologies 5067-1504
FlashGel® QuantLadder, 250 µL	Lonza 50475

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Prepare a 2 × 25 bp mate-paired library

Required Applied Biosystems reagent kits

Item (Part number)‡	Components	Kit components used in…
SOLiD™ Mate-Paired Library Plus Oligos Kit (4425772)	SOLiD™ Library Oligos Kit 1 – P1 Adaptor (ds)	Ligation of adaptors
	SOLiD™ Library Oligos Kit 1 – P2 Adaptor (ds)	
	SOLiD™ Library Oligos Kit 1 – Library PCR Primer 1	Library amplification
	SOLiD™ Library Oligos Kit 1– Library PCR Primer 2	
	SOLiD™ Library Oligos Kit 2 – EcoP15I CAP Adaptor (ds)	Ligation of EcoP15I CAP Adaptors to DNA
	SOLiD™ Library Oligos Kit 2 – Internal Adaptor (ds)	DNA circularization
	SOLiD™ Library Oligos Kit 2 – LMP CAP Adaptor (ds)	2 × 50 bp Mate-Paired Library Preparation
	SOLiD™ Library PCR Master Mix	Library amplification
SOLiD™ Buffer Kit (4387918)	1X Bead Wash Buffer (4389758)	Binding of DNA to streptavidin beads
	1X Bind & Wash Buffer (4389760)	
	1X Low Salt Binding Buffer (4389762)	Templated bead preparation (see Applied Biosystems SOLiD™ 3 Templated Bead Preparation Guide)
	1X Low TE Buffer (4389764)	
	1X TEX Buffer (4389766)	
	2-Butanol§ (4389770)	

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

§ The tube is labeled as “butanol” in the kit.

Optional Applied Biosystems reagent kits

Item (Part number)‡	Components	Kit components used in…
SOLiD™ Mate-Paired Library Oligos Kit (4400468)	SOLiD™ Library Oligos Kit 1 – P1 Adaptor (ds)	Ligation of adaptors
	SOLiD™ Library Oligos Kit 1 – P2 Adaptor (ds)	
	SOLiD™ Library Oligos Kit 1 – Library PCR Primer 1	Library amplification
	SOLiD™ Library Oligos Kit 1– Library PCR Primer 2	
	SOLiD™ Library Oligos Kit 2 – EcoP15I CAP Adaptor (ds)	Ligation of EcoP15I CAP Adaptors to DNA
	SOLiD™ Library Oligos Kit 2 – Internal Adaptor (ds)	DNA circularization
	SOLiD™ Library Oligos Kit 2 – LMP CAP Adaptor (ds)	2 × 25 bp Mate-Paired Library Preparation

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Required equipment

Product Name‡	Vendor
HydroShear® from Genomic Solutions®§	• Applied Biosystems 4392889 (115 V) • Applied Biosystems 4392890 (230 V)
Covaris™ S2 System (110 V for U.S. customers) (220 V for international customers) The system includes: • Covaris™ S2 sonicator • Latitude™ laptop from Dell® Inc. • MultiTemp III Thermostatic Circulator • Covaris-2 series Machine Holder for (one) 1.5-mL microcentrifuge tube • Covaris-2 series Machine Holder for (one) 0.65-mL microcentrifuge tube • Covaris-2 series Machine Holder for (one) 13 mm × 65 mm tube For system materials summary, see "Covaris™ S2 System Materials Summary," *SOLiD™ 3 System Site Preparation Guide*.	• Applied Biosystems 4387833 (110 V) • Applied Biosystems 4392718 (220 V) or Covaris™ Inc.
Microcentrifuge 5417R, refrigerated, without rotor	• Eppendorf# 022621807 (120 V/60 Hz) • Eppendorf§ 022621840 (230 V/50 Hz)
FA-45-24-11, fixed-angle rotor, 24 × 1.5/2 mL, including aluminum lid, aerosol-tight	Eppendorf§ 022636006

Product Name‡	Vendor
96-well GeneAmp® PCR System 9700 (thermal cycler)	• Applied Biosystems N8050200 (Base) • Applied Biosystems 4314443 (Block)‡
NanoDrop® ND-1000 Spectrophotometer (computer required)	Thermo Scientific ND-1000
2100 Bioanalyzer Agilent DNA 1000 Kit	• Agilent Technologies G2938C • Agilent Technologies 5067-1504
Labquake Rotisserie Rotator, Barnstead/Thermolyne	VWR 56264-312
6-Tube Magnetic Stand	Applied Biosystems AM10055
0.8% E-Gel Starter Pak	Invitrogen™ Corporation G5018-08
FlashGel® DNA starter pack	Lonza 57026
Gel Imaging System	MLS‡‡
Tabletop Centrifuge	MLS
Gel boxes and power supplies for agarose gels	MLS
Vortexer	MLS
Picofuge	MLS
Incubator (16 °C)	MLS
Incubator (37 °C)	MLS
Incubator (65 °C)	MLS
Scale	MLS
Pipettors, 2 µL	MLS
Pipettors, 20 µL	MLS
Pipettors, 200 µL	MLS
Pipettors, 1000 µL	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ For more information on HydroShear® materials, refer to the manufacturer's documentation.
# Or equivalent but validation of the equipment for library preparation is required.
‡‡ For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Optional equipment

Product name‡	Vendor
2100 Bioanalyzer	Agilent Technologies G2938C

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Required consumables

Item ‡	Source
GeneAmp® dNTP Blend, 100 mM	Applied Biosystems N8080261
3 M Sodium acetate, pH 5.5	Applied Biosystems AM9740
• 10× TAE, 1 L *and/or* • 10× TBE, 1 L	• Applied Biosystems AM9869 • Applied Biosystems AM9863
Agarose-LE§	Applied Biosystems AM9040
Gel Loading Solution (All-purpose)	Applied Biosystems AM8556
Nuclease-free Water, 1 L	Applied Biosystems AM9932
5 M Sodium chloride, 100 mL	Applied Biosystems AM9760G
1 M Magnesium chloride	Applied Biosystems AM9530G
TE, pH 8.0	Applied Biosystems AM9858
500 mM EDTA	Applied Biosystems AM9261
Covaris Tubes and Caps, 125	Applied Biosystems 4399054
10× NEBuffer 2	New England BioLabs® Inc. B7002S
100× BSA	New England BioLabs® Inc. B9001S
DNA Polymerase I, *E. coli*, 10 U/µL	New England BioLabs® Inc. M0209L
DNA Polymerase I, Klenow large fragment, 5 U/µL	New England BioLabs® Inc. M0210-S
S-adenosylmethionine (SAM), 32 mM	New England BioLabs® Inc. B9003S

Item ‡	Source
Quick Ligation™ Kit	New England BioLabs® Inc. M2200L
EcoP15I, 10 U/µL	New England BioLabs® Inc. R0646L
Sinefungin	Sigma-Aldrich S8559
Isopropyl alcohol	Sigma-Aldrich I9516
Glycerol, 99% pure	Sigma-Aldrich G5516-1L
Ethylene glycol	American Bioanalytical AB00455-01000
Ethidium bromide	American Bioanalytical AB00512-00010
End-It™ DNA End-Repair Kit	Epicentre® ER0720
Plasmid-Safe™ ATP-Dependent DNase	Epicentre® E3110K
TrackIt™ 25 bp Ladder	Invitrogen™ Corporation 10488-022
1 kb Ladder	Invitrogen™ Corporation 15615-016
Dynabeads® MyOne Streptavidin C1	Invitrogen™ Corporation 650-02
0.8% E-Gel	Invitrogen™ Corporation G6000-08
6% PAGE gels	Invitrogen™ Corporation EC6365Box
Cloned Pfu polymerase, 2.5 U/µL	Stratagene 600153
QIAquick® Gel Extraction Kit	Qiagen 28706
FlashGel® DNA Cassette, 2.2%, 12 + 1 wells, single tier, 9 pk	Lonza 57031
FlashGel® DNA Marker 50 bp to 1.5 kb, 500 µL, or 100 bp to 4 kb, 500 µL	• Lonza 57033 • Lonza 50473
FlashGel® Loading Dye, 5 × 1 mL	Lonza 50462

Item ‡	Source
0.5-mL LoBind Tubes	Eppendorf 022431005
1.5-mL LoBind Tubes	Eppendorf 022431021
2.0-mL LoBind Tubes	Eppendorf 022431048
Hydrochloric Acid, 0.20 N	VWR VW8888-0
Sodium Hydroxide, 0.20 N	VWR VW8889-0
CF-1 Calibration Fluid Kit	Thermo Scientific CF-1
PR-1 Conditioning Kit#	Thermo Scientific PR-1
500 mM Tris-HCl, pH 7.5	MLS‡‡
Filtered pipettor tips	MLS
Razor blades	MLS
PCR strip tubes	MLS
15-mL conical polypropylene tubes	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

§ For library gel purification, Reliant® Precast 4% NuSieve® 3:1 Plus Agarose Gel (Lonza, 54927) or 3% Mini ReadyAgarose Gel (Bio-Rad, 161-3018) may be substituted. For size selection using 1% agarose gels, 1% Mini ReadyAgarose Gel (Bio-Rad, 161-3016) may be substituted.

The NanoDrop® Conditioning Kit is useful for "reconditioning" the sample measurement pedestals to a hydrophobic state if they become "unconditioned" (see NanoDrop user's manual for more information). The PR-1 kit consists of a container of specially formulated polishing compound and a supply of convenient applicators.

‡‡ For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Optional consumables

Product name‡	Vendor
Agilent DNA 1000 Kit	Agilent Technologies 5067-1504
FlashGel® QuantLadder, 250 µL	Lonza 50475

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Prepare a barcoded fragment library

Required Invitrogen reagent kits

Item (Invitrogen part number)‡§	Components	Kit components used in…
SOLiD™ Fragment Library Construction Kit with SizeSelect™ Gels (S3100101)#	SOLiD™ Fragment Library Enzyme/Core Kit: • 5X End Polishing Buffer • dNTP, 10 mM • End Polishing Enzyme 1 • End Polishing Enzyme 2	DNA end repair
	• 5X Ligase Buffer • T4 DNA Ligase	Ligation of P1 and P2 Adaptors
	Platinum® PCR Amplification Mix	Nick translation/library amplification
	PureLink™ PCR Purification Kit	DNA end repair, ligation of P1 and P2 Adaptors, and nick translation/library amplification
	E-Gel® SizeSelect™ 2%, 10 gels	Size selection

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ Invitrogen products can be ordered at **www.invitrogen.com**.
# For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Optional Invitrogen reagent kits

Item (part number)‡	Components	Kit components used in…
SOLiD™ Fragment Library Construction Kit (S3100102)§	SOLiD™ Fragment Library Enzyme/Core Kit: • 5X End Polishing Buffer • dNTP, 10 mM • End Polishing Enzyme 1 • End Polishing Enzyme 2	DNA end repair
	• 5X Ligase Buffer • T4 DNA Ligase	Ligation of P1 and P2 Adaptors
	Platinum® PCR Amplification Mix	Nick translation/library amplification
	PureLink™ PCR Purification Kit	DNA end repair, ligation of P1 and P2 Adaptors, and nick translation/library amplification

Item (part number)‡	Components	Kit components used in…
SOLiD™ Fragment Library Construction Reagents (S3100105)	SOLiD™ Fragment Library Enzyme/Core Kit: • 5× End Polishing Buffer • dNTP, 10 mM • End Polishing Enzyme 1 • End Polishing Enzyme 2	DNA end repair
	• 5× Ligase Buffer • T4 DNA Ligase	Ligation of P1 and P2 Adaptors
	– Platinum® PCR Amplification Mix	Nick translation/library amplification

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

§ For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Required oligonucleotides

Item‡	Source	Sequence
Multiplex P1 Adaptor (ds)	Any oligonucleotide vendor	See Appendix D
Multiplex P2 Adaptor – Barcode 1 (ds)	Any oligonucleotide vendor	See Appendix D
Multiplex P2 Adaptor – Barcode 2 (ds)	Any oligonucleotide vendor	See Appendix D
Multiplex P2 Adaptor – Barcode 3 (ds)	Any oligonucleotide vendor	See Appendix D
Multiplex P2 Adaptor – Barcode 4 (ds)	Any oligonucleotide vendor	See Appendix D
Multiplex P2 Adaptor – Barcode 5 (ds)	Any oligonucleotide vendor	See Appendix D
Multiplex P2 Adaptor – Barcode 6 (ds)	Any oligonucleotide vendor	See Appendix D
Multiplex P2 Adaptor – Barcode 7 (ds)	Any oligonucleotide vendor	See Appendix D
Multiplex P2 Adaptor – Barcode 8 (ds)	Any oligonucleotide vendor	See Appendix D
Multiplex P2 Adaptor – Barcode 9 (ds)	Any oligonucleotide vendor	See Appendix D
Multiplex P2 Adaptor – Barcode 10 (ds)	Any oligonucleotide vendor	See Appendix D
Multiplex P2 Adaptor – Barcode 11 (ds)	Any oligonucleotide vendor	See Appendix D
Multiplex P2 Adaptor – Barcode 12 (ds)	Any oligonucleotide vendor	See Appendix D
Multiplex P2 Adaptor – Barcode 13 (ds)	Any oligonucleotide vendor	See Appendix D
Multiplex P2 Adaptor – Barcode 14 (ds)	Any oligonucleotide vendor	See Appendix D

Item‡	Source	Sequence
Multiplex P2 Adaptor – Barcode 15 (ds)	Any oligonucleotide vendor	See Appendix D
Multiplex P2 Adaptor – Barcode 16 (ds)	Any oligonucleotide vendor	See Appendix D
Multiplex Library PCR Primer 1 (ss)	Any oligonucleotide vendor	See Appendix D
Library PCR Primer 2 (ss)	Any oligonucleotide vendor	See Appendix D

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Required equipment

Item‡	Source
Covaris™ S2 System (110 V for U.S. customers) (220 V for international customers) The system includes: • Covaris™ S2 sonicator • Latitude™ laptop from Dell® Inc. • MultiTemp III Thermostatic Circulator • Covaris-2 series Machine Holder for (one) 1.5-mL microcentrifuge tube • Covaris-2 series Machine Holder for (one) 0.65-mL microcentrifuge tube • Covaris-2 series Machine Holder for (one) 13 mm × 65 mm tube For system materials summary, see "Covaris™ S2 System Materials Summary," *SOLiD™ 3 System Site Preparation Guide*.	• Applied Biosystems 4387833 (110 V) • Applied Biosystems 4392718 (220 V) or Covaris™ Inc.
Covaris™ microTube loading and unloading station	Covaris 500142
Covaris™-2 series Machine Holder for (one) microTube	Covaris 500114
Covaris™ S2 System Pump Kit, with water fill level label	Covaris 500165
Microcentrifuge 5417R, refrigerated, without rotor	• Eppendorf§ 022621807 (120 V/60 Hz) • Eppendorf‡ 022621840 (230 V/50 Hz)
FA-45-24-11, fixed-angle rotor, 24 × 1.5/2 mL, including aluminum lid, aerosol-tight	Eppendorf‡ 022636006

Item‡	Source
96-well GeneAmp® PCR System 9700 (thermal cycler)	• Applied Biosystems N8050200 (Base) • Applied Biosystems 4314443 (Block)‡
NanoDrop® ND-1000 Spectrophotometer (computer required)	Thermo Scientific ND-1000
6-Tube Magnetic Stand	Applied Biosystems AM10055
E-Gel® iBase™ and E-Gel® Safe Imager™ Combo Kit	Invitrogen G6465
Vortexer	MLS
Picofuge	MLS
Pipettors, 2 µL	MLS
Pipettors, 20 µL	MLS
Pipettors, 200 µL	MLS
Pipettors, 1000 µL	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ Or equivalent but validation of the equipment for library preparation is required.

Optional equipment

Item‡	Source
2100 Bioanalyzer	• Agilent Technologies G2938C
Qubit™ Quantitation Starter Kit	Invitrogen Q32860

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Required consumables

Item‡	Source
1× Low TE Buffer	Applied Biosystems 4389764
Nuclease-free Water, 1 L	Applied Biosystems AM9932
Covaris microTubes	Covaris 520045
Isopropyl alcohol	Sigma-Aldrich I9516
Ethylene glycol	American Bioanalytical AB00455-01000

Item‡	Source
50-bp ladder	Invitrogen 10416-014
0.5-mL LoBind Tubes	Eppendorf 022431005
1.5-mL LoBind Tubes	Eppendorf 022431021
CF-1 Calibration Fluid Kit	Thermo Scientific CF-1
PR-1 Conditioning Kit§	Thermo Scientific PR-1
Filtered pipettor tips	MLS#
PCR strip tubes	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

§ The NanoDrop® Conditioning Kit is useful for "reconditioning" the sample measurement pedestals to a hydrophobic state if they become "unconditioned" (see NanoDrop user's manual for more information). The PR-1 kit consists of a container of specially formulated polishing compound and a supply of convenient applicators.

For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Optional consumables

Product name‡	Vendor
Agilent DNA 1000 Kit	Agilent Technologies 5067-1504

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

B SOLiD™ 3 System Library Quantitation

This appendix covers:

Overview

A key component of SOLiD™ sequencing is emulsion PCR (ePCR), which allows for monoclonal amplification of individual species of template DNA from a complex library pool. During ePCR, multiple copies of single DNA sequence are coated onto a single 1 µm magnetic bead within one water-in-oil emulsion droplet. For optimal monoclonal amplification, precise quantitation of template is critical.

Quantitative PCR (qPCR) is the preferred method of nucleic acid quantitation for a wide range of applications. qPCR offers two important advantages in template quantitation:

- **qPCR provides better precision and accuracy.** ePCR performance is correlated to the absolute number of input template. qPCR provides superior accuracy over other quantitation methods in measuring the number of amplifiable templates in the constructed libraries, thus it is a reliable alternative to library titration analysis assays for the SOLiD™ 3 System.
- **qPCR can detect extremely low quantities of DNA.** Due to the high sensitivity of qPCR, you can dilute libraries to very low concentrations for quantitation and to increase detection specificity.

This appendix covers two types of assays for absolute quantification by qPCR: SOLiD™ TaqMan® Gene Expression Assay and SYBR® Green Assay.

The TaqMan Gene Expression Assay uses a fluorogenic probe to detect specific PCR product as it accumulates during PCR cycles. The assay provides more specificity and accuracy in detecting amplifiable templates over non-probe based quantitation methods for SOLiD™ sequencing.

In qPCR with the TaqMan Gene Expression Assay, the library template is amplified by PCR in the presence of a probe labeled with FAM dye and a dye quencher. A fluorescent signal is generated by the FAM dye, which detaches from the probe as the AmpliTaq Gold® DNA polymerase extends the 3′ strand (see Figure 20 on page 157).

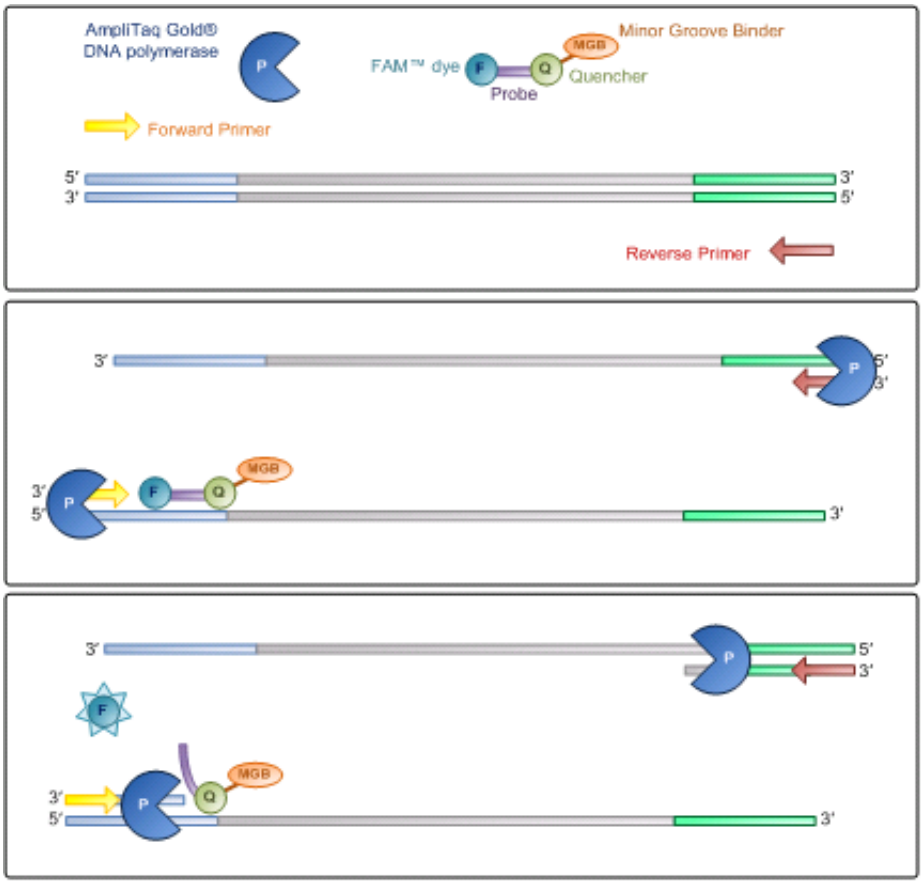


Figure 20 Scheme of TaqMan® Gene Expression Assay for SOLiD™ libraries.

The SYBR® Green Assay uses a fluorescent dye, which binds double-stranded DNA. When the DNA is denatured, the SYBR Green dye is released and the fluorescence is significantly reduced. After denaturation and release of the SYBR Green dye, primers anneal to the template, and the PCR product is generated. After polymerization is complete, the SYBR Green dye binds to the double-stranded PCR product, resulting in a net increase in fluorescence (see Figure 21 on page 158 and Figure 22 on page 159).

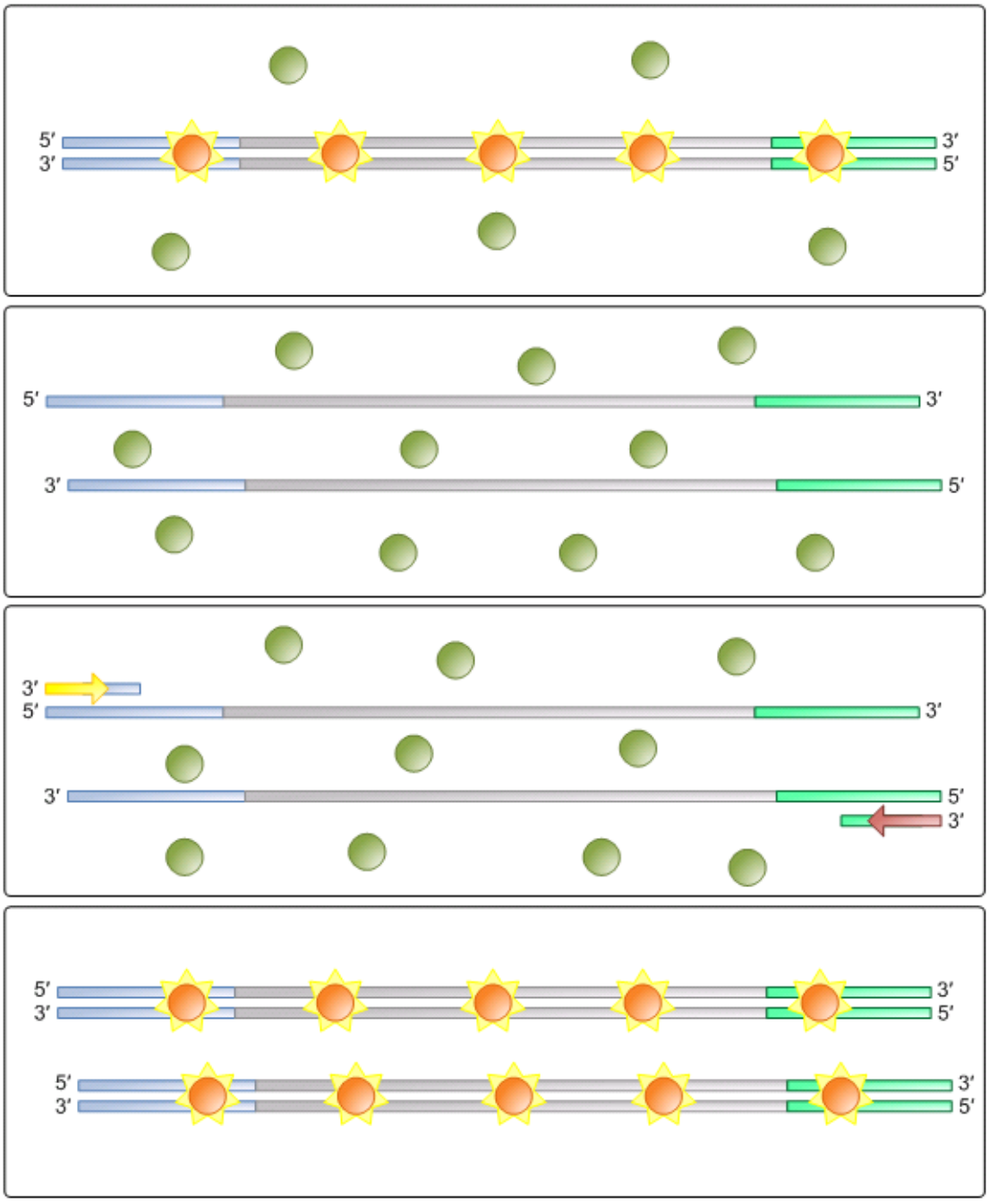


Figure 21 Scheme of SYBR® Green assay.

The SYBR Green Assay is also designed to be compatible with both fragment and mate-paired libraries. The forward and reverse primers in the SYBR Green Assay are specific to the P1 and P2 Adaptor sequences in SOLiD libraries (see Figure 22 on page 159).

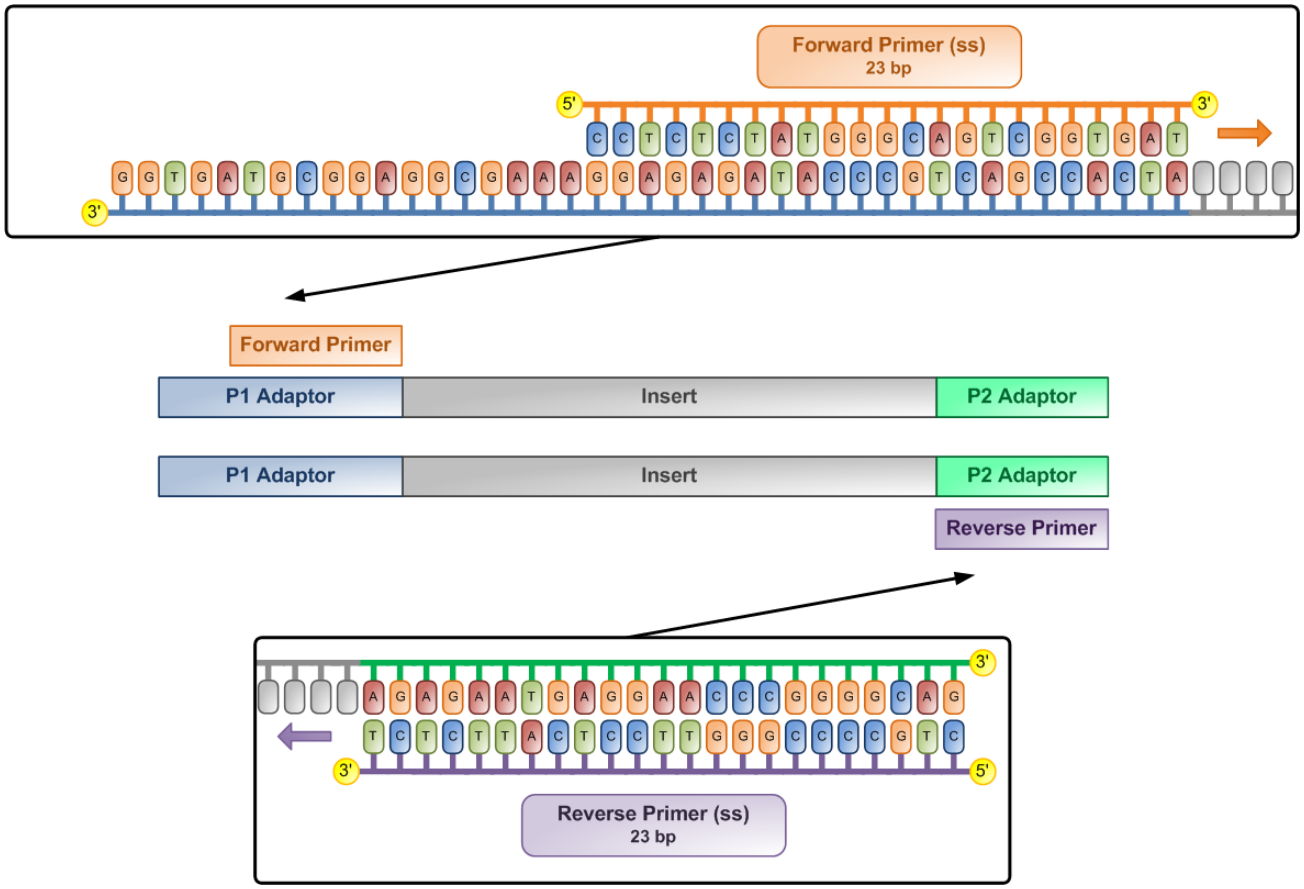


Figure 22 SYBR® Green assay primers design.

The TaqMan assay provides better specificity and greater accuracy. The SYBR Green dye binds non-specifically to double-stranded DNA, but, since no probe is required, SYBR dye costs less to use.

For accurate library quantitation, quantitative PCR is strongly recommended. But, if a real-time PCR system is not available, library quantitation can be performed using at least one of the following alternative methods. For each method, follow the manufacturer's instructions (see Table 59).

Table 59 Library quantitation by other methods other than by qPCR

Quantitation method	Sensitivity
Lonza 2.2% FlashGel® with FlashGel® QuantLadder	3 ng/µL
Invitrogen Qubit™	200 pg/µL
Agilent Bioanalyzer DNA 1000 Assay	100 pg/µL

Section B.1 The TaqMan® Gene Expression Assay

Materials and equipment required

Required Applied Biosystems reagent kits

Item (Part number)‡	Components	Kit components used in…
SOLiD™ Fragment Library Oligo Kit (4401151)	SOLiD™ Library Oligos Kit 1 – Library PCR Primer 1	Library amplification
	SOLiD™ Library Oligos Kit 1 – Library PCR Primer 2	
	SOLiD™ Library Oligos Kit 1 – P1 Adaptor (ds)	Library preparation
	SOLiD™ Library Oligos Kit 1 – P2 Adaptor (ds)	

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Required equipment

Item‡	Source
7500 Fast Real-Time PCR System with Dell™ Tower	• Applied Biosystems 4351107
96-well GeneAmp® PCR System 9700 (thermal cycler)	• Applied Biosystems N8050200 (Base) • Applied Biosystems 4314443 (Block)‡
Microcentrifuge 5417R, refrigerated, without rotor	• Eppendorf§ 022621807 (120 V/60 Hz) • Eppendorf‡ 022621840 (230 V/50 Hz)
FA-45-24-11, fixed-angle rotor, 24 × 1.5/2 mL, including aluminum lid, aerosol-tight	Eppendorf‡ 022636006
FlashGel® DNA starter pack	Lonza 57026
Gel imager system	MLS
Tabletop Centrifuge	MLS
Vortexer	MLS
Picofuge	MLS
Pipettors, 20 µL	MLS
Pipettors, 200 µL	MLS
Pipettors, 1000 µL	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ Or equivalent but validation of the equipment for library preparation is required.

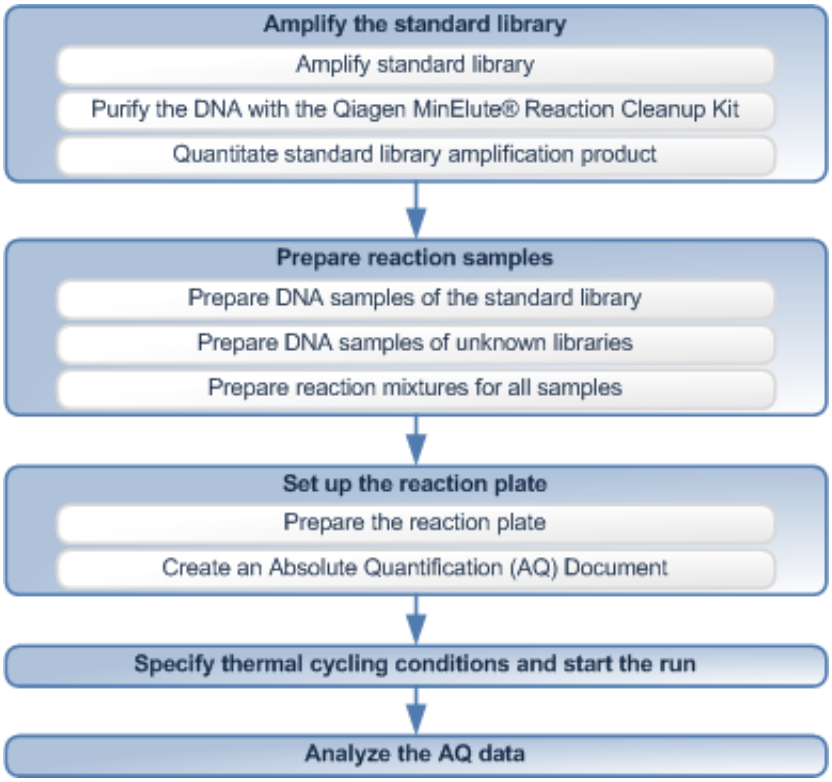
Required consumables[1]

Item‡	Source
MicroAmp® Fast Optical 96-well reaction plate with barcode, 0.1 mL	Applied Biosystems 4346906
TaqMan® Fast Universal PCR Master Mix, 2-Pack, 2 × 5 mL®	Applied Biosystems 4366072
SOLiD™ TaqMan® Gene Expression Assay, 20X, Assay ID: Ac00010015_a1	Applied Biosystems 4331182
SOLiD™ DH10B Fragment Control Library	Applied Biosystems 4392545
Nuclease-free Water, 1 L	Applied Biosystems AM9932
MicroAmp® Optical Adhesive Film	Applied Biosystems 4360954
3 M Sodium Acetate, pH 5.5	Applied Biosystems AM9740
Invitrogen Platinum® PCR SuperMix High Fidelity	Invitrogen™ Corporation 12532-016
0.5-mL LoBind Tubes	Eppendorf 022431005
1.5-mL LoBind Tubes	Eppendorf 022431021
MinElute® Reaction Cleanup Kit	Qiagen 28204
FlashGel® QuantLadder, 250 µL	Lonza 50475
FlashGel® DNA Cassette, 2.2%, 12 + 1 wells, single tier, 9 pk	Lonza 57031
FlashGel® Loading Dye, 5 × 1 mL	Lonza 50462
Filtered pipettor tips	MLS§
Ice	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

§ For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Workflow



Amplify the standard library

It is important to pick a library that amplifies well on templated beads to use as the standard for the generation of qPCR standard curves. For this qPCR standard, the SOLiD™ DH10B Fragment Library Control is recommended. In order to obtain accurate and consistent qPCR results, the standard library needs to be stored at no less than 1 ng/µL concentration at -20 °C to avoid degradation.

Prepare the reaction samples

The standard library and the unknown library are diluted in preparation of the quantitative PCR reaction. It is suggested that unknown library samples are prepared at a 50 pg/µL concentration (based on other quantitation methods) and stored at -20 °C. Prepare enough volume for PCR reactions and aliquot into LoBind tubes. Use the same diluted samples for both qPCR quantification and subsequent ePCR reactions. After the samples have been diluted, they are mixed with the qPCR reagents. The reaction volume for 7500 Fast Real-Time PCR System is 20 µL per well (for both standard and fast mode). All samples including NTC should be set up in triplicate to increase accuracy.

Set up the reaction plate

The reaction samples are transferred to a Fast Optical 96-well plate and the sample information is entered.

Specify the thermal cycling conditions and start the run

The run conditions are entered, and the qPCR run is started.

Analyze the AQ data

The TaqMan Assay measures the number of DNA molecules rather than the mass. The quantities entered for standards are in units of copies/µL. Therefore, the quantitation results of the unknown libraries are also given in units of copies/µL and need to be converted to pg/µL.

Tips

General

- Perform all steps requiring 0.5-mL and 1.5-mL tubes with Eppendorf LoBind tubes.
- Avoid multiple thaws of samples.

Amplify the standard library

Amplify the standard library

1. Combine (see Table 60):

Table 60 Prepare the PCR master mix

Component	Volume (µL)
DH10B Fragment Control Library, 5 ng/µL	1
Invitrogen Platinum® PCR SuperMix High Fidelity	100
Library PCR Primer 1, 50 µM	2
Library PCR Primer 2, 50 µM	2
Nuclease-free water	5
Total	110

2. Set up the PCR conditions on the GeneAmp® PCR System 9700:
   - PCR thermal cycling program (see Table 61):

Table 61 PCR conditions to amplify the library

Stage	Temp (°C)	Time
Hold	95	5 min
5 cycles	95	15 sec
	62	15 sec
	70	1 min
Hold	70	5 min
Hold	4	∞

   - Ramp speed: **Standard**
   - Reaction volume: **110 µL**

3. Place the 96-well plate in a GeneAmp® PCR System 9700, then start the run.

Purify the DNA with the Qiagen MinElute® Reaction Cleanup Kit

1. Add 3 volumes of Buffer ERC to the PCR product. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is
≤ 7.5.

2. Apply about 700 µL of PCR product in Buffer ERC to the column(s). The maximum amount of DNA that can be applied to a MinElute® column is 5 µg. Use more columns if necessary.

3. Let the column(s) stand for 2 minutes at room temperature.

4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the MinElute® column(s) back into the same collection tube.
6. Add 750 µL of Buffer PE to wash the column(s).
7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat this step to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 20 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. If necessary, pool the eluted DNA.

Quantitate the standard library amplification product

1. Load 5 µL of the FlashGel® QuantLadder in one well of a 2.2% Lonza FlashGel® cassette.
2. Load and run 5 µL of two different dilutions of library sample. The total amount of DNA loaded per sample must be in the 5 to 20 ng range.
3. Run the gel for 6 minutes at 275 V.
4. Use a gel imager system to accurately quantitate the DNA using the FlashGel QuantLadder as a reference. Do not use the top band (30 ng) of the FlashGel QuantLadder to generate a standard curve. The loading dye co-migrates with the top band and interferes with its quantitation.
5. Dilute the purified library to 1 ng/µL and divide the library into 5-µL aliquots in 0.5-mL LoBind Tubes.
6. Store standard library stock samples at 1 ng/µL at -20 °C.

Prepare the reaction samples

Prepare DNA samples of the standard library

1. Thaw one tube of 1 ng/µL standard library on ice. Pipette 1 µL of standard library and make a fresh dilution at 50 pg/µL by adding 19 µL of nuclease-free water. Put the rest of the stock back into the freezer.
2. Make a fresh serial dilution from the 50 pg/µL dilution for each qPCR run. Vortex and pulse-spin the dilutions. Store the samples on ice. To prepare typical standard dilutions see Table 62.

Table 62 Prepare standard dilutions of the library

	Dilutions from 50 pg/µL stock	Concentration (pg/µL)	Quantity‡ (copies/µL)	Components
Standard 1	1:50	1	5.92×10^6	1 µL of 50 pg/µL stock + 49 µL of nuclease-free water
Standard 2	1:500	0.1	5.92×10^5	5 µL of Standard 1 + 45 µL of nuclease-free water
Standard 3	1:5000	0.01	5.92×10^4	5 µL of Standard 2 + 45 µL of nuclease-free water
Standard 4	1:50000	0.001	5.92×10^3	5 µL of Standard 3 + 45 µL of nuclease-free water

‡ This value is calculated based on the size of the SOLiD™ DH10B Fragment Control Library (150 bp). When a library of a different size is used as the standard, this number needs to be changed.

Prepare DNA samples of unknown libraries

1. Make a dilution of your unknown libraries targeting 50 pg/µL based on other quantitation methods. Aliquot in small volumes for routine usage and store at – 20 °C. Avoid multiple freezes and thaws.
2. Make a 1:1000 dilution of each unknown library in a 1.5-mL LoBind tube. Include a negative control (NTC) with no template.

Prepare reaction mixtures for all samples

1. Calculate and prepare the appropriate volume of PCR master mix (see the formula and example below and Table 63 on page 168).

Total number of samples = 4 standard + 1 NTC + # unknown samples

X = (Total number of samples + 1) × 3.5

Example:

For 4 unknown libraries to be quantified

X = (4 + 1 + 4 + 1) × 3.5 = 35

Table 63 Calculate the volume of PCR master mix

Components	Typical reaction volumes (µL)	PCR Master Mix (µL)	Example PCR Master Mix (µL)
TaqMan® Fast Universal PCR Master Mix	10	*X* × 10	350
SOLiD™ TaqMan® Gene Expression Assay 20X, Assay ID: Ac00010015_a1	1	*X* × 1	35
Total	11	*X* × 11	385

2. Label a 1.5-mL LoBind tube for each standard or unknown library and add 31.5 µL of sample. Add 31.5 µL of nuclease-free water to the NTC tube.

3. In each tube, add 38.5 µL of PCR Master Mix. Mix well, then leave the tube(s) on ice.

Set up the reaction plate

Prepare the reaction plate

IMPORTANT! Ensure that you use the MicroAmp® Fast Optical 96-well reaction plate with barcode, 0.1 mL for the 7500 Fast Real-Time PCR system.

1. Make a template for the 96-well plate indicating the position for each library sample (see Figure 23).

Figure 23 Sample 96-well template.

2. Aliquot 20 µL of each mixed sample into the appropriate wells according to the template.

3. Seal the plate with optical adhesive film.

4. Keep the plate on ice until you are ready to load it onto the instrument. Spin the plate for 30 seconds at 100 × g before loading.

Create an Absolute Quantification (AQ) Plate document

Refer to chapter 2 in the *Applied Biosystems 7500/7500 Fast Real-Time PCR System Standard Curve Experiments Getting Started Guide* (PN 4387779).

Enter the quantities for 4 standards as 5.92×10^6, 5.92×10^5, 5.92×10^4, and 5.92×10^3. The quantity for the unknown library is calculated based on these numbers in the units of copies/µL.

Specify thermal cycling conditions, then start the run

Refer to chapter 2 in the *Applied Biosystems 7500/7500 Fast Real-Time PCR System Standard Curve Experiments Getting Started Guide* (PN 4387779).

1. Select either the **Graphical View** tab or **Tabular View** tab.
2. Enter **20** µL in the **Reaction Volume per Well** field.
3. Add **Stage** as needed.
   See Table 64 and Table 65 for fast and standard mode run cycles, respectively.

Table 64 Typical run conditions for *fast* mode

Stage	Temp (°C)	Time
1 cycle	95	20 sec
40 cycles	95	3 sec
	60	30 sec

Table 65 Typical run conditions for *standard* mode

Stage	Temp (°C)	Time
1 cycle	95	10 min
40 cycles	95	15 sec
	60	60 sec

4. Click **Save** to save the created plate document.
5. Load the reaction plate into the instrument.
6. Click **Start Run**. The running time is 35 minutes for fast mode and 1.5 hours for standard mode.

Analyze the AQ data

1. After the qPCR run is finished, click **Analysis**. Select the wells in **View Plate Layout** by *click+drag*. Click **Amplification Plot** or **Standard Curve** to view the analysis results (for examples, see Figure 24 and Figure 25 on page 172).

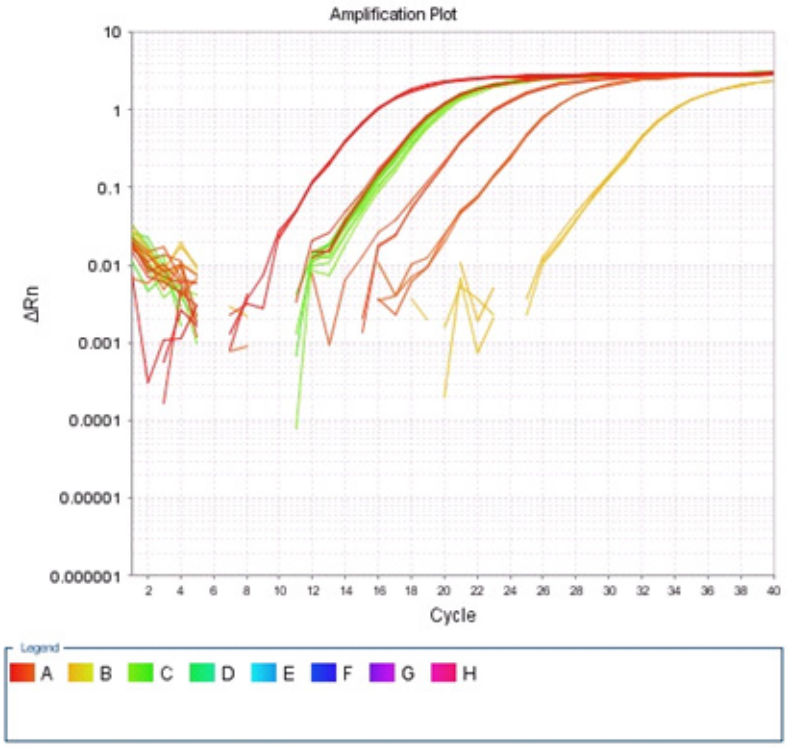


Figure 24 Sample amplification plot.

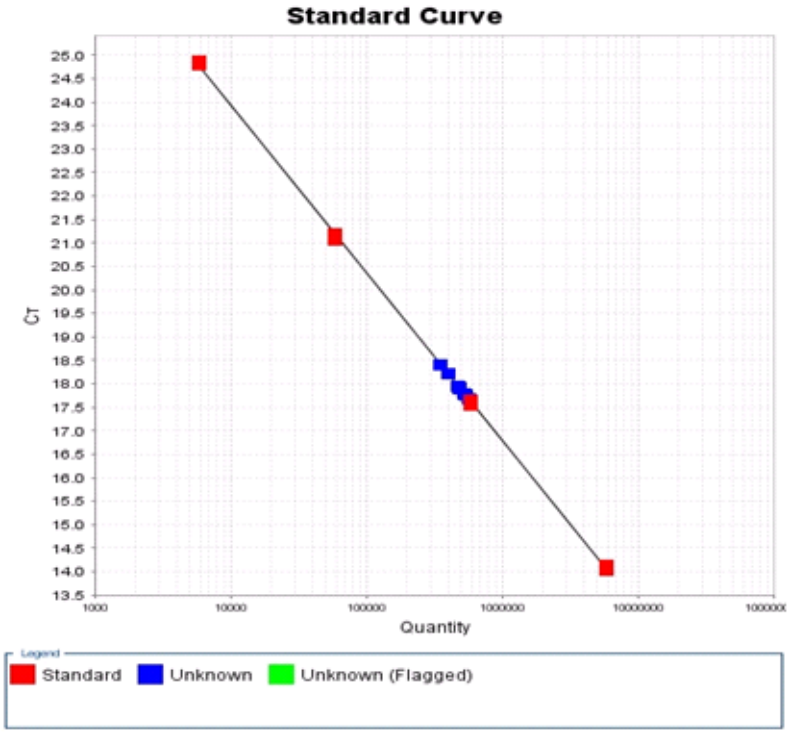


Figure 25 Sample standard curve.

2. Calculate the stock concentration of unknown libraries by entering the *Mean Qty* in the following equation:

$$\text{Library Concentration (pg/µL)} = \frac{\text{Mean Qty (copies/µL)} \times \text{fold dilution}}{6.022 \times 10^{23}\ \text{copies/mol}} \times \text{Size} \times \frac{660\ \text{pg}}{1\ \text{pmol}} \times \frac{10^{12}\ \text{pmol}}{1\ \text{mol}}$$

Example:

For a fragment library (assuming average size is 175 bp) with Mean Qty = 4.5×10^5 copies/µL

$$\text{Library Concentration (pg/µL)} = \frac{4.5 \times 10^5\ \text{copies/µL} \times 1000}{6.022 \times 10^{23}\ \text{copies/mol}} \times 175 \times \frac{660\ \text{pg}}{1\ \text{pmol}} \times \frac{10^{12}\ \text{pmol}}{1\ \text{mol}}$$

$$= 86.3\ \text{pg/µL}$$

Note: To determine the library concentration to be used for emulsion PCR, refer to Appendix B of the *Applied Biosystems SOLiD™ 3 System Templated Bead Preparation Guide* (PN 4407421).

Section B.2 The SYBR® Green Assay

Materials and equipment required

Required Applied Biosystems reagent kits

Item (Part number)‡	Components	Kit components used in…
SOLiD™ Fragment Library Oligos Kit (4401151)	SOLiD™ Library Oligos Kit 1 – Library PCR Primer 1	Library amplification
	SOLiD™ Library Oligos Kit 1 – Library PCR Primer 2	
	SOLiD™ Library Oligos Kit 1 – P1 Adaptor (ds)	Library preparation
	SOLiD™ Library Oligos Kit 1 – P2 Adaptor (ds)	

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Required equipment

Item‡	Source
Real-Time PCR System: • StepOne™ Real-Time PCR System • StepOnePlus™ Real-Time PCR System	Applied Biosystems • 4376357 • 4379216 (Upgrade only)
96-well GeneAmp® PCR System 9700 (thermal cycler)	Applied Biosystems • N8050200 (Base) • 4314443 (Block)
Microcentrifuge 5417R, refrigerated, without rotor	• Eppendorf§ 022621807 (120 V/60 Hz) • Eppendorf§ 022621840 (230 V/50 Hz)
FA-45-24-11, fixed-angle rotor, 24 × 1.5/2 mL, including aluminum lid, aerosol-tight	Eppendorf§ 022636006
FlashGel® DNA starter pack	Lonza 57026
Gel imager system	MLS
Picofuge	MLS
Vortexer	MLS
Picofuge	MLS
Pipettors, 20 µL	MLS
Pipettors, 200 µL	MLS
Pipettors, 1000 µL	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ Or equivalent but validation of the equipment for library preparation is required.

Required consumables

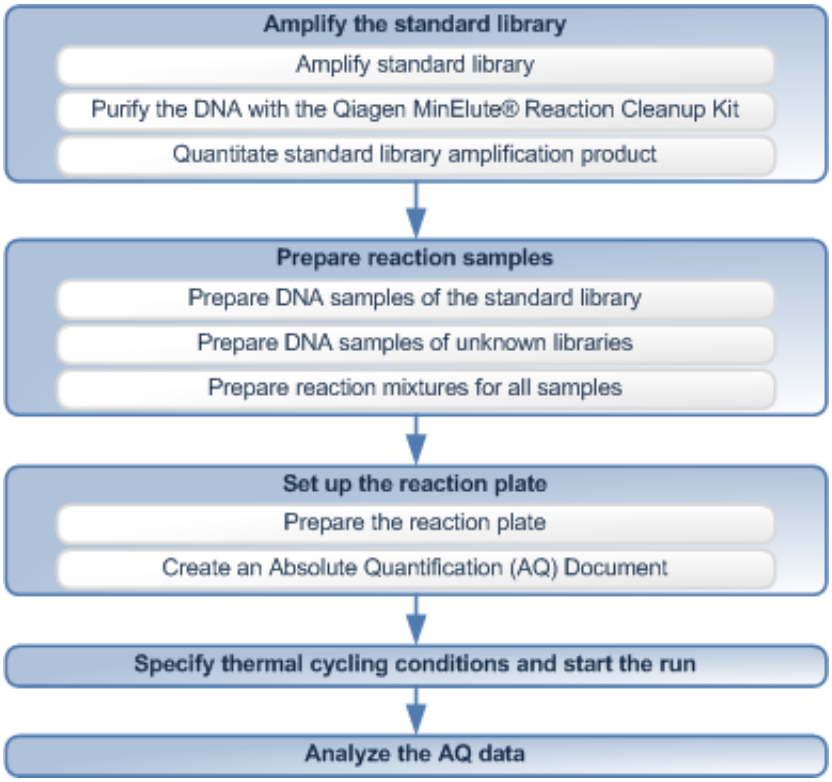
Item‡	Source
MicroAmp® Fast Optical 96-well reaction plate with barcode, 0.1 mL	Applied Biosystems 4346906
Fast SYBR®Green Master Mix, 1-pack, 1 × 5 mL	Applied Biosystems 4385612
SOLiD™ DH10B Fragment Control Library	Applied Biosystems 4392545
MicroAmp® Optical Adhesive Film	Applied Biosystems 4360954
3 M Sodium acetate, pH 5.5	Applied Biosystems AM9740
Invitrogen™ Platinum® PCR SuperMix High Fidelity	Invitrogen™Corporation 12532-016
MinElute® Reaction Cleanup Kit	Qiagen 28204
FlashGel® QuantLadder, 250 µL	Lonza 50475
FlashGel® DNA Cassette, 2.2%, 12 + 1 wells, single tier, 9 pk	Lonza 57031
FlashGel® Loading Dye, 5 × 1 mL	Applied Biosystems 4392543
Nuclease-free Water, 1 L	Applied Biosystems AM9932
0.5-mL LoBind Tubes	Eppendorf 022431005
1.5-mL LoBind Tubes	Eppendorf 022431021
PCR strip tubes	MLS
Filtered pipettor tips	MLS
Ice	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Required oligonucleotides

Sequence name	Oligonucleotide sequence	Sequence length (bp)
Forward Primer	5′ – CCT CTC TAT GGG CAG TCG GTG AT – 3′	23
Reverse Primer	5′ – CTG CCC CGG GTT CCT CAT TCT CT – 3′	23

Workflow



Amplify the standard library

It is important to pick a library that amplifies well on templated beads to use as the standard for the generation of qPCR standard curves. For this qPCR standard, the SOLiD™ DH10B Fragment Library Control is recommended. In order to obtain accurate and consistent qPCR results, the standard library needs to be stored at no less than 1 ng/µL concentration at -20 °C to avoid degradation.

Prepare reaction samples

You should make fresh dilutions of the stock for each qPCR run. The standard library and the unknown library are diluted in preparation of the quantitative PCR reaction. Applied Biosystems suggests that unknown library samples are prepared at a 50 pg/µL concentration (based on other quantitation methods) and stored at – 20 °C. Prepare enough volume for ePCR reactions and aliquot into LoBind tubes. Use the same diluted samples for both qPCR quantification and subsequent ePCR reactions. After the samples have been diluted, they are mixed with the qPCR reagents. All samples including the non-template control should be set up in triplicate to increase accuracy.

Set up the reaction plate

The reaction samples are transferred to an Optical 96-well plate and the sample information is entered.

Specify the thermal cycling conditions and start the run

The run conditions are entered, then the qPCR run is started.

Analyze AQ data The SYBR Green Assay measures the number of DNA molecules rather than the mass. The quantities entered for standards are in units of copies/µL. Therefore, the quantitation results of the unknown libraries are also given in unites of copies/µL and need to be converted to pg/µL.

Tips

General

- Perform all steps requiring 0.5-mL and 1.5-mL tubes with Eppendorf LoBind tubes.
- Avoid multiple thaws of samples.

Amplify the standard library

Amplify the standard library

1. Combine (see Table 66):

Table 66 Prepare the PCR master mix

Component	Volume (µL)
DH10B Fragment Control Library, 5 ng/µL	1
Invitrogen Platinum® PCR SuperMix High Fidelity	100
Library PCR Primer 1, 50 µM	2
Library PCR Primer 2, 50 µM	2
Nuclease-free water	5
Total	110

2. Set up the PCR conditions on the GeneAmp® PCR System 9700:
   - PCR thermal cycling program:

Stage	Temp (°C)	Time
Hold	95	5 min
5 cycles	95	15 sec
	62	15 sec
	70	1 min
Hold	70	5 min
Hold	4	∞

   - Ramp speed: **Standard**
   - Reaction volume: **110 µL**

3. Place the 96-well plate in a GeneAmp® PCR System 9700, then start the run.

Purify the DNA with the Qiagen MinElute® Reaction Cleanup Kit

1. Add 3 volumes of Buffer ERC to the PCR product. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.

2. Apply about 700 µL of PCR product in Buffer ERC to the column(s). The maximum amount of DNA that can be applied to a MinElute® column is 5 µg. Use more columns if necessary.

3. Let the column(s) stand for 2 minutes at room temperature.

4. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.

5. Repeat steps 2 and 4 until the entire sample has been loaded onto the column(s). Place the MinElute® column(s) back into the same collection tube(s).
6. Add 750 µL of Buffer PE to wash the column(s).
7. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.
8. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
9. Add 20 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.
10. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.
11. If needed, pool the eluted DNA.

Quantitate the standard library amplification product

1. Load 5 µL of the FlashGel® QuantLadder in one well of a 2.2% Lonza FlashGel® cassette.
2. Load and run 5 µL of two different dilutions of library sample. The total amount of DNA loaded per sample must be in the 5 to 20 ng range.
3. Run the gel for 6 minutes at 275 V.
4. Use a gel imager system to accurately quantitate the DNA using the FlashGel QuantLadder as a reference. Do not use the top band (30 ng) of the FlashGel QuantLadder to generate a standard curve. The loading dye co-migrates with the top band and interferes with its quantitation.
5. Dilute the purified library to 1 ng/µL and divide the library into 5-µL aliquots in 0.5-mL LoBind Tubes.
6. Store standard library stock samples at 1 ng/µL at -20 °C.

Prepare reaction samples

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

CAUTION! CHEMICAL HAZARD. Power SYBR® Master Mix, 2×.

Prepare DNA samples of the standard library

1. Thaw one tube of 1 ng/µL standard library on ice. Pipette 1 µL of standard library and make a fresh dilution at 50 pg/µL by adding 19 µL of nuclease-free water. Put the rest of the stock back into the freezer.
2. Make a fresh serial dilution from the 50 pg/µL dilution for each qPCR run. Vortex and pulse-spin the dilutions. Store the samples on ice. To prepare typical standard dilutions see Table 67.

Table 67 Prepare standard dilutions of the library

	Dilutions from 50 pg/µL stock	Concentration (pg/µL)	Quantity‡ (copies/µL)	Components
Standard 1	1:50	1	5.92×10^6	1 µL of 50 pg/µL stock + 49 µL of nuclease-free water
Standard 2	1:500	0.1	5.92×10^5	5 µL of Standard 1 + 45 µL of nuclease-free water
Standard 3	1:5000	0.01	5.92×10^4	5 µL of Standard 2 + 45 µL of nuclease-free water
Standard 4	1:50000	0.001	5.92×10^3	5 µL of Standard 3 + 45 µL of nuclease-free water

‡ This value is calculated based on the size of the SOLiD™ DH10B Fragment Control Library (150 bp). When a library of a different size is used as the standard, this number needs to be changed.

Prepare DNA samples of unknown libraries

1. Make a dilution of your unknown libraries targeting 50 pg/µL based on other quantitation methods. Aliquot in small volumes for routine usage and store at – 20 °C. Avoid multiple freezes and thaws.
2. Make a 1:1000 dilution of each unknown library in a 1.5-mL LoBind tube. Include a negative control (NTC) with no template.

Prepare reaction mixtures for all samples

1. Dilute Forward Primer and Reverse Primer each to a 2.5 µM stock solution using nuclease-free water.
2. Calculate and prepare the appropriate volume of PCR master mix (see Table 68 on page 180):

Total number of samples = 4 standard + 1 NTC + # unknown samples

X = (Total number of samples + 1) × 3.5

Example:

For 4 unknown libraries to be quantified

X = (4 + 1 + 4 + 1) × 3.5 = 35

Table 68 Calculate the volume of PCR master mix

Components	Typical reaction volumes (µL)	PCR Master Mix (µL)	Example PCR Master Mix (µL)
Fast SYBR® Green Master Mix	10	X × 10	350
Forward Primer, 2.5 µM	1	X × 1	35
Reverse Primer, 2.5 µM	1	X × 1	35
Nuclease-free water	7	X × 7	245
Total	19	X × 19	665

3. Label a 1.5-mL LoBind tube for each standard or unknown library and add 3.5 µL of sample. Add 3.5 µL of nuclease-free water to the NTC tube.

4. In each tube, add 66.5 µL of PCR Master Mix. Mix well, then leave on ice.

Set up the reaction plate

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

CAUTION! CHEMICAL HAZARD. Power SYBR® Green Master Mix, 2×.

Prepare the reaction plate

IMPORTANT! Ensure that you use the Fast Optical 96-well reaction plate for the StepOne™ or StepOne™.

1. Make a template for the 96-well plate indicating the position for each library sample (see Figure 26).

Figure 26 Sample 96-well template.

2. Aliquot 20 µL out of each mixed sample into the appropriate wells according to the template.

3. Seal the plate with optical adhesive film.

4. Keep the plate on ice until you are ready to load it onto the instrument. Spin the plate for 30 seconds at 100 × g before loading.

Create an Absolute Quantification (AQ) Plate document

Refer to chapters 2 and 3 in the *Applied Biosystems 7500/7500 Fast Real-Time PCR System Standard Curve Experiments Getting Started Guide* (PN 4387779).

Enter the quantities for 4 standards as 5.92×10^6, 5.92×10^5, 5.92×10^4, and 5.92×10^3. The quantity for the unknown library is calculated based on these numbers in the units of copies/µL.

Specify thermal cycling conditions and start the run

Refer to chapters 2 and 3 in the *Applied Biosystems StepOne™/StepOne Plus™ Real-Time PCR System Standard Curve Experiments Getting Started Guide* (PN 4379704).

1. Select either the **Graphical View** tab or **Tabular View** tab.
2. Enter **20** µL in the **Reaction Volume per Well** field.
3. Add **Stage** as needed.
   See Table 69 for the typical fast mode.

   Table 69 Typical run conditions for *fast* mode

Stage	Temp (°C)	Time
1 cycle	95	20 sec
40 cycles	95	3 sec
	60	30 sec

4. Click **Save** to save the created plate document.
5. Load the reaction plate into the instrument.
6. Click **Start Run**. The running time is 35 minutes for fast mode.

Analyze the AQ data

1. After the qPCR run is finished, click **Analysis**. Select the wells in **View Plate Layout** by *click+drag*. Click **Amplification Plot** or **Standard Curve** to view the analysis results (for examples, see Figure 27 and Figure 28 on page 185).

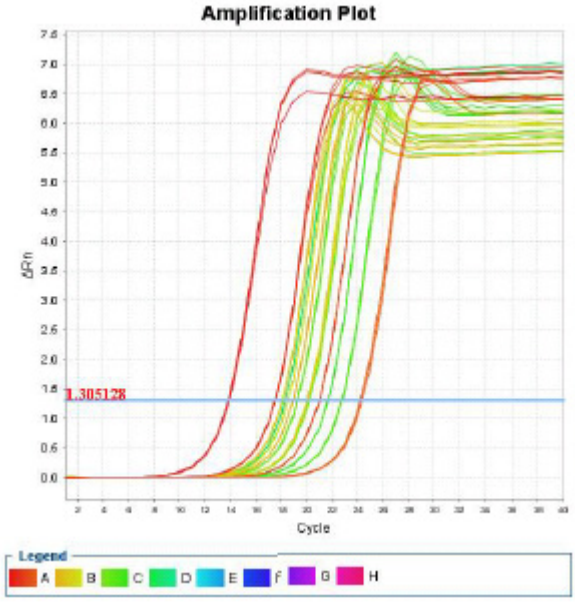


Figure 27 Sample amplification plot.

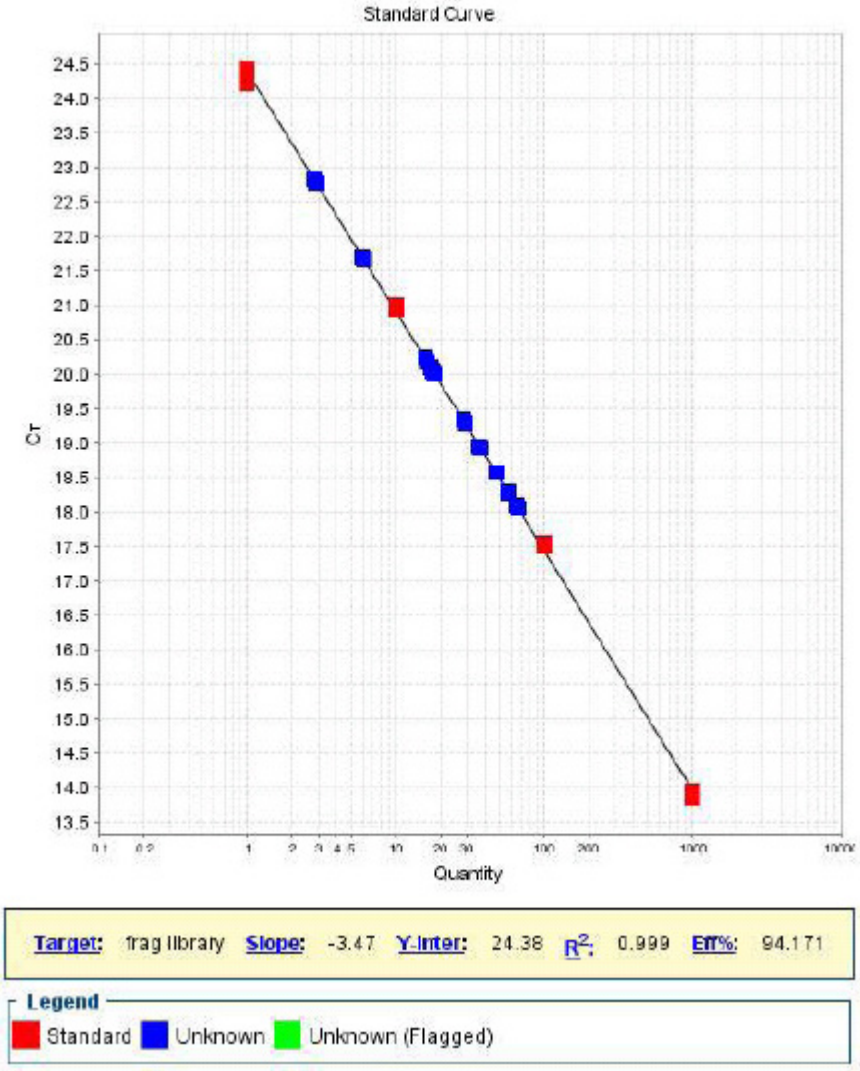


Figure 28 Sample standard curve.

2. Calculate the stock concentration of unknown libraries by inputting the *Mean Qty* in the following equation:

$$\text{Library Concentration (pg/µL)} = \frac{\text{Mean Qty (copies/µL)} \times \text{fold dilution}}{6.022 \times 10^{23}\ \text{copies/mol}} \times \text{Size} \times \frac{660\ \text{pg}}{1\ \text{pmol}} \times \frac{10^{12}\ \text{pmol}}{1\ \text{mol}}$$

Example:

For a fragment library (assuming average size is 175 bp) with Mean Qty = 4.5×10^5 copies/µL

$$\text{Library Concentration (pg/µL)} = \frac{4.5 \times 10^5\ \text{copies/µL} \times 1000}{6.022 \times 10^{23}\ \text{copies/mol}} \times 175 \times \frac{660\ \text{pg}}{1\ \text{pmol}} \times \frac{10^{12}\ \text{pmol}}{1\ \text{mol}}$$

$$= 86.3\ \text{pg/µL}$$

Note: To determine the library concentration to be used for emulsion PCR, refer to Appendix B of the *Applied Biosystems SOLiD™ 3 System Templated Bead Preparation Guide* (PN 4407421).

C

Supplemental Procedures

This appendix covers:

Hybridization of oligonucleotides

Oligonucleotide hybridization is required to hybridize single-stranded oligonucleotides to create double-stranded oligonucleotides.

Materials and equipment required

Required equipment

Item‡	Source
96-well GeneAmp® PCR System 9700 (thermal cycler)	• Applied Biosystems PN N8050200 (base) • Applied Biosystems PN 4314443 (block)
Pipettors	MLS§

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Required consumables

Item‡	Source
5× T4 DNA Ligase Buffer	Invitrogen™ Corporation PN 46300-018
Oligonucleotides	MLS§
PCR strip tubes	MLS
Filtered pipettor tips	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Procedure

1. Prepare 1 mM stock of individual oligonucleotides.
2. Mix equal volumes of 1 mM oligonucleotide A and B. Add enough 5× Ligase Buffer for a final concentration of 1× Ligase Buffer.
3. Hybridize the oligonucleotides by running the following program on a PCR machine (see Table 70 on page 189):

Table 70 Hybridization protocol

Temperature (°C)	Time (minutes)
95	5
72	5
60	5
50	3
40	3
30	3
20	3
10	3
4	∞

STOPPING POINT. After hybridization, store hybridized oligonucleotides at – 20 °C until ready for use.

Quantitate the DNA with the NanoDrop® ND-1000 Spectrophotometer

The Thermo Scientific NanoDrop® 1000 Spectrophotometer measures nucleic acid samples up to 3700 ng/µL without dilution.

Materials and equipment required

Required equipment

Item‡	Source
NanoDrop® ND-1000 Spectrophotometer (computer required)	Thermo Scientific ND-1000
Pipettors (20 µL)	MLS§

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Required consumables

Item‡	Source
Nuclease-free water (1 L)	Applied Biosystems PN AM9932
CF-1 Calibration Fluid Kit§	Thermo Scientific CF-1
PR Conditioning Kit	Thermo Scientific PR-1
Filtered pipettor tips	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ The NanoDrop® Conditioning Kit is useful for “reconditioning” the sample measurement pedestals to a hydrophobic state if they become “unconditioned.” (See the NanoDrop user's manual for more information.) The PR-1 kit consists of a container of specially formulated polishing compound and a supply of convenient applicators.

Procedure

1. Ensure that the NanoDrop ND-1000 Spectrophotometer is properly calibrated. Use the CF-1 Calibration Fluid Kit if necessary.

2. Open the NanoDrop ND-1000 Spectrophotometer software to display a dialog box (see Figure 29 on page 191).

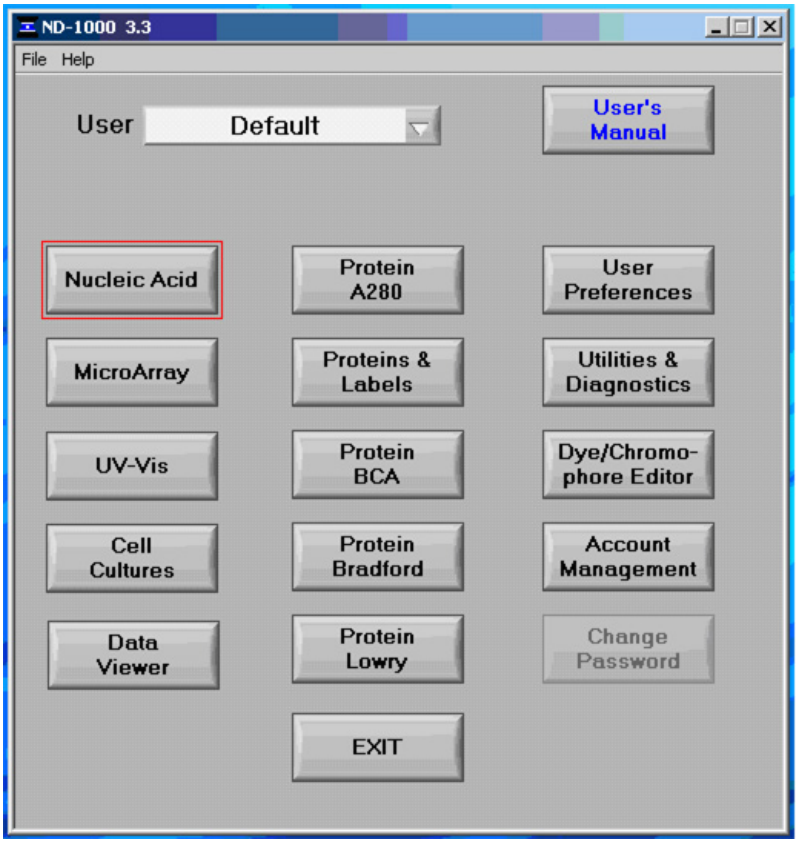


Figure 29 NanoDrop® ND-1000 Spectrophotometer software dialog box (from http://nanodrop.com/nd-1000-software.html).

3. Select the **Nucleic Acid** button.

4. Lift the sampling arm and load 2 µL of nuclease-free water onto the lower measurement pedestal and lower the sampling arm (see Figure 30 on page 192).

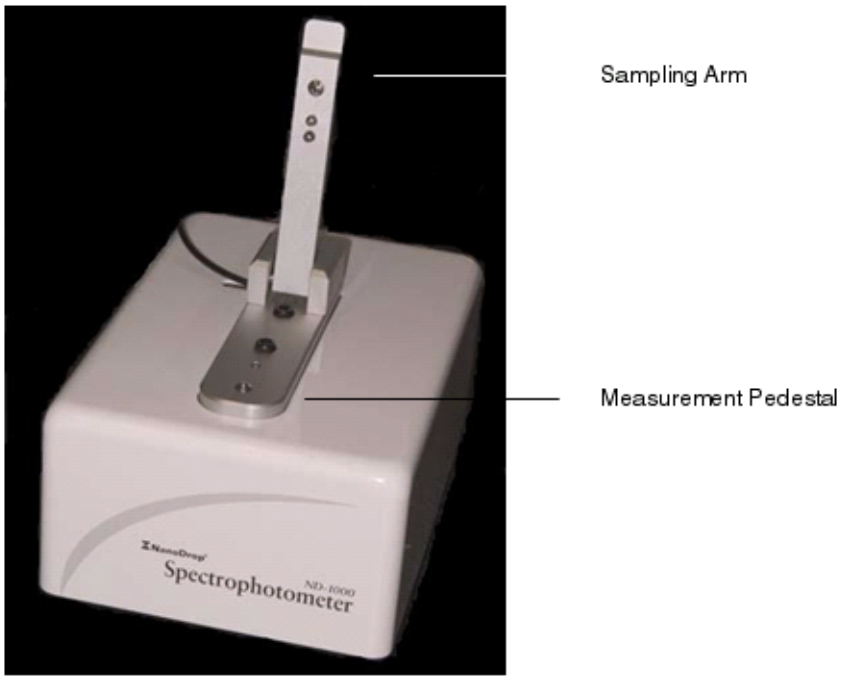


Figure 30 Components of the NanoDrop® ND-1000 Spectrophotometer.

5. In the dialog box, click **OK** and allow the instrument to initialize.
6. Lift the sampling arm and use a Kimwipe® to remove water from the measurement pedestal and the sampling arm.
7. Load 2 µL of the same buffer that was used to resuspend or elute the DNA onto the measurement pedestal and lower the sampling arm.
8. Click **Blank** and allow the instrument to take a measurement (see Figure 31 on page 193).

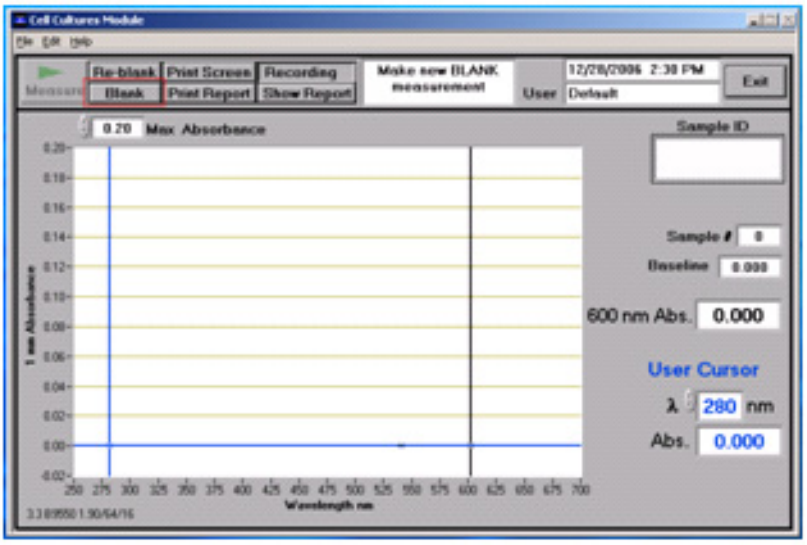


Figure 31 NanoDrop® ND-1000 Spectrophotometer software measurement dialog box.

9. Lift the sampling arm and wipe away the buffer from both the upper and lower measurement pedestals with a Kimwipe. The instrument is now ready to take readings.

10. Load 2 µL of DNA sample onto the lower measurement pedestal and lower the sampling arm.

Phenol-chloroform-isoamyl alcohol extraction

Phenol:chloroform:isoamyl alcohol extraction can be used to isolate DNA. Qiagen MaXtract High Density Tubes can be used in an alternative procedure (see "Phenol-chloroform-isoamyl alcohol extraction with MaXtract" on page 196).

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. Phenol-chloroform-isoamyl alcohol.

Materials and equipment required

Required equipment

Item‡	Source
Microcentrifuge 5417R, refrigerated, without rotor	• Eppendorf§ 022621807 (120 V/60 Hz) • Eppendorf‡ 022621840 (230 V/50 Hz))
FA-45-24-11, fixed-angle rotor 24 × 1.5/2 mL, including aluminum lid, aerosol-tight	Eppendorf 022636006
Pipettors	MLS#

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ Or equivalent but validation of the equipment for library preparation is required.
# For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Required consumables

Item‡	Source
Phenol:chloroform:isoamyl alcohol, with pH 7.9 buffer	Applied Biosystems PN AM9730
1.5-mL LoBind Tubes	Eppendorf 022431021
Filtered pipettor tips	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Procedure

1. Add an equal volume of cold phenol:chloroform:isoamyl alcohol to the sample and vortex.
2. Centrifuge at room temperature at 21,000 × g (minimum 14,000 × g) for 5 minutes.
3. Transfer the upper aqueous layer to a new tube.

4. Discard the phenol:chloroform:isoamyl alcohol layer in hazardous waste.
5. Proceed to "Isopropanol precipitation" on page 200.

Phenol-chloroform-isoamyl alcohol extraction with MaXtract

Phenol:chloroform:isoamyl alcohol extraction can be used to isolate DNA. Qiagen MaXtract High Density Tubes can be used for increased recovery.

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. Phenol-chloroform-isoamyl alcohol.

Materials and equipment required

Required equipment

Item‡	Source
Microcentrifuge 5417R, refrigerated, without rotor	• Eppendorf§ 022621807 (120 V/60 Hz) • Eppendorf‡ 022621840 (230 V/50 Hz))
FA-45-24-11, fixed-angle rotor 24 × 1.5/2 mL, including aluminum lid, aerosol-tight	Eppendorf 022636006
Pipettors	MLS#

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ Or equivalent but validation of the equipment for library preparation is required.
# For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Required consumables

Item‡	Source
MaXtract High Density Tubes	Qiagen 129046
Phenol:chloroform:isoamyl alcohol, with pH 7.9 buffer	Applied Biosystems PN AM9730
1.5-mL LoBind Tubes	Eppendorf 022431021
Filtered pipettor tips	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Procedure

1. Centrifuge the MaXtract tube at 21,000 × g (minimum 14,000 × g) for 20 seconds.
2. Add an equal volume of cold phenol:chloroform:isoamyl alcohol to the aqueous sample.
3. Mix by inversion.
4. Transfer the solution to the pre-spun MaXtract tube.
5. Centrifuge at room temperature at 21,000 × g (minimum 14,000 × g) for 5 minutes.
6. Transfer the upper aqueous layer to a new tube.
7. Discard the MaXtract tube with phenol:chloroform:isoamyl layer in hazardous waste.
8. Proceed to "Isopropanol precipitation" on page 200.

PAGE gel DNA elution

Materials and equipment required

Required equipment

Item‡	Source
Microcentrifuge 5417R, refrigerated, without rotor	• Eppendorf§ 022621807 (120 V/60 Hz) • Eppendorf‡ 022621840 (230 V/50 Hz))
FA-45-24-11, fixed-angle rotor 24 × 1.5/2 mL, including aluminum lid, aerosol-tight	Eppendorf 022636006
Pipettors	MLS#

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ Or equivalent but validation of the equipment for library preparation is required.
# For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Required consumables

Item‡	Source
TE, pH 8.0	Applied Biosystems PN AM9858
7.5 M Ammonium acetate	Sigma-Aldrich A-2706
100× BSA	New England®, Inc. B9001S
0.45 µm Nanosep® spin columns	VWR ODM45C34
0.5-mL LoBind Tubes	Eppendorf 022431005
1.5-mL LoBind Tubes	Eppendorf 022431021
21-gauge needle	MLS
Razor blades	MLS
Filtered pipettor tips	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Procedure

1. Excise the appropriate-sized band using a clean razor blade.
2. Using a 21-gauge needle, make a hole in the bottom of a 0.5-mL LoBind tube.
3. Place the gel piece(s) in the 0.5-mL LoBind tube.
4. Place the 0.5-mL LoBind tube with the gel in a 1.5-mL LoBind tube and centrifuge at ≥10,000 × g (13,000 rpm) for 3 minutes to shred the gel and collect in the bottom tube.
5. If some gel pieces remain in the 0.5-mL LoBind tube, repeat the centrifugation step using a new 1.5-mL LoBind tube and then pool the tubes.
6. Add enough PAGE Elution Buffer (1 volume of 7.5 M ammonium acetate in 5 volumes of 1✕ TE) to soak the gel pieces completely with a thin layer of liquid on top.
7. Incubate at room temperature for 20 minutes. The length of elution time can be increased 2 to 3 times for maximum elution.
8. Transfer supernatant to a new 1.5-mL LoBind tube.
9. Add 250 µL of PAGE Elution Buffer to the gel pieces.
10. Incubate at 37 °C for 40 minutes.
11. Pool all of the liquids, including the first elution, into a 0.45-µm filter Nanosep® spin column with a 1.5-mL LoBind tube as the collection tube. Centrifuge the column at ≥10,000 × g (13,000 rpm) for 5 minutes.
12. Proceed to "Isopropanol precipitation" on page 200.

Isopropanol precipitation

Isopropanol precipitation can be used to purify and/or concentrate DNA.

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. 3 M Sodium acetate.

Materials and equipment required

Required equipment

Item‡	Source
Microcentrifuge 5417R, refrigerated, without rotor	• Eppendorf§ 022621807 (120 V/60 Hz) • Eppendorf‡ 022621840 (230 V/50 Hz))
FA-45-24-11, fixed-angle rotor 24 × 1.5/2 mL, including aluminum lid, aerosol-tight	Eppendorf 022636006
Pipettors	MLS#

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ Or equivalent but validation of the equipment for library preparation is required.
# For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Required consumables

Item‡	Source
• 7.5 M Ammonium acetate, or • 3 M Sodium acetate, pH 5.5	• Sigma-Aldrich, A-2706 • Applied Biosystems, PN AM9740
Glycogen, 5 mg/mL	Applied Biosystems PN AM9510
Isopropyl alcohol	Sigma-Aldrich I9516
Ethanol	E7023
Filtered pipettor tips	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Procedure

1. Add either an equal volume of 7.5 M ammonium acetate or 1/10 volume of 3 M sodium acetate (pH 5.5) to the aqueous phase.
2. Add 1/100 volume of glycogen.
3. Add 0.7 volume of 100% isopropyl alcohol at room temperature. Vortex well.
4. Incubate at room temperature for 5 minutes to precipitate.
5. Centrifuge the solution at 21,000 × g (minimum 14,000 × g) for 15 minutes
6. Remove the supernatant.
7. Wash the DNA pellet three times with 200 µL 70% ethanol to remove salts. Ensure all the isopropanol is completely removed. If the pellet is dispersed during the wash, then centrifuge at 21,000 × g (minimum 14,000 × g) for 2 minutes.
8. Completely remove the 70% ethanol by a short centrifugation step and a pipette tip.
9. Air-dry the sample for 2 to 5 minutes.

Confirm complete methylation of DNA fragments

To confirm complete methylation of DNA fragments, the following is compared on a quality control gel: (1) unmethylated, unsheared genomic DNA, (2) unmethylated, unsheared EcoP15I-digested genomic DNA, (3) methylated, sheared genomic DNA, and (4) methylated, sheared, EcoP15I-digested DNA.

For the following hazards, see the complete safety alert descriptions in "Safety alerts" on page 249:

WARNING! CHEMICAL HAZARD. Gel loading solution.

Materials and equipment required

Required equipment

Item‡	Source
Microcentrifuge 5417R, refrigerated, without rotor	• Eppendorf§ 022621807 (120 V/60 Hz) • Eppendorf‡ 022621840 (230 V/50 Hz))
FA-45-24-11, fixed-angle rotor 24 × 1.5/2 mL, including aluminum lid, aerosol-tight	Eppendorf 022636006
Incubator (37 °C)	MLS
Pipettors	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.
§ Or equivalent but validation of the equipment for library preparation is required.

Required consumables

Item‡	Source
Gel Loading Solution, All-Purpose	Applied Biosystems PN AM8556
EcoP15I, 10 U/µL	New England®, Inc. R0646L
100X BSA	New England®, Inc. B9001S
Sinefungin	Sigma-Aldrich S8559
1 kb ladder	Invitrogen™ Corporation 15615-016
0.8% E-Gel Starter Pack	Invitrogen™ Corporation G5018-08
1.5-mL LoBind Tubes	Eppendorf 022431021

Item‡	Source
QIAquick® Gel Extraction Kit	Qiagen 28706
Filtered pipettor tips	MLS

‡ Applied Biosystems has validated this protocol using this specific material. Substitution may adversely affect system performance.

Procedure

1. Combine and mix the following components in two separate microcentrifuge tubes (one for unmethylated, unsheared DNA and one for methylated, sheared DNA) (see Table 71):

Table 71 Mix to digest circularized DNA with EcoP15I

Component	Amount
DNA	0.5 µg
NEBuffer 3, 10×	10
100× BSA	1
10 mM Sinefungin	1
10× ATP	20
EcoP15I Enzyme, 10 U/µL	1
Nuclease-free water	Variable
Total	100

2. Incubate the digestion reaction mixtures at 37 °C for 2 hours.
3. Add 3 volumes of Buffer QG and 1 volume of isopropyl alcohol to the digested DNA. If the color of the mixture is orange or violet, add 10 µL of sodium acetate, pH 5.5 and mix. The color turns yellow. The pH required for efficient adsorption of the DNA to the membrane is ≤ 7.5.
4. Apply 750 µL of digested DNA in Buffer QG to the column(s). The maximum amount of DNA that can be applied to a QIAquick® column is 10 µg. Use more columns if necessary.
5. Let the column(s) stand for 2 minutes at room temperature.
6. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute and discard the flow-through.
7. Repeat steps 4 and 6 until the entire sample has been loaded onto the column(s). Place the QIAquick® column(s) back into the same collection tube(s).
8. Add 750 µL of Buffer PE to wash the column(s).
9. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 2 minutes, then discard the flow-through. Repeat to remove residual wash buffer.

10. Air-dry the column(s) for 2 minutes to evaporate any residual alcohol. Transfer the column(s) to clean 1.5-mL LoBind tube(s).

11. Add 25 µL of Buffer EB to the column(s) to elute the DNA and let the column(s) stand for 2 minutes.

12. Centrifuge the column(s) at ≥10,000 × g (13,000 rpm) for 1 minute.

13. If necessary, pool the eluted DNA.

14. Add 2.5 µL of 10✕ Gel Loading Solution to the purified DNA.

15. Load 1 µL of 1 kb DNA ladder onto a 0.8% E-Gel®. Load 11 µL of dye-mixed sample per well. There should be at least one lane in between the ladder well and the sample wells to avoid contamination of the sample with ladder.

16. Run the gel and confirm that the sheared, methylated DNA is the expected size relative to the controls (see Figure 32).

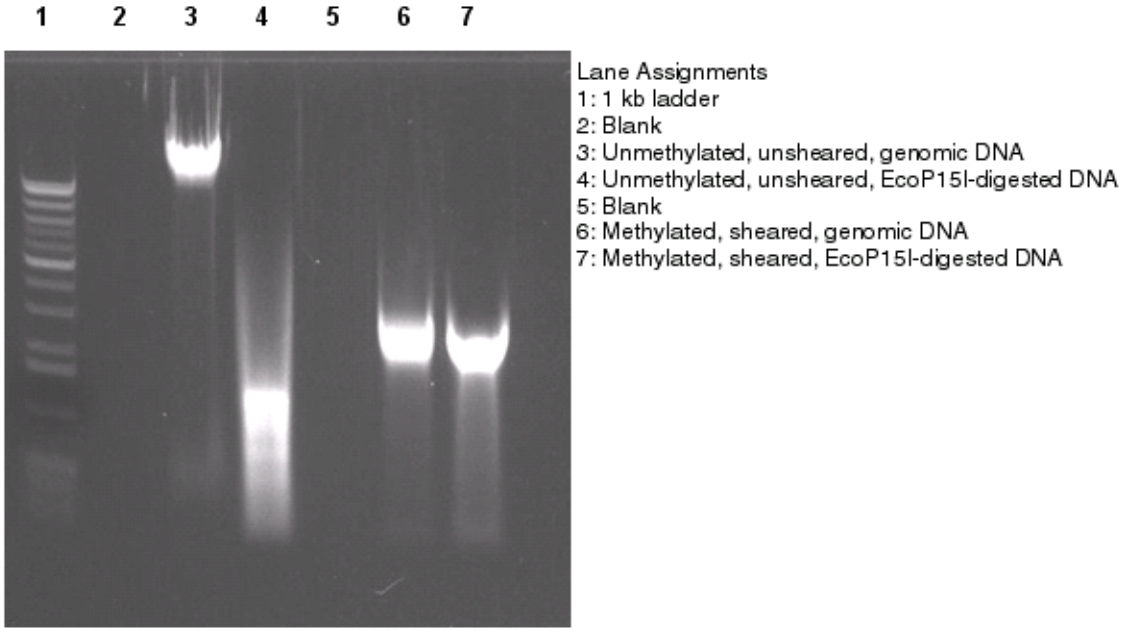


Figure 32 Methylation confirmation gel.

D Oligonucleotide Sequences

This appendix covers:

Fragment library construction oligonucleotides

All library construction oligonucleotides needed for fragment library construction are included in the SOLiD™ Fragment Library Oligos Kit (PN 4401151). If you are using individual oligo components to make double-stranded P1 and P2 Adaptors, follow the procedure, "Hybridization of oligonucleotides" on page 188. The hybridization step is not necessary if you are using oligonucleotides from the SOLiD™ Fragment Library Oligos Kit.

Oligonucleotides needed for fragment library construction

- P1 Adaptor, 50 µM
- P2 Adaptor, 50 µM
- Library PCR Primer 1, 50 µM
- Library PCR Primer 2, 50 µM

Mate-paired library construction oligonucleotides

All library construction oligonucleotides needed for mate-paired library construction are included in the SOLiD™ Mate-Paired Library Oligos Plus Kit (PN 4425772) or SOLiD™ Mate-Paired Library Oligos Kit (PN 4400468). If you are using individual oligo components to make double-stranded P1 and P2 Adaptors, follow the "Hybridization of oligonucleotides" on page 188. The hybridization step is not necessary if you are using oligonucleotides from the SOLiD™ Mate-Paired Library Oligos Plus Kit or SOLiD™ Mate-Paired Library Oligos Kit.

Oligonucleotides needed for 2 × 50 bp mate-paired library construction

- LMP CAP Adaptor, 50 µM
- Internal Adaptor, 2 µM
- P1 Adaptor, 50 µM
- P2 Adaptor, 50 µM
- Library PCR Primer 1, 50 µM
- Library PCR Primer 2, 50 µM

Oligonucleotides needed for 2 × 25 bp mate-paired library construction

- EcoP15I CAP Adaptor, 50 µM
- Internal Adaptor, 2 µM
- P1 Adaptor, 50 µM
- P2 Adaptor, 50 µM
- Library PCR Primer 1, 50 µM
- Library PCR Primer 2, 50 µM

Barcoded fragment library construction oligonucleotides

All library construction oligonucleotides need to be ordered from an oligonucleotide vendor. Follow the "Hybridization of oligonucleotides" on page 188 to make double-stranded Multiplex P1 and P2 Adaptors from single-stranded oligo components.

Oligonucleotides needed for barcoded library construction

- Multiplex P1 Adaptor, 50 µM
- Multiplex P2 Adaptor - Barcode 1, 50 µM
- Multiplex P2 Adaptor - Barcode 2, 50 µM
- Multiplex P2 Adaptor - Barcode 3, 50 µM
- Multiplex P2 Adaptor - Barcode 4, 50 µM
- Multiplex P2 Adaptor - Barcode 5, 50 µM
- Multiplex P2 Adaptor - Barcode 6, 50 µM
- Multiplex P2 Adaptor - Barcode 7, 50 µM
- Multiplex P2 Adaptor - Barcode 8, 50 µM
- Multiplex P2 Adaptor - Barcode 9, 50 µM
- Multiplex P2 Adaptor - Barcode 10, 50 µM
- Multiplex P2 Adaptor - Barcode 11, 50 µM
- Multiplex P2 Adaptor - Barcode 12, 50 µM
- Multiplex P2 Adaptor - Barcode 13, 50 µM
- Multiplex P2 Adaptor - Barcode 14, 50 µM
- Multiplex P2 Adaptor - Barcode 15, 50 µM
- Multiplex P2 Adaptor - Barcode 16, 50 µM
- Multiplex P2 Adaptor - Barcode 17, 50 µM
- Multiplex P2 Adaptor - Barcode 18, 5 µM
- Multiplex P2 Adaptor - Barcode 19, 50 µM
- Multiplex P2 Adaptor - Barcode 20, 50 µM
- Multiplex Library PCR Primer 1, 50 µM
- Library PCR Primer 2, 50 µM

Oligonucleotide sequences

Barcode sequences are in red.

Sequence name	Oligonucleotide sequence	Sequence length (bp)
P1 Adaptor	5' - CCA CTA CGC CTC CGC TTT CCT CTC TAT GGG CAG TCG GTG AT - 3' 3' - TT GGT GAT GCG GAG GCG AAA GGA GAG ATA CCC GTC AGC CAC TA - 5'	41/43
P2 Adaptor	5' - AGA GAA TGA GGA ACC CGG GGC AG TT - 3' 3' - TCT CTT ACT CCT TGG GCC CCG TC- 5'	25/23
Library PCR Primer 1	5' - CCA CTA CGC CTC CGC TTT CCT CTC TAT G - 3'	28
Library PCR Primer 2	5' - CTG CCC CGG GTT CCT CAT TCT - 3'	21
EcoP15I CAP Adaptor	5' Phos - CTG CTG TAC - 3' 3' - GAC GAC A - Phos 5'	9/7
LMP CAP Adaptor	5' Phos - CTG CTG TAC - 3' 3' - GAC GAC A - 5'	9/7
Internal Adaptor	Biotin ▼ 5' Phos - CGT ACA TCC GCC TTG GCC GT - 3' 3' - TG GCA TGT AGG CGG AAC CGG - Phos 5'	20/20
Multiplex P1 Adaptor	5' - CCT CTC TAT GGG CAG TCG GTG AT - 3' 3' - TT GGA GAG ATA CCC GTC AGC CAC TA - 5'	23/25
Multiplex P2 Adaptor – Barcode 1	5' - CGC CTT GGC CGT ACA GCA GCC TCT TAC ACA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CGG AGA ATG TGT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 2	5' - CGC CTT GGC CGT ACA GCA GAC CAC TCC CTA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CTG GTG AGG GAT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 3	5' - CGC CTT GGC CGT ACA GCA GTA TAA CCT ATA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CAT ATT GGA TAT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 4	5' - CGC CTT GGC CGT ACA GCA GGA CCG CAT CCA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CCT GGC GTA GGT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 5	5' - CGC CTT GGC CGT ACA GCA GCT TAC ACC ACA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CGA ATG TGG TGT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 6	5' - CGC CTT GGC CGT ACA GCA GTG TCC CTC GCA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CAC AGG GAG CGT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 7	5' - CGC CTT GGC CGT ACA GCA GGG CAT AAC CCA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CCC GTA TTG GGT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 8	5' - CGC CTT GGC CGT ACA GCA GAT CCT CGC TCA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CTA GGA GCG AGT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 9	5' - CGC CTT GGC CGT ACA GCA GGT CGC AAC CTA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CCA GCG TTG GAT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 10	5' - CGC CTT GGC CGT ACA GCA GAG CTT ACC GCA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CTC GAA TGG CGT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 11	5' - CGC CTT GGC CGT ACA GCA GCG TGT CGC ACA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CGC ACA GCG TGT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52

Oligonucleotide sequences – continued

Multiplex P2 Adaptor – Barcode 12	5' - CGC CTT GGC CGT ACA GCA GTT TTC CTC TTA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CAA AAG GAG AAT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 13	5' - CGC CTT GGC CGT ACA GCA GGC CTT ACC GCA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CCG GAA TGG CGT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 14	5' - CGC CTT GGC CGT ACA GCA GTC TGC CGC ACA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CAG ACG GCG TGT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 15	5' - CGC CTT GGC CGT ACA GCA GCA TTC AAC TCA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CGT AAG TTG AGT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 16	5' - CGC CTT GGC CGT ACA GCA GAA CGT CTC CCA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CTT GCA GAG GGT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 17	5' - CGC CTT GGC CGT ACA GCA GCT CCC ACC TCA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CGA GGG TGG AGT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 18	5' - CGC CTT GGC CGT ACA GCA GGT GTC CCA CCA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CCA CAG GGT GGT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 19	5' - CGC CTT GGC CGT ACA GCA GTT AGT ATA ACA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CAA TCA TAT TGT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex P2 Adaptor – Barcode 20	5' - CGC CTT GGC CGT ACA GCA GAT TAT CTC GCA GAG AAT GAG GAA CCC GGG GCA GTT - 3' 3' - GCG GAA CCG GCA TGT CGT CTA ATA GAG CGT CTC TTA CTC CTT GGG CCC CGT C- 5'	54/52
Multiplex Library PCR Primer 1	5' - CCA CTA CGC CTC CGC TTT CCT CTC TAT GGG CAG TCG GTG AT - 3'	41

E Formulas and calculations

This appendix covers:

Fragment library

Ligation of P1 and P2 Adaptors

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{\text{Average insert size}}$$

$$x_1 \text{ pmol DNA for adaptor ligation} = \#\text{ µg DNA} \times \frac{X \text{ pmol}}{1 \text{ µg DNA}}$$

$$x_2 \text{ pmol adaptor needed} = x_1 \text{ pmol} \times 30$$

$$Y \text{ µL adaptor needed} = x_2 \text{ pmol} \times \frac{1 \text{ µL}}{50 \text{ pmol}}$$

2 × 50 bp mate-paired library

Ligation of LMP CAP Adaptors

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{\text{Average insert size}}$$

$$x_1 \text{ pmol DNA for adaptor ligation} = \#\text{ µg DNA} \times \frac{X \text{ pmol}}{1 \text{ µg DNA}}$$

$$x_2 \text{ pmol adaptor needed} = x_1 \text{ pmol} \times 100$$

$$Y \text{ µL adaptor needed} = x_2 \text{ pmol} \times \frac{1 \text{ µL}}{50 \text{ pmol}}$$

DNA circularization

The formula to determine dilution of ligation reaction and achieve intramolecular ligation [Francis S. Collins and Sherman M. Weissman, "Directional Cloning of DNA Fragments at a Large Distance from an Initial Probe: A Circularization method," *PNAS* **81** (1984): 6812-6816] is:

$$J = \frac{63.4}{(\text{DNA size in kb})^{1/2}}$$

$$I\ \text{ng/µL} = \frac{J}{\text{Targeted circularization efficiency}} - J$$

$$= \text{Final concentration of DNA for circularization}$$

Example:

For an insert size of 1 to 2 kb

$$J = \frac{63.4}{(1.5)^{1/2}} = 51.8$$

$$I\ \text{ng/µL} = \frac{51.8}{0.95} - 51.8 = 2.74\ \text{ng/µL}$$

The final concentration of DNA required for circularization can be calculated using the formula above. The circularization reaction volume per µg of DNA can then be calculated (see Table 72).

Table 72 Circularization reaction volumes

Insert Size	Final concentration of DNA for circularization	Calculation	Circularization reaction volume per 1 µg DNA
600 to 800 bp	4.3 ng/µL	1000 ng ÷ 4.3 ng/µL	235 µL
800 to 1000 bp	3.75 ng/µL	1000 ng ÷ 3.75 ng/µL	270 µL
1 to 2 kb	2.74 ng/µL	1000 ng ÷ 2.74 ng/µL	365 µL
2 to 3 kb	2.1 ng/µL	1000 ng ÷ 2.1 ng/µL	500 µL
3 to 4 kb	1.8 ng/µL	1000 ng ÷ 1.8 ng/µL	560 µL
4 to 5 kb	1.6 ng/µL	1000 ng ÷ 1.6 ng/µL	625 µL
5 to 6 kb	1.4 ng/µL	1000 ng ÷ 1.4 ng/µL	720 µL

The amount of Internal Adaptor (ds) needed for circularization can be calculated as follows:

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{\text{Average insert size}}$$

$$x_1 \text{ pmol DNA for adaptor ligation} = \#\text{ µg DNA} \times \frac{X \text{ pmol}}{1 \text{ µg DNA}}$$

$$x_2 \text{ pmol adaptor needed} = x_1 \text{ pmol} \times 3$$

$$Y \text{ µL adaptor needed} = x_2 \text{ pmol} \times \frac{1 \text{ µL}}{2 \text{ pmol}}$$

Example:
For 20 µg of DNA with an insert size of 1 to 2 kb

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{1500} = 1.0 \text{ pmol/µg DNA}$$

$$Y \text{ µL adaptor needed} = 20 \text{ µg DNA} \times \frac{1.0 \text{ pmol}}{1 \text{ µg DNA}} \times 3 \times \frac{1 \text{ µL adaptor needed}}{2 \text{ pmol}}$$

$$= 30 \text{ µL adaptor needed}$$

Ligation of P1 and P2 Adaptors

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{\text{Circularized DNA size}}$$

$$x_1 \text{ pmol DNA for adaptor ligation} = \#\text{ µg DNA} \times \frac{X \text{ pmol}}{1 \text{ µg DNA}}$$

$$x_2 \text{ pmol adaptor needed} = x_1 \text{ pmol} \times 30$$

$$Y \text{ µL adaptor needed} = x_2 \text{ pmol} \times \frac{1 \text{ µL}}{50 \text{ pmol}}$$

2 × 25 bp mate-paired library

Ligation of EcoP15I CAP Adaptors

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{\text{Average insert size}}$$

$$x_1 \text{ pmol DNA for adaptor ligation} = \#\text{ µg DNA} \times \frac{X \text{ pmol}}{1 \text{ µg DNA}}$$

$$x_2 \text{ pmol adaptor needed} = x_1 \text{ pmol} \times 100$$

$$Y \text{ µL adaptor needed} = x_2 \text{ pmol} \times \frac{1 \text{ µL}}{50 \text{ pmol}}$$

DNA circularization

The formula to determine dilution of ligation reaction and achieve intramolecular ligation [Francis S. Collins and Sherman M. Weissman, “Directional Cloning of DNA Fragments at a Large Distance from an Initial Probe: A Circularization method,” *PNAS* **81** (1984): 6812-6816] is:

$$J = \frac{63.4}{(\text{DNA size in kb})^{1/2}}$$

$$I \text{ ng/µL} = \frac{J}{\text{Targeted circularization efficiency}} - J$$

$$= \text{Final concentration of DNA for circularization}$$

Example:

For an insert size of 1 to 2 kb

$$J = \frac{63.4}{(1.5)^{1/2}} = 51.8$$

$$I \text{ ng/µL} = \frac{51.8}{0.95} - 51.8 = 2.74 \text{ ng/µL}$$

The final concentration of DNA required for circularization can be calculated using the formula above. The circularization reaction volume per µg of DNA can then be calculated (see Table 73 on page 216).

Table 73 Circularization reaction volumes

Insert Size	Final concentration of DNA for circularization	Calculation	Circularization reaction volume per 1 µg DNA
600 to 800 bp	4.3 ng/µL	1000 ng ÷ 4.3 ng/µL	235 µL
800 to 1000 bp	3.75 ng/µL	1000 ng ÷ 3.75 ng/µL	270 µL
1 to 2 kb	2.74 ng/µL	1000 ng ÷ 2.74 ng/µL	365 µL
2 to 3 kb	2.1 ng/µL	1000 ng ÷ 2.1 ng/µL	500 µL
3 to 4 kb	1.8 ng/µL	1000 ng ÷ 1.8 ng/µL	560 µL
4 to 5 kb	1.6 ng/µL	1000 ng ÷ 1.6 ng/µL	625 µL
5 to 6 kb	1.4 ng/µL	1000 ng ÷ 1.4 ng/µL	720 µL

The amount of Internal Adaptor (ds) needed for circularization can be calculated as follows:

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{\text{Average insert size}}$$

$$x_1 \text{ pmol DNA for adaptor ligation} = \#\text{ µg DNA} \times \frac{X \text{ pmol}}{1 \text{ µg DNA}}$$

$$x_2 \text{ pmol adaptor needed} = x_1 \text{ pmol} \times 3$$

$$Y \text{ µL adaptor needed} = x_2 \text{ pmol} \times \frac{1 \text{ µL}}{2 \text{ pmol}}$$

Example:
For 20 µg of DNA with an insert size of 1 to 2 kb

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{1500} = 1.0 \text{ pmol/µg DNA}$$

$$Y \text{ µL adaptor needed} = 20 \text{ µg DNA} \times \frac{1.0 \text{ pmol}}{1 \text{ µg DNA}} \times 3 \times \frac{1 \text{ µL adaptor needed}}{2 \text{ pmol}}$$

$$= 30 \text{ µL adaptor needed}$$

Ligation of P1 and P2 Adaptors

$$X \text{ pmol/µg DNA} = 1 \text{ µg DNA} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{86 \text{ bp}} = 17.6 \text{ pmol/µg DNA}$$

$$x_1 \text{ µg DNA for linker ligation} = \#\text{ µg DNA} \div \text{Fold reduction}$$

$$x_2 \text{ pmol DNA available for adaptor ligation} = x_1 \text{ µg} \times \frac{17.6 \text{ pmol}}{1 \text{ µg DNA}}$$

$$x_3 \text{ pmol adaptor needed} = x_2 \text{ pmol} \times 60$$

$$Y \text{ µL adaptor needed} = x_3 \text{ pmol} \times \frac{1 \text{ µL}}{50 \text{ pmol}}$$

F Checklists and workflow tracking forms

This appendix covers:

Workflow checklists: prepare a standard fragment library

	Equipment	Reagents	Preparation steps
Shear the DNA	☐ Covaris™ S2 System ☐ Covaris microTube adaptor ☐ Covaris microTube loading station ☐ Pipettors	☐ 1× Low TE Buffer ☐ Ethylene glycol ☐ Covaris™ microTube ☐ 1.5-mL LoBind tubes ☐ Ethylene glycol ☐ Filtered pipettor tips	☐ Degas the water in the Covaris™ S2 System 30 minutes prior to use. ☐ Supplement the circulated water chiller with 20% ethylene glycol.
End-repair the DNA	☐ Microcentrifuge ☐ NanoDrop™ ND-1000 Spectrophotometer ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ 5× End-Polishing Buffer ☐ dNTP Mix ☐ End Polishing Enzyme 1 ☐ End Polishing Enzyme 2 ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ PureLink™ PCR Purification Kit ☐ Filtered pipettor tips	☐ Thaw 5× End-Polishing Buffer and dNTP Mix on ice.
Ligate P1 and P2 Adaptors to the DNA	☐ Microcentrifuge ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ P1 Adaptor (ds) (50 µM) ☐ P2 Adaptor (ds) (50 µM) ☐ 5× T4 Ligase Buffer ☐ T4 Ligase ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ PureLink™ PCR Purification Kit ☐ Filtered pipettor tips	☐ Thaw P1 and P2 Adaptors on ice. ☐ Thaw5× T4 Ligase Buffer on ice.
Size-select the DNA	☐ iBase™ ☐ E-gel Safe Imager™ ☐ Pipettors	☐ E-Gel® 2% SizeSelect™ gel ☐ 50 bp DNA Ladder ☐ Nuclease-free water ☐ Filtered pipettor tips	☐ Thaw 50 bp DNA Ladder on ice.
Nick-translate and amplify the library	☐ Thermal cycler ☐ Microcentrifuge ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ Library PCR Primer 1 ☐ Library PCR Primer 2 ☐ Platinum® PCR Amplification Mix ☐ PureLink™ PCR Purification Kit ☐ 1.5-mL LoBind tube ☐ PCR strip tubes ☐ Filtered pipettor tips	☐ Thaw Library PCR Primers 1 and 2 on ice. ☐ Thaw Platinum® PCR Amplification Mix on ice.
Quantitate	☐ Real-time PCR system	☐ TaqMan or SYBR Green assay	

Workflow checklists: prepare an express fragment library

	Equipment	Reagents	Preparation steps
Shear the DNA	☐ Covaris™ S2 System ☐ Covaris microTube adaptor ☐ Covaris microTube loading station ☐ Pipettors	☐ 1× Low TE Buffer ☐ Ethylene glycol ☐ Covaris™ microTube ☐ 1.5-mL LoBind tubes ☐ Ethylene glycol ☐ Filtered pipettor tips	☐ Degas the water in the Covaris™ S2 System 30 minutes prior to use. ☐ Supplement the circulated water chiller with 20% ethylene glycol.
End-repair the DNA	☐ Microcentrifuge ☐ NanoDrop™ ND-1000 Spectrophotometer ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ 5× End-Polishing Buffer ☐ dNTP Mix ☐ End Polishing Enzyme 1 ☐ End Polishing Enzyme 2 ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ PureLink™ PCR Purification Kit ☐ Filtered pipettor tips	☐ Thaw 5× End-Polishing Buffer and dNTP Mix on ice.
Ligate P1 and P2 Adaptors to the DNA	☐ Microcentrifuge ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ P1 Adaptor (ds) (50 µM) ☐ P2 Adaptor (ds) (50 µM) ☐ 5× T4 Ligase Buffer ☐ T4 Ligase ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ PureLink™ PCR Purification Kit ☐ Filtered pipettor tips	☐ Thaw P1 and P2 Adaptors on ice. ☐ Thaw5× T4 Ligase Buffer on ice.
Nick-translate, then amplify the library	☐ Thermal cycler ☐ Microcentrifuge ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ Library PCR Primer 1 ☐ Library PCR Primer 2 ☐ Platinum® PCR Amplification Mix ☐ PureLink™ PCR Purification Kit ☐ 1.5-mL LoBind tube ☐ PCR strip tubes ☐ Filtered pipettor tips	☐ Thaw Library PCR Primers 1 and 2 on ice. ☐ Thaw Platinum® PCR Amplification Mix on ice.
Quantitate	☐ Real-time PCR system	☐ TaqMan or SYBR Green assay	

Workflow tracking: prepare standard and express fragment libraries

Sample:

Quantitation	
Step	**Quantity of DNA**
Starting Amount	
End-Repair	
Quantitative PCR	

Lot number	
Step	**Lot number**
SOLiD™ Library Oligos Kit 1	
P1 Adaptor	
P2 Adaptor	
Library PCR Primer 1	
Library PCR Primer 2	

Sample:

Quantitation	
Step	**Quantity of DNA**
Starting Amount	
End-Repair	
Quantitative PCR	

Lot number	
Step	**Lot number**
SOLiD™ Library Oligos Kit 1	
P1 Adaptor	
P2 Adaptor	
Library PCR Primer 1	
Library PCR Primer 2	

Sample:

Quantitation	
Step	**Quantity of DNA**
Starting Amount	
End-Repair	
Quantitative PCR	

Lot number	
Step	**Lot number**
SOLiD™ Library Oligos Kit 1	
P1 Adaptor	
P2 Adaptor	
Library PCR Primer 1	
Library PCR Primer 2	

Sample:

Quantitation	
Step	**Quantity of DNA**
Starting Amount	
End-Repair	
Quantitative PCR	

Lot number	
Step	**Lot number**
SOLiD™ Library Oligos Kit 1	
P1 Adaptor	
P2 Adaptor	
Library PCR Primer 1	
Library PCR Primer 2	

Workflow checklists: prepare a 2 × 50 bp mate-paired library

	Equipment	Reagents	Preparation steps
Shear the DNA with Covaris™ S2 System	☐ Covaris™ S2 System ☐ Microcentrifuge ☐ NanoDrop™ ND-1000 Spectrophotometer ☐ Pipettors	☐ 1 M Tris, pH 8.0 ☐ Nuclease-free water ☐ Ethylene glycol ☐ Glycerol ☐ Covaris™ Tubes and Caps ☐ 1.5-mL LoBind tubes ☐ QIAquick® Gel Extraction Kit ☐ Filtered pipettor tips	☐ Degas the water in the Covaris™ S2 System 30 minutes prior to use. ☐ Supplement the circulated water chiller with 20% ethylene glycol.
Shear the DNA with HydroShear®	☐ HydroShear® ☐ Microcentrifuge ☐ NanoDrop™ ND-1000 Spectrophotometer ☐ Pipettors	☐ Nuclease-free water ☐ 0.2 N HCl ☐ 0.2 N NaOH ☐ 1.5-mL LoBind tubes ☐ QIAquick® Gel Extraction Kit ☐ Filtered pipettor tips	
End-repair the DNA	☐ Microcentrifuge ☐ NanoDrop™ ND-1000 Spectrophotometer ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ End-It™ DNA End-Repair Kit ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ QIAquick® Gel Extraction Kit ☐ Filtered pipettor tips	☐ Thaw End-It™ DNA End-Repair Kit reagents on ice.
Ligate LMP CAP Adaptors to the DNA	☐ Microcentrifuge ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ LMP CAP Adaptor (ds) (50 µM) ☐ Quick Ligation™ Kit ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ QIAquick® Gel Extraction Kit ☐ Filtered pipettor tips	☐ Thaw LMP CAP Adaptor on ice. ☐ Thaw Quick Ligation™ Kit reagents on ice.
Size-select the DNA	☐ Gel box and power supply for agarose gel ☐ Gel imaging system ☐ Microcentrifuge ☐ Vortexer ☐ Picofuge ☐ Pipettors ☐ Scale ☐ NanoDrop™ ND-1000 Spectrophotometer	☐ 1×TAE or TBE buffer ☐ Agarose ☐ Gel Loading Solution ☐ 1 kb DNA Ladder ☐ Ethidium bromide ☐ Nuclease-free water ☐ Razor blades ☐ 15-mL conical polypropylene tubes ☐ QIAquick® Gel Extraction Kit ☐ Isopropyl alcohol ☐ 1.5-mL LoBind tubes	☐ Prepare 1× TAE or 1× TBE buffer. ☐ Prepare a 0.8% or 1.0% agarose gel.
Circularize the DNA	☐ Microcentrifuge ☐ NanoDrop™ ND-1000 Spectrophotometer ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ Quick Ligation™ Kit ☐ Internal Adaptor (ds) ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ QIAquick® Gel Extraction Kit ☐ Filtered pipettor tips	☐ Thaw Internal Adaptor on ice. ☐ Thaw Quick Ligation™ Kit on ice.

	Equipment	Reagents	Preparation steps
Treat the DNA with Plasmid-Safe™ DNase	☐ Microcentrifuge ☐ NanoDrop™ ND-1000 Spectrophotometer ☐ Incubator (37 °C) ☐ Incubator (70 °C) ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ Plasmid-Safe™ ATP-Dependent DNase Kit ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ QIAquick® Gel Extraction Kit ☐ Filtered pipettor tips	☐ Thaw Plasmid-Safe™ ATP-Dependent DNase Kit on ice.
Nick-translate the library	☐ Microcentrifuge ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ dNTP Mix (100 mM, 25 mM each) ☐ 10× NEBuffer 2 ☐ DNA Polymerase I (10 U/µL) ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ QIAquick® Gel Extraction Kit ☐ Isopropyl alcohol ☐ Filtered pipettor tips ☐ Ice	☐ Thaw dNTP Mix and 10× NEBuffer 2 on ice.
Digest the DNA	☐ Incubator (37 °C) ☐ Microcentrifuge ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ T7 exonuclease ☐ 10× NEBuffer 4 ☐ 10× S1 Nuclease Buffer ☐ S1 Nuclease ☐ 5 M Sodium chloride ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ QIAquick® Gel Extraction Kit ☐ Isopropyl alcohol ☐ Filtered pipettor tips ☐ Ice	☐ Thaw NEBuffer 4 and Nuclease Buffer on ice.
End-repair the digested DNA	☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ 500 mM Tris-HCl (pH 7.5) ☐ 5 M Sodium chloride ☐ 500 mM EDTA ☐ End-It™ DNA End-Repair Kit ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ QIAquick® Gel Extraction Kit ☐ Filtered pipettor tips	☐ Thaw End-It™ DNA End-Repair Kit reagents on ice.
Bind the library molecules to beads	☐ Vortexer ☐ Picofuge ☐ 6 Tube Magnetic Rack ☐ Rotator ☐ Pipettors	☐ 100× BSA ☐ Dynal® MyOne™ streptavidin C1 beads ☐ 1× Bead Wash Buffer ☐ 1× Bind & Wash Buffer ☐ Quick Ligase Buffer ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ Filtered pipettor tips	☐ Thaw 100× BSA and Quick Ligase Buffer on ice.

	Equipment	Reagents	Preparation steps
Ligate P1 and P2 Adaptors the DNA	☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ Quick Ligation™ Kit ☐ P1 Adaptor (ds) ☐ P2 Adaptor (ds) ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ Filtered pipettor tips	☐ Thaw P1 Adaptor (ds) and P2 Adaptor (ds) on ice. ☐ Thaw Quick Ligation™ Kit on ice.
Wash the DNA-bound beads	☐ Vortexer ☐ Picofuge ☐ 6 Tube Magnetic Rack ☐ Rotator ☐ Pipettors	☐ 1× Bead Wash Buffer ☐ 1× Bind & Wash Buffer ☐ 10× NEBuffer 2 ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ Filtered pipettor tips	☐ Thaw 100× BSA and 10× NEBuffer 2 on ice. ☐ Prepare 1× NEBuffer 2 if necessary.
Nick-translate library	☐ Incubator (16 °C) ☐ 6 Tube Magnetic Rack ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ dNTP Mix (100 mM 25 mM each) ☐ DNA Polymerase I (10 U/µL) ☐ Buffer EB (Qiagen) ☐ 1.5-mL LoBind tubes ☐ Filtered pipettor tips	☐ Thaw dNTP Mix on ice.
Amplify the library	☐ Thermal cycler ☐ FlashGel® apparatus ☐ Pipettors	☐ Library PCR Primer 1 ☐ Library PCR Primer 2 ☐ Library PCR Master Mix ☐ Pfu polymerase ☐ Nuclease-free water ☐ FlashGel® Loading Dye ☐ FlashGel® DNA Marker ☐ 2.2% FlashGel® ☐ PCR strip tubes ☐ Filtered pipettor tips	☐ Thaw Library PCR Primers 1 and 2 on ice. ☐ Rinse FlashGel® wells with water.
Gel-purify the library	☐ Gel box and power supply for agarose gel ☐ Gel imaging system ☐ Microcentrifuge ☐ Vortexer ☐ Picofuge ☐ Pipettors ☐ Scale	☐ 1×TAE or TBE buffer ☐ Agarose ☐ Gel Loading Solution ☐ TrackIt™ 25 bp Ladder ☐ Ethidium bromide ☐ Nuclease-free water ☐ Razor blades ☐ 15-mL conical polypropylene tubes ☐ QIAquick® Gel Extraction Kit ☐ Isopropyl alcohol ☐ 1.5-mL LoBind tubes ☐ Filtered pipettor tips	☐ Prepare 1× TAE or 1× TBE buffer. ☐ Prepare a 4% agarose gel.
Quantitate	☐ Real-time PCR system	☐ TaqMan or SYBR Green assay	

Workflow tracking: prepare a 2 × 50 bp mate-paired library

Sample:

Quantitation	
Step	**Quantity of DNA**
Starting Amount	
Shearing the DNA	
End-Repair	
Size-Selection	
Plasmid-Safe™ DNase Treatment	
Quantitative PCR	

Lot number	
Step	**Lot number**
SOLiD™ Library Oligos Kit 1	
P1 Adaptor	
P2 Adaptor	
Library PCR Primer 1	
Library PCR Primer 2	
SOLiD™ Library Oligos Kit 2	
LMP CAP Adaptor	
Internal Adaptor	
Library PCR Master Mix	

Sample:

Quantitation	
Step	**Quantity of DNA**
Starting Amount	
Shearing the DNA	
End-Repair	
Size-Selection	
Plasmid-Safe™ DNase Treatment	
Quantitative PCR	

Lot number	
Step	**Lot number**
SOLiD™ Library Oligos Kit 1	
P1 Adaptor	
P2 Adaptor	
Library PCR Primer 1	
Library PCR Primer 2	
SOLiD™ Library Oligos Kit 2	
LMP CAP Adaptor	
Internal Adaptor	
Library PCR Master Mix	

Sample:

Quantitation	
Step	**Quantity of DNA**
Starting Amount	
Shearing the DNA	
End-Repair	
Size-Selection	
Plasmid-Safe™ DNase Treatment	
Quantitative PCR	

Lot number	
Step	**Lot number**
SOLiD™ Library Oligos Kit 1	
P1 Adaptor	
P2 Adaptor	
Library PCR Primer 1	
Library PCR Primer 2	
SOLiD™ Library Oligos Kit 2	
LMP CAP Adaptor	
Internal Adaptor	
Library PCR Master Mix	

Sample:

Quantitation	
Step	**Quantity of DNA**
Starting Amount	
Shearing the DNA	
End-Repair	
Size-Selection	
Plasmid-Safe™ DNase Treatment	
Quantitative PCR	

Lot number	
Step	**Lot number**
SOLiD™ Library Oligos Kit 1	
P1 Adaptor	
P2 Adaptor	
Library PCR Primer 1	
Library PCR Primer 2	
SOLiD™ Library Oligos Kit 2	
LMP CAP Adaptor	
Internal Adaptor	
Library PCR Master Mix	

Workflow checklists: prepare a 2 × 25 bp mate-paired library

	Equipment	Reagents	Preparation Steps
Shear the DNA with Covaris™ S2 System	☐ Covaris™ S2 System ☐ Microcentrifuge ☐ NanoDrop™ ND-1000 Spectrophotometer ☐ Pipettors	☐ 1 M Tris, pH 8.0 ☐ Nuclease-free water ☐ Ethylene glycol ☐ Glycerol ☐ Covaris™ Tubes and Caps ☐ 1.5-mL LoBind tubes ☐ QIAquick® Gel Extraction Kit ☐ Filtered pipettor tips	☐ Degas the water in the Covaris™ S2 System 30 minutes prior to use. ☐ Supplement the circulated water chiller with 20% ethylene glycol.
Shear the DNA with HydroShear®	☐ HydroShear® ☐ Microcentrifuge ☐ NanoDrop™ ND-1000 Spectrophotometer ☐ Pipettors	☐ Nuclease-free water ☐ 0.2 N HCl ☐ 0.2 N NaOH ☐ 1.5-mL LoBind tubes ☐ QIAquick® Gel Extraction Kit ☐ Filtered pipettor tips	
End-repair the DNA	☐ Microcentrifuge ☐ NanoDrop™ ND-1000 Spectrophotometer ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ End-It™ DNA End-Repair Kit ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ QIAquick® Gel Extraction Kit ☐ Filtered pipettor tips	☐ Thaw End-It™ DNA End-Repair Kit reagents on ice.
Methylate the genomic EcoP15I sites	☐ Incubator (37 °C) ☐ Microcentrifuge ☐ NanoDrop™ ND-1000 Spectrophotometer ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ 10× NEBuffer 3 ☐ 100× BSA ☐ DNA Polymerase I (10 U/µL) ☐ S-adenosylmethionine ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ QIAquick® Gel Extraction Kit ☐ Filtered pipettor tips	☐ Thaw 10× NEBuffer 3, 100× BSA, and S-adenosylmethionine on ice.
Ligate EcoP15I Adaptors to methylated DNA	☐ Microcentrifuge ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ EcoP15I CAP Adaptor (ds) (50 µM) ☐ Quick Ligation™ Kit ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ QIAquick® Gel Extraction Kit ☐ Filtered pipettor tips	☐ Thaw EcoP15I CAP Adaptor on ice. ☐ Thaw Quick Ligation Kit reagents on ice.
Size-select the DNA	☐ Gel box and power supply for agarose gel ☐ Gel imaging system ☐ Microcentrifuge ☐ Vortexer ☐ Picofuge ☐ Pipettors ☐ Scale ☐ NanoDrop™ ND-1000 Spectrophotometer	☐ 1×TAE or TBE buffer ☐ Agarose ☐ Gel Loading Solution ☐ 1 kb DNA Ladder ☐ Ethidium bromide ☐ Nuclease-free water ☐ Razor blades ☐ 15-mL conical polypropylene tubes ☐ QIAquick® Gel Extraction Kit ☐ Isopropyl alcohol ☐ 1.5-mL LoBind tubes	☐ Prepare 1× TAE or 1× TBE buffer. ☐ Prepare a 0.8% or 1.0% agarose gel.
Circularize the DNA	☐ Microcentrifuge ☐ NanoDrop™ ND-1000 Spectrophotometer ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ Quick Ligation™ Kit ☐ Internal Adaptor (ds) ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ QIAquick® Gel Extraction Kit ☐ Filtered pipettor tips	☐ Thaw Internal Adaptor on ice. ☐ Thaw Quick Ligation™ Kit on ice.

	Equipment	Reagents	Preparation Steps
Treat the DNA with Plasmid-Safe™ DNase	☐ Microcentrifuge ☐ NanoDrop™ ND-1000 Spectrophotometer ☐ Incubator (37 °C) ☐ Incubator (70 °C) ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ Plasmid-Safe™ ATP-Dependent DNase Kit ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ QIAquick® Gel Extraction Kit ☐ Filtered pipettor tips	☐ Thaw Plasmid-Safe™ ATP-Dependent DNase Kit on ice.
Digest the DNA	☐ Incubator (37 °C) ☐ Incubator (65 °C) ☐ Vortexer ☐ Scale ☐ Picofuge ☐ Pipettors	☐ 10× NEBuffer 3 ☐ 100× BSA ☐ Sinefungin ☐ 10× ATP ☐ EcoP15I Enzyme (10 U/µL) ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ Filtered pipettor tips ☐ Ice	☐ Prepare 10 mM Sinefungin ☐ Thaw 10× NEBuffer 3, 100× BSA, 10× ATP on ice
End-repair with Klenow	☐ Incubator (65 °C) ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ dNTP Mix (100 mM, 25 mM each) ☐ DNA polymerase, Klenow large fragment ☐ 1.5-mL LoBind tubes ☐ Filtered pipettor tips ☐ Ice	☐ Thaw dNTP Mix on ice
Bind the library molecules to beads	☐ Vortexer ☐ Picofuge ☐ 6 Tube Magnetic Rack ☐ Rotator ☐ Pipettors	☐ 100× BSA ☐ Dynal® MyOne™ streptavidin C1 beads ☐ 1× Bead Wash Buffer ☐ 1× Bind & Wash Buffer ☐ Quick Ligase Buffer ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ Filtered pipettor tips	☐ Thaw 100× BSA and Quick Ligase Buffer on ice.
Ligate P1 and P2 Adaptors the DNA	☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ Quick Ligation™ Kit ☐ P1 Adaptor (ds) ☐ P2 Adaptor (ds) ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ Filtered pipettor tips	☐ Thaw P1 Adaptor (ds) and P2 Adaptor (ds) on ice. ☐ Thaw Quick Ligation™ Kit on ice.
Wash the DNA-bound beads	☐ Vortexer ☐ Picofuge ☐ 6 Tube Magnetic Rack ☐ Rotator ☐ Pipettors	☐ 1× Bead Wash Buffer ☐ 1× Bind & Wash Buffer ☐ 10× NEBuffer 2 ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ Filtered pipettor tips	☐ Thaw 100× BSA and 10× NEBuffer 2 on ice. ☐ Prepare 1× NEBuffer 2 if necessary.
Nick-translate library	☐ Incubator (16 °C) ☐ 6 Tube Magnetic Rack ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ dNTP Mix (100 mM 25 mM each) ☐ DNA Polymerase I (10 U/µL) ☐ Buffer EB (Qiagen) ☐ 1.5-mL LoBind tubes ☐ Filtered pipettor tips	☐ Thaw dNTP Mix on ice.

	Equipment	Reagents	Preparation Steps
Amplify the library	☐ Thermal cycler ☐ FlashGel® apparatus ☐ Pipettors	☐ Library PCR Primer 1 ☐ Library PCR Primer 2 ☐ Library PCR Master Mix ☐ Pfu polymerase ☐ Nuclease-free water ☐ FlashGel® Loading Dye ☐ FlashGel® DNA Marker ☐ 2.2% FlashGel® ☐ PCR strip tubes ☐ Filtered pipettor tips	☐ Thaw Library PCR Primers 1 and 2 on ice. ☐ Rinse FlashGel® wells with water.
Gel-purify the library	☐ Gel box and power supply for agarose gel ☐ Gel imaging system ☐ Microcentrifuge ☐ Vortexer ☐ Picofuge ☐ Pipettors ☐ Scale	☐ 1×TAE or TBE buffer ☐ Agarose ☐ Gel Loading Solution ☐ TrackIt™ 25 bp Ladder ☐ Ethidium bromide ☐ Nuclease-free water ☐ Razor blades ☐ 15-mL conical polypropylene tubes ☐ QIAquick® Gel Extraction Kit ☐ Isopropyl alcohol ☐ 1.5-mL LoBind tubes ☐ Filtered pipettor tips	☐ Prepare 1× TAE or 1× TBE buffer. ☐ Prepare a 4% agarose gel.
Quantitate	☐ Real-time PCR system	☐ TaqMan or SYBR Green assay	

Workflow tracking: prepare a 2 × 25 bp mate-paired library

Sample:

Quantitation	
Step	**Quantity of DNA**
Starting Amount	
Shearing the DNA	
End-Repair	
Methylation of Genomic EcoP15I	
Size-Selection	
Plasmid-Safe™ DNase Treatment	
Quantitative PCR	

Lot number	
Step	**Lot number**
SOLiD™ Library Oligos Kit 1	
P1 Adaptor	
P2 Adaptor	
Library PCR Primer 1	
Library PCR Primer 2	
SOLiD™ Library Oligos Kit 2	
EcoP15I CAP Adaptor	
Internal Adaptor	

Sample:

Quantitation	
Step	**Quantity of DNA**
Starting Amount	
Shearing the DNA	
End-Repair	
Methylation of Genomic EcoP15I	
Size-Selection	
Plasmid-Safe™ DNase Treatment	
Quantitative PCR	

Lot number	
Step	**Lot number**
SOLiD™ Library Oligos Kit 1	
P1 Adaptor	
P2 Adaptor	
Library PCR Primer 1	
Library PCR Primer 2	
SOLiD™ Library Oligos Kit 2	
EcoP15I CAP Adaptor	
Internal Adaptor	

Sample:

Quantitation	
Step	**Quantity of DNA**
Starting Amount	
Shearing the DNA	
End-Repair	
Methylation of Genomic EcoP15I	
Size-Selection	
Plasmid-Safe™ DNase Treatment	
Quantitative PCR	

Lot number	
Step	**Lot number**
SOLiD™ Library Oligos Kit 1	
P1 Adaptor	
P2 Adaptor	
Library PCR Primer 1	
Library PCR Primer 2	
SOLiD™ Library Oligos Kit 2	
EcoP15I CAP Adaptor	
Internal Adaptor	

Sample:

Quantitation	
Step	**Quantity of DNA**
Starting Amount	
Shearing the DNA	
End-Repair	
Methylation of Genomic EcoP15I	
Size-Selection	
Plasmid-Safe™ DNase Treatment	
Quantitative PCR	

Lot number	
Step	**Lot number**
SOLiD™ Library Oligos Kit 1	
P1 Adaptor	
P2 Adaptor	
Library PCR Primer 1	
Library PCR Primer 2	
SOLiD™ Library Oligos Kit 2	
EcoP15I CAP Adaptor	
Internal Adaptor	

Workflow checklists: prepare a barcoded fragment library

	Equipment	Reagents	Preparation steps
Shear the DNA	☐ Covaris™ S2 System ☐ Covaris microTube adaptor ☐ Covaris microTube loading station ☐ Pipettors	☐ 1× Low TE Buffer ☐ Ethylene glycol ☐ Covaris™ microTube ☐ 1.5-mL LoBind tubes ☐ Ethylene glycol ☐ Filtered pipettor tips	☐ Degas the water in the Covaris™ S2 System 30 minutes prior to use. ☐ Supplement the circulated water chiller with 20% ethylene glycol.
End-repair the DNA	☐ Microcentrifuge ☐ NanoDrop™ ND-1000 Spectrophotometer ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ 5× End-Polishing Buffer ☐ dNTP Mix ☐ End Polishing Enzyme 1 ☐ End Polishing Enzyme 2 ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ PureLink™ PCR Purification Kit ☐ Filtered pipettor tips	☐ Thaw 5× End-Polishing Buffer and dNTP Mix on ice.
Ligate P1 and P2 Adaptors to the DNA	☐ Microcentrifuge ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ Multiplex P1 Adaptor (ds) (50 µM) ☐ Multiplex P2 Adaptor (ds) (50 µM) ☐ 5× T4 Ligase Buffer ☐ T4 Ligase ☐ Nuclease-free water ☐ 1.5-mL LoBind tubes ☐ PureLink™ PCR Purification Kit ☐ Filtered pipettor tips	☐ Thaw P1 and P2 Adaptors on ice. ☐ Thaw5× T4 Ligase Buffer on ice.
Nick-translate, then amplify the library	☐ Thermal cycler ☐ Microcentrifuge ☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ Multiplex Library PCR Primer 1 ☐ Library PCR Primer 2 ☐ Platinum® PCR Amplification Mix ☐ PureLink™ PCR Purification Kit ☐ 1.5-mL LoBind tube ☐ PCR strip tubes ☐ Filtered pipettor tips	☐ Thaw Library PCR Primers 1 and 2 on ice. ☐ Thaw Platinum® PCR Amplification Mix on ice.
Quantitate	☐ Real-time PCR system	☐ TaqMan or SYBR Green assay	
Pool the barcoded libraries	☐ Vortexer ☐ Picofuge ☐ Pipettors	☐ 1.5-mL LoBind tubes ☐ Filtered pipettor tips	
Gel-purify the libraries	☐ iBase™ ☐ E-gel Safe Imager™ ☐ Pipettors	☐ E-Gel® 2% SizeSelect™ gel ☐ 50 bp DNA Ladder ☐ Nuclease-free water ☐ Filtered pipettor tips	☐ Thaw 50 bp DNA Ladder on ice.

Workflow tracking: prepare a barcoded fragment library

Sample:			**Barcode:**
Quantitation		**Lot number**	
Step	**Quantity of DNA**	**Step**	**Lot number**
Starting Amount		Multiplex P1 Adaptor	
End-Repair		Multiplex P2 Adaptor	
Quantitative PCR		Multiplex Library PCR Primer 1	
		Library PCR Primer 2	

Sample:			**Barcode:**
Quantitation		**Lot number**	
Step	**Quantity of DNA**	**Step**	**Lot number**
Starting Amount		Multiplex P1 Adaptor	
End-Repair		Multiplex P2 Adaptor	
Quantitative PCR		Multiplex Library PCR Primer 1	
		Library PCR Primer 2	

Sample:			**Barcode:**
Quantitation		**Lot number**	
Step	**Quantity of DNA**	**Step**	**Lot number**
Starting Amount		Multiplex P1 Adaptor	
End-Repair		Multiplex P2 Adaptor	
Quantitative PCR		Multiplex Library PCR Primer 1	
		Library PCR Primer 2	

Sample:			**Barcode:**
Quantitation		**Lot number**	
Step	**Quantity of DNA**	**Step**	**Lot number**
Starting Amount		Multiplex P1 Adaptor	
End-Repair		Multiplex P2 Adaptor	
Quantitative PCR		Multiplex Library PCR Primer 1	
		Library PCR Primer 2	

Sample:			**Barcode:**
Quantitation		**Lot Number**	
Step	**Quantity of DNA**	**Step**	**Lot number**
Starting Amount		Multiplex P1 Adaptor	
End-Repair		Multiplex P2 Adaptor	
Quantitative PCR		Multiplex Library PCR Primer 1	
		Library PCR Primer 2	

G Covaris™ S2 System

This appendix covers:

Operation notes

Fill the tank

Fill the tank with fresh deionized water to the proper fill line. The water should cover the visible part of the tube.

Degas the water

Degas the water for 30 minutes. To maintain degassed water, keep the pump continuously on during operation and sample processing.

Set the chiller

Set the chiller temperature to between 2 to 5 °C to ensure that the temperature reading in the water bath displays 5 °C. The circulated water chiller should be supplemented with 20% ethylene glycol.

Perform required maintenance of the Covaris™ S2 System

The Covaris S2 System requires regular maintenance to work properly. Perform the tasks in the table below (see Table 74):

Table 74 Required maintenance of the Covaris™ S2 System

Required maintenance task	Frequency to perform task
Degas water for 30 minutes prior to use	Before every use
Change water	Daily
Clean with bleach	Every two weeks

Covaris™ S2 Programs

***Fragment* library preparation (standard, express, and barcoded)**

Program the Covaris™ S2 System:

- Number of Cycles: **6**
- Bath Temperature: **5** °C
- Bath Temperature Limit: **30** °C
- Mode: **Frequency sweeping**
- Water Quality Testing Function: **Off**
- Duty cycle: **20%**
- Intensity: **5**
- Cycles/burst: **200**
- Time: **60 seconds**

IMPORTANT! Set the chiller temperature to between 2 to 5 °C to ensure that the temperature reading in the water bath displays 5 °C. The circulated water chiller should be supplemented with 20% ethylene glycol.

Mate-paired library preparation

Table 75 Recommended shearing conditions or desired mate-paired library insert sizes.

Insert size	Shearing method	Shearing conditions
600 to 800 bp	Covaris™ Shearing in 20% glycerol (13 mm × 65 mm borosilicate tube)	• Number of Cycles: **75** • Bath Temperature: **5 °C** • Bath Temperature Limit: **12 °C** • Mode: **Frequency sweeping** • Water Quality Testing Function: **Off** • Duty cycle: **2%** • Intensity: **7** • Cycles/burst: **200** • Time: **10 seconds**
800 to 1000 bp	Covaris™ Shearing in 20% glycerol (13 mm × 65 mm borosilicate tube)	• Number of Cycles: **30** • Bath Temperature: **5 °C** • Bath Temperature Limit: **12 °C** • Mode: **Frequency sweeping** • Water Quality Testing Function: **Off** • Duty cycle: **2%** • Intensity: **5** • Cycles/burst: **200** • Time: **10 seconds**

H Instrument Warranty Information

This appendix covers:

Computer configuration

Applied Biosystems supplies or recommends certain configurations of computer hardware, software, and peripherals for use with its instrumentation. Applied Biosystems reserves the right to decline support for or impose extra charges for supporting nonstandard computer configurations or components that have not been supplied or recommended by Applied Biosystems. Applied Biosystems also reserves the right to require that computer hardware and software be restored to the standard configuration prior to providing service or technical support. For systems that have built-in computers or processing units, installing unauthorized hardware or software may void the Warranty or Service Plan.

Limited product warranty

Applied Biosystems warrants that all standard components of the SOLiD™ 3 Analyzer, IKA® ULTRA-TURRAX® Tube Drive, the Covaris™ S2 System, APC UPS, and the recirculating chiller will be free of defects in materials and workmanship for a period of one (1) year from the date the warranty period begins. Applied Biosystems will repair or replace, at its discretion, all defective components during this warranty period. Applied Biosystems warrants the Genomic Solutions Hydroshear will be free of defects in materials and workmanship for a period of one (1) year from the date the warranty period begins. Applied Biosystems will replace a defective Hydroshear during the warranty period. The following parts of the Hydroshear are use- replaceable and not covered by the warranty on the Hydroshear: shearing assembly, syringes, syringe adapters, syringe shields, and output tubing. Applied Biosystems reserves the right to use new, repaired, or refurbished instruments or components for warranty and post-warranty service agreement replacements. Repair or replacement of products or components that are under warranty does not extend the original warranty period.

Applied Biosystems warrants that all optional accessories supplied with its SOLiD 3 Analyzer, such as peripherals, printers, and special monitors, will be free of defects in materials and workmanship for a period of ninety (90) days from the date the warranty begins. Applied Biosystems will repair or replace, at its discretion, defective accessories during this warranty period. After this warranty period, Applied Biosystems will pass on to the buyer, to the extent that it is permitted to do so, the warranty of the original manufacturer for such accessories.

With the exception of consumable and maintenance items, replaceable products or components used on or in the instrument are themselves warranted to be free of defects in materials and workmanship for a period of ninety (90) days.

Applied Biosystems warrants that chemicals and other consumable products will be free of defects in materials and workmanship when received by the buyer, but not thereafter, unless otherwise specified in documentation accompanying the product.

Applied Biosystems warrants that for a period of ninety (90) days from the date the warranty period begins, the tapes, diskettes, or other media bearing the operating software of the product, if any, will be free of defects in materials and workmanship under normal use. If there is a defect in the media covered by the above warranty and the media is returned to Applied Biosystems within the ninety (90) day warranty period, Applied Biosystems will replace the defective media.

Unless indicated herein, Applied Biosystems makes no warranty whatsoever in regard to products or parts furnished by third parties, including but not limited to the non-APC- branded UPS or APC UPS, Covaris S2, Genomic Solutions Hydroshear, Reciruclating Chiller, and IKA ULTRA-TURRAX purchased or obtained from a third party. Such products or parts will be subject to the warranties, if any, of their respective manufacturers to the extent they are ‘transferable or otherwise available to Applied Biosystems' buyer.

Applied Biosystems at its sole discretion may refuse to provide buyer with support or service for buyer's use of Covaris S2 in a method not described in a SOLiD System protocol.

Applied Biosystems does not warrant that the operation of the instrument or its operating software will be uninterrupted or be error-free.

Warranty period effective date

Any applicable warranty period under these sections begins on the earlier of the date of installation or ninety (90) days from the date of shipment for hardware and software installed by Applied Biosystems personnel. For all hardware and software installed by the buyer or anyone other than Applied Biosystems, and for all other products, the applicable warranty period begins the date the product is delivered to the buyer.

Warranty claims

Warranty claims must be made within the applicable warranty period, or, for chemicals or other consumable products, within thirty (30) days after receipt by the buyer unless otherwise specified in the documentation accompanying the product.

Warranty exceptions

The above warranties do not apply to defects resulting from misuse, neglect, or accident, including without limitation: operation with incompatible solvents or samples in the system; operation outside of the environmental or use specifications or not in conformance with the instructions for the instrument system, software, or accessories; improper or inadequate maintenance by the user; installation of software or interfacing, or use in combination with software or products, not supplied or authorized by Applied Biosystems; modification or repair of the product not authorized by Applied

Biosystems; relocation or movement of the instrument by buyer or by any third party not acting on behalf of Applied Biosystems; or intrusive activity, including without limitation, computer viruses, hackers or other unauthorized interactions with instrument or software that detrimentally affects normal operations.

Parts in contact with any liquid are considered wetted and may be deemed user-replaceable and not be covered by the above warranties, including, but not limited to, seals, filters, gaskets, shearing assemblies, valves, syringes, syringe adapters, syringe shields, and output tubing.

Warranty limitations

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Some countries or jurisdictions limit the scope of or preclude limitations or exclusion of warranties, of liability, such as liability for gross negligence or willful misconduct, or of remedies or damages, as or to the extent set forth above. In such countries and jurisdictions, the limitation or exclusion of warranties, liability, remedies or damages set forth above shall apply to the fullest extent permitted by law, and shall not apply to the extent prohibited by law.

Damages, claims, and returns

Damages

If shipping damage to the product is discovered, contact the shipping carrier and request inspection by a local agent. Secure a written report of the findings to support any claim. Do not return damaged goods to Applied Biosystems without first securing an inspection report and contacting Applied Biosystems Technical Support for a Return Authorization (RA) number.

Claims

After a damage inspection report is received by Applied Biosystems, Applied Biosystems will process the claim unless other instructions are provided.

Returns

Do not return any material without prior notification and authorization.

If for any reason it becomes necessary to return material to Applied Biosystems, contact Applied Biosystems Technical Support or your nearest Applied Biosystems subsidiary or distributor for a return authorization (RA) number and forwarding address. Place the RA number in a prominent location on the outside of the shipping container, and return the material to the address designated by the Applied Biosystems representative.

I Safety

This appendix covers:

Instrumentation safety

Note: For important instrument safety information, refer to the *Applied Biosystems SOLiD™ 3 System Instrument Operation Guide* (PN 4407430). For general safety information, see the "Preface" on page ix.

General instrument safety

Operating the instrument

Ensure that anyone who operates the instrument has:

- Received instructions in both general safety practices for laboratories and specific safety practices for the instrument.
- Read and understood all applicable Material Safety Data Sheets (MSDSs). See "About MSDSs" on page 245.

Cleaning or decontaminating the instrument

CAUTION! Before using a cleaning or decontamination method other than those recommended by the manufacturer, verify with the manufacturer that the proposed method will not damage the equipment.

Physical hazard safety

Moving parts

WARNING! PHYSICAL INJURY HAZARD. Moving parts can crush and cut. Keep hands clear of moving parts while operating the instrument. Disconnect power before servicing the instrument.

WARNING! PHYSICAL INJURY HAZARD. Do not operate the instrument without the arm shield in place. Keep hands out of the deck area when the instrument is spotting.

Solvents and pressurized fluids

WARNING! PHYSICAL INJURY HAZARD. Always wear eye protection when working with solvents or any pressurized fluids.

WARNING! PHYSICAL INJURY HAZARD. To avoid hazards associated with high-pressure fluids in polymeric tubing:

- Be aware that PEEK™ tubing is a polymeric material. Use caution when working with any polymer tubing that is under pressure.

  Always wear eye protection when near pressurized polymer tubing.
- Extinguish all nearby flames if you use flammable solvents.
- Do not use PEEK tubing that has been severely stressed or kinked.
- Do not use PEEK tubing with tetrahydrofuran or nitric and sulfuric acids.
- Be aware that methylene chloride and dimethyl sulfoxide cause PEEK tubing to swell and greatly reduce the rupture pressure of the tubing.
- Be aware that high solvent flow rates (~40 mL/min) may cause a static charge to build up on the surface of the tubing. Electrical sparks may result.

Chemical safety

General chemical safety

WARNING! CHEMICAL HAZARD. Before handling any chemicals, refer to the Material Safety Data Sheet (MSDS) provided by the manufacturer, and observe all relevant precautions.

WARNING! CHEMICAL HAZARD. All chemicals in the instrument, including liquid in the lines, are potentially hazardous. Always determine what chemicals have been used in the instrument before changing reagents or instrument components. Wear appropriate eyewear, protective clothing, and gloves when working on the instrument.

WARNING! CHEMICAL HAZARD. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

WARNING! CHEMICAL STORAGE HAZARD. Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

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. (See "About MSDSs" on page 245.)
- 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

The MSDS for any chemical supplied by Applied Biosystems is available to you free 24 hours a day. To obtain MSDSs:

1. Go to **www.appliedbiosystems.com**, click **Support**, then select **MSDS**.
2. In the Keyword Search field, enter the chemical name, product name, MSDS part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose

   Note: For the MSDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

Chemical waste safety

Chemical waste hazards

CAUTION! HAZARDOUS WASTE. Refer to Material Safety Data Sheets and local regulations for handling and disposal.

WARNING! CHEMICAL WASTE HAZARD. Wastes produced by Applied Biosystems instruments are potentially hazardous and can cause injury, illness, or death.

WARNING! CHEMICAL STORAGE HAZARD. Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

Chemical waste safety guidelines

To minimize the hazards of chemical waste:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- 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.
- Handle chemical wastes in a fume hood.
- After emptying a waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.

- Ensure that the instrument waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

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

Safety alerts

Chemical alerts

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

General alerts for all chemicals

Avoid contact with (skin, eyes, and/or clothing). Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Specific chemical alerts

WARNING! CHEMICAL HAZARD. AmpliTaq® DNA Polymerase may cause eye, skin, and respiratory tract irritation. May be harmful if swallowed. Avoid breathing vapor. Use with adequate ventilation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

WARNING! CHEMICAL HAZARD. 1×Bead Wash Buffer causes severe eye irritation. It also causes skin, and respiratory tract irritation. Avoid breathing vapor. Use with adequate ventilation.

CAUTION! CHEMICAL HAZARD. 1× Bind & Wash Buffer may cause eye, skin, and respiratory tract irritation. It may be harmful if swallowed. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

WARNING! CHEMICAL HAZARD. Ethidium bromide causes eye, skin, and respiratory tract irritation. It is a known mutagen. It can change genetic material in a living cell and has the potential to cause cancer. It also causes eye, skin, and respiratory tract irritation. Avoid breathing vapor. Use with adequate ventilation.

WARNING! CHEMICAL HAZARD. Gel loading solution.

CAUTION! CHEMICAL HAZARD. Glycerol causes eye, skin, and respiratory tract irritation. Avoid breathing vapor. Use with adequate ventilation. May be harmful if swallowed.

CAUTION! CHEMICAL HAZARD. 1× Low Salt Binding Buffer may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

WARNING! CHEMICAL HAZARD. Magnesium chloride causes eye, skin, and respiratory tract irritation. Avoid breathing vapor. Use with adequate ventilation.

WARNING! CHEMICAL HAZARD. Phenol-chloroform-isoamyl alcohol (with pH 7.9 buffer) is extremely toxic. Phenol and chloroform are highly corrosive to the skin and eyes. Chloroform is a potential human carcinogen.

WARNING! CHEMICAL HAZARD. 3 M Sodium acetate causes eye, skin, and respiratory tract irritation. Avoid breathing vapor. Use with adequate ventilation.

WARNING! CHEMICAL HAZARD. SYBR® Green PCR Master Mix may cause eye, skin, and respiratory tract irritation. It is readily absorbed through the skin and is a combustible liquid and vapor. Keep away from heat and flame. This

product contains material which may cause liver and blood damage.

WARNING! CHEMICAL HAZARD. 10× TAE causes eye, skin, and respiratory tract irritation. Avoid breathing vapor. Use with adequate ventilation.

WARNING! CHEMICAL HAZARD. 10× TBE causes eye, skin, and respiratory irritation. May be harmful if swallowed.

WARNING! CHEMICAL HAZARD. TRIS (Tris (hydroxymethyl)aminomethane) may cause eye, skin, and respiratory tract irritation.

Glossary

barcode	Unique sequence identifier added to the sample during library construction
barcoded fragment library	Fragment library with a barcode sequence appended to the 3′ end of the sheared DNA fragments
EcoP15I CAP Adaptor	Double-stranded oligonucleotide 7 to 9 bases long containing the Eco15I restriction sequence that is ligated to a sheared DNA insert during 2 × 25 bp mate-paired library construction
fragment library	Library consisting of a sheared DNA fragment with P1 and P2 Adaptors ligated to the 5′ end and 3′ end, respectively
Internal Adaptor	Double-stranded oligonucleotide 20 bases long, used to circularize DNA during mate-paired library construction
library	Set of DNA tags prepared from the same biological sample, to be sequenced on the SOLiD™ System
Library PCR Primer 1	Single-stranded oligonucleotide used in library amplification and corresponding to the P1 Adaptor sequence
Library PCR Primer 2	Single-stranded oligonucleotide used in library amplification and corresponding to the P2 Adaptor sequence
LMP CAP Adaptor	Double-stranded oligonucleotide 7 to 9 bases long, with a phosphate missing from one of the ends. The adaptor is ligated to a sheared DNA insert during 2 × 25 bp mate-paired library construction.
mate-paired library	Library consisting of two DNA tags a known distance apart linked by an internal adaptor with P1 and P2 Adaptors ligated to the 5′ end and 3′ end, respectively
Multiplex Library PCR Primer 1	Single-stranded oligonucleotide used in barcoded fragment library amplification and corresponding to the Multiplex P1 Adaptor sequence
Multiplex P1 Adaptor	Double-stranded oligonucleotide ligated at the 5′ end of the barcoded fragment library

Multiplex P2 Adaptor	Double-stranded oligonucleotide ligated at the 3′ end of the barcoded fragment library; contains the barcode sequence
multiplexing	Method to analyze multiple biological samples in a single spot using barcodes
P1 Adaptor	Double-stranded oligonucleotide ligated at the 5′ end of the library
P2 Adaptor	Double-stranded oligonucleotide ligated at the 3′ end of the library
tag	A length of DNA to be sequenced
templated bead preparation	Process of adding library template to beads by emulsion PCR, enriching the beads to remove beads without template, and modifying the 3′ end of the template on the beads to prepare for bead deposition and sequencing

Related documentation

Document	Part number	Description
Applied Biosystems SOLiD™ 3 System Library Preparation Quick Reference Card	4407414	Provides brief, step-by-step procedures for preparing libraries.
Applied Biosystems SOLiD™ 3 System Templated Bead Preparation Guide	4407421	Describes how to prepare templated beads by emulsion PCR (ePCR), required before sequencing on the SOLiD™ 3 System.
Applied Biosystems SOLiD™ 3 System Templated Bead Preparation Quick Reference Card	4407429	Provides brief, step-by-step procedures for preparing templated beads by emulsion PCR (ePCR), required before sequencing on the SOLiD™ 3 System.
Applied Biosystems SOLiD™ 3 System Instrument Operation Guide	4407430	Describes how to load and run the SOLiD™ 3 System for sequencing.
Applied Biosystems SOLiD™ 3 System Instrument Operation Quick Reference Card	4407431	Provides brief, step-by-step procedures for loading and running the SOLiD™ 3 System.
SOLiD™ 3 System Site Preparation Guide	4386998	Provides all the information that you need to set up the SOLiD™ 3 System.
Applied Biosystems SOLiD™ 3 System SETS Software Getting Started Guide	4389302	Describes how to monitor the run, modify run settings, and/or perform data analysis for the SOLiD™ 3 System.
Applied Biosystems SOLiD™ 3 System Instrument Control Software (ICS) Help	—	Provides convenient information for setting up a run on the SOLiD™ 3 System (see the Instrument Control Software).
SOLiD™ Analysis Tools (SAT) User Guide	4392959	Provides in-depth information on sequencing analysis with the SOLiD™ 3 System.

Note: For additional documentation, see "How to obtain support" on page x.

Send us your comments

Applied Biosystems welcomes your comments and suggestions for improving its user documents. You can e-mail your comments to:

techpubs@appliedbiosystems.com

IMPORTANT! The e-mail address above is for submitting comments and suggestions relating *only* to documentation. To order documents, download PDF files, or for help with a technical question, see "How to obtain support" on page x.

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Part Number 4407413 Rev. B 02/2009

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