iGENOMX

Version 1.04

User Manual:

RIPTIDE™ HIGH THROUGHPUT RAPID DNA LIBRARY PREP (96-WELL PLATE FORMAT)

Compatible with Illumina sequencing ■ For use with 50 ng of input DNA ■ Contains reagents for 960 reactions

Contents

I. Introduction

The iGenomX RipTide High Throughput Rapid DNA Library Prep kit is designed for the preparation of 96 next-generation sequencing DNA libraries at a time. The first step of the prep, a polymerase-mediated primer extension reaction, is performed in a 96-well plate. Following this step, reaction products are combined into one pool and all subsequent steps are performed with the single pool. A schematic depicting the sequence of steps in the library preparation procedure is shown in Figure 1.

For users working with hundreds of samples, it is possible to prepare multiple 96-well plates in parallel or serial for the primer extension reaction. After primer extension, the reaction products of each 96-well plate are combined and all downstream steps are performed with a single pool for each plate. The pooling of samples makes it practical to handle multiple pools (i.e., a few or several hundred samples) simultaneously. An index barcode, added to the library during the PCR step, acts as a plate identifier and permits the pooling of multiple 96-reaction library preps on a single Illumina flow cell. Reagents for up to ten 96-sample pools are provided with one kit.

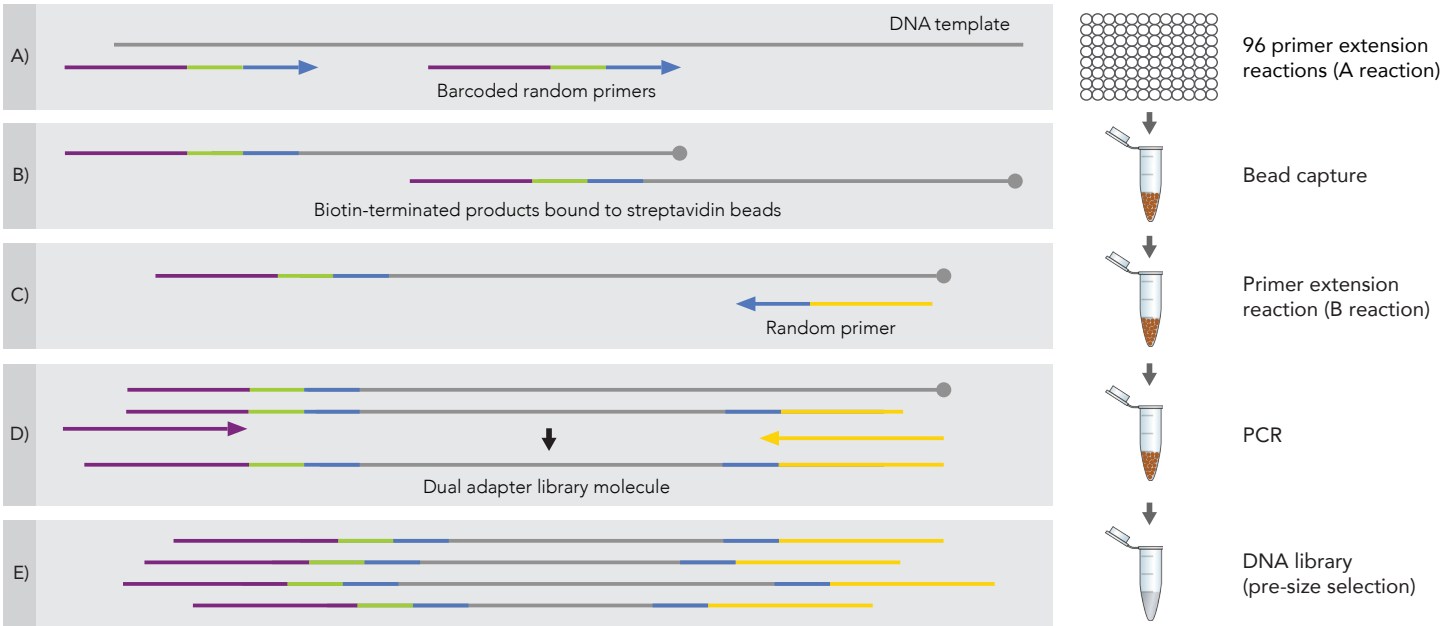

Figure 1: A schematic of the RipTide High Throughput Rapid DNA Library Prep method. (A) The DNA of interest is denatured and barcoded random primers with partial Illumina P5 adapter sequences (termed "Primer A") are used for primer extension with a DNA polymerase. Since each primer extension reaction occurs in one well of a 96-well plate and each well contains a uniquely barcoded primer, the library generated from each well is uniquely identifiable. The nucleotide mix contains a small fraction of dideoxynucleotides, which causes the primer extension reaction to self-terminate at lengths amenable to sequencing. (B) Because the dideoxynucleotides are covalently bound to biotin, the products of primer extension can be captured by streptavidin beads while other DNA fragments are washed away. (C) A second round of primer extension with a random primer with partial P7 adapter sequence (termed "Primer B") is performed on the bead-captured DNA molecules to create the complementary strand. The newly created strand has Illumina adapter sequences at both ends of the molecule. (D) PCR is performed with full length Illumina adapters. (E) This generates a double-stranded DNA library, which must undergo a final size selection before it can be loaded on a sequencing instrument. Blue lines in panels A - E refer to the random sequence in the primers; green lines indicate the sample barcode; purple and yellow lines refer to the Illumina P5 and P7 adapter sequences, respectively.

II. Primer A design

Primer A is the name given to the barcoded random primer employed in the initial primer extension step of the RipTide library prep. The primer contains a partial Illumina P5 adapter sequence at the 5′ end. The adapter sequence is followed in the 3′ direction by an eight nucleotide barcode sequence and a twelve nucleotide random sequence. The random sequence is necessary for random priming along the sample DNA template. The barcode acts as a sample identifier; there are 96 such barcodes, each unique to one well of the 96-well plate. A schematic of the A primer is shown below:

In contrast, the B primer, which is employed in the second primer extension step of the prep to generate a strand complementary to the A reaction product, does not contain a barcode sequence. It has a partial Illumina P7 adapter sequence at the 5′ end and an adjacent eight nucleotide random sequence at the 3′ end.

Two A primer plates are provided with the kit. Each plate has an identical set of eight nucleotide barcodes. The only difference between the plates is within the random sequence at the 3′ end of the A primer. In the first plate, labeled "Low GC Primer A Plate", the A primer contains, on average, less than 50% GC content in the 3′ random sequence. In contrast, in the second plate, labeled "High GC Primer A Plate", the GC content of the random sequence of the A primer is, on average, greater than 50%. The GC content of the 3′ end of the A primer affects sequencing coverage in a manner dependent on sample GC content. The availability of two sets of A primers, each with different GC content, gives the user the flexibility to customize the library prep to the GC content of the sample of interest. This is discussed further in the next section.

III. Recommendations for working with DNA of low and/or high GC content

The RipTide DNA Library Prep can be "tuned" to the GC composition of most genomes in order to improve sequence coverage of the genome of interest. Tuning is performed by changing the GC composition of the random sequence of the primer used in the initial primer extension reaction (the A primer). We provide two Primer A sets with the kit to give the user the option of tuning the library prep to GC-rich or AT-rich samples.

iGenomX provides the following guidelines for use of the two primer sets:

a. Use low GC primers for genomes of low GC content (< 50% GC).

b. Use high GC primers for genomes of high GC content (≥ 50% GC).

c. Use a combination of both primers in equal proportions for most plasmids. A similar 1:1 combination of primers can also be used for genomes of intermediate GC content (40% - 60% GC).

d. Use a combination of both primers in equal proportions for unknown samples or complex samples containing DNA from multiple sources. Alternatively, test each sample separately with the low GC and high GC primers (i.e., prepare two libraries for each sample). Be aware that a combination of low GC and high GC primers produces a better representative library of a DNA sample containing a mixture of low and high GC genomes than either the low GC primer or high GC primer alone.

Preheating the DNA sample:

The RipTide library prep protocol states that all DNA samples be heated to 98°C for 1 minute prior to the A reaction [see Step 1h of the protocol (Section IX)]. However, this step is not strictly required for samples of <50% GC content and if the user is working exclusively with low GC samples, this step may be omitted. There is no obvious benefit or detriment to heating low GC samples for a minute prior to library preparation. In contrast, this denaturation step substantially improves sequencing data generated from samples with high GC content (>50% GC) and the preheating of such samples is highly recommended.

Exceptionally high GC content:

For samples with exceptionally high GC content (average GC content >65%), iGenomX also suggests doubling the quantity of DNA used as input in the library prep (100 ng per reaction (i.e., per well) instead of 50 ng). Increasing input DNA helps to raise library yields from such samples, which often can be challenging to prepare for sequencing. Furthermore, when libraries are prepared from multiple sources with diverse GC content in a single 96-well plate, raising the input quantity of exceptionally high GC samples helps to improve the uniformity of read counts between samples.

IV. PCR cycles

Eight cycles of PCR during the amplification step of the RipTide library prep is appropriate in most circumstances. If library yields are larger or smaller than preferred, the user may alter the number of cycles. The appropriate number of PCR cycles depends on the GC content of the sample, the particular type of size selection employed in the final step of the protocol and, obviously, the yield desired by the user.

If working with a genome of low GC content and performing 8 cycles of PCR (as recommended in the protocol) and a single 1.4X SPRI bead selection, the user may obtain as much as 5 µg of library. However, a single SPRI bead selection step is far less stringent than two-step SPRI bead selection and, with the latter, the library yield may be reduced to as little as 150 ng (depending on the particular size selection method used). This is primarily because the single bead selection step produces a library with a wide range of fragment sizes (200 bp – 2500 bp), whereas the two-step bead selection protocol is designed to produce fragments of a specific size. Library yield is also inversely correlated with the GC content of the sample; thus, when working with DNA of high GC content, the yield of the library may be half or one-third of what it would be with low GC DNA.

Plasmid and metagenomic samples can be challenging templates from which to prepare RipTide libraries. If preparing RipTide libraries with these templates for the first time, it is suggested that one or two additional PCR cycles be considered for a total of 9 or 10 cycles of amplification.

V. DNA quality

High molecular weight DNA is recommended as input in the RipTide library prep. Library yields will be negatively affected if heavily fragmented DNA, DNA with substantial polysaccharide contamination or damaged or crosslinked DNA is used as input material.

VI. Genome size

The RipTide DNA Library Prep kit is designed for the sequencing to high depth of small genomes (bacterial, viral, archaeal and small fungal genomes) and plasmids, and for low depth sequencing (i.e., genome skimming) of larger genomes.

VII. Read structure

The schematic below (Figure 2) shows the RipTide library DNA fragment structure and corresponding expected read structure generated after Illumina paired-end sequencing of library molecules. Read 1 begins with eight nucleotides identifying the sample barcode, followed by twelve nucleotides corresponding to the sequence derived from the random sequence at the 3′ end of Primer A. Subsequent nucleotides are derived from the template sequence. Read 2 begins with twelve nucleotides derived from the random sequence at the 3′ end of Primer B. They are followed by template-derived nucleotides.

In the barcode demultiplexing software (more detail provided below), the read structure can be written as:

Read 1 = 8B12M+T
Read 2 = 8M+T

B refers to the in-line sample barcode, M signifies “molecular index” (i.e., sequence derived from the random sequence of the A or B primer) and T refers to the template sequence.

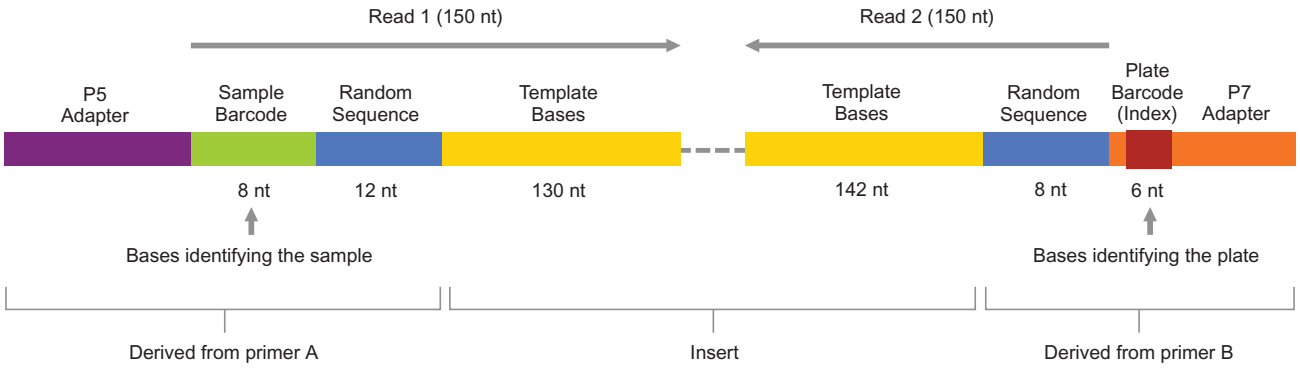

Figure 2: Logical read structure (Illumina 2 x 150 bp paired-end sequencing).

VIII. Sample demultiplexing

Open source software is available on GitHub to demultiplex the in-line sample barcodes present in Read 1. The software and associated technical documentation are available to download from the following web pages:

Software:
https://fulcrumgenomics.github.io/fgbio/

Tool Documentation:
http://fulcrumgenomics.github.io/fgbio/tools/latest/DemuxFastqs.html

Read Structure Validation:
http://fulcrumgenomics.github.io/fgbio/validate-read-structure.html

Sample Sheet Validation:
http://fulcrumgenomics.github.io/fgbio/validate-sample-sheet.html

A file containing information about the well-position and sequence of the 96 sample barcodes is available on the iGenomX website (https://igenomx.com).

IX. Protocol

This protocol allows users to perform 96 next-generation sequencing library preparations at a time. The first step of the protocol is performed in a 96-well plate with 96 individually barcoded primers that are provided with the accompanying kit. In subsequent steps, the 96 reactions are pooled and treated as a single sample.

1. Primer Extension and Termination: the "A" reaction

Each initial primer extension reaction or "A" reaction contains the following components:

2 µL	**dNTP Mix I**
1 µL	**10X Enzyme I Buffer**
2 µL	**Primer A**
1 µL	**Enzyme I**
x µL	DNA (50 ng)
y µL	**Nuclease-Free Water**
10 µL	Total

Maintain **Enzyme I** at -20°C until required. Thaw and maintain on ice the other kit reagents listed above. Follow the instructions in Step 1d before thawing the **96-well Primer A Plate**.

a. Prepare the purified DNA samples for sequencing by measuring their concentration and diluting them to a single concentration that is consistent with the volume and DNA input requirements of this library prep. A minimum sample DNA input quantity of 50 ng per reaction (or well) is recommended for the library prep. The reaction volume per well is 10 µL of which 6 µL consist of essential reagents (**dNTP Mix I**, **10X Enzyme I Buffer**, **Primer A** and **Enzyme I**). Thus, up to 4 µL of sample DNA can be added to each reaction (i.e., each well) at a starting concentration of no less than 12.5 ng/µL (12.5 ng/µL x 4 µL = 50 ng).

> **Note:** For exceptionally high GC samples (>65% GC), we recommend using a minimum of 100 ng of DNA. Please see Section III of this manual for details.

b. Transfer the total quantity of each DNA sample intended as input for the library prep (along with 10% - 20% extra material) to a tube or plate appropriate for a thermal cycler or heat block. The DNA will be heat denatured in this tube or plate immediately before the initial primer extension reaction (see Step 1h).

c. Obtain a 96-well plate compatible with the thermal cycler that the user intends to use in the first step of this protocol.

d. Choose which **96-well Primer A Plate** to use for the library prep (**Low GC Primer A Plate**, **High GC Primer A Plate** or both). Refer to Sections II and III of this manual to decide which primers are appropriate for your samples. General guidelines are provided below:

   i. Use low GC primers for genomes of low GC content (< 50% GC)

   ii. Use high GC primers for genomes of high GC content (≥ 50% GC)

   iii. Use a combination of both primers in equal proportions for most plasmids. A similar 1:1 combination of primers can also be used for genomes of intermediate GC content (40% - 60% GC).

   iv. Use a combination of both primers in equal proportions for unknown samples or complex samples containing DNA from multiple sources. Alternatively, test each sample separately with the low GC and high GC primers (i.e., prepare two libraries for each sample).

   For a combination of low GC and high GC primers, it is recommended that the user obtain a 96-well plate and transfer a small volume of the primers from one **96-well Primer A Plate** to the new plate followed by the same volume of primers from the second **96-well Primer A Plate**. Ensure that each primer is transferred to the same position from where it originated (i.e., primer in well A1 of both **96-well Primer A Plates** is transferred to well A1, primer in well B1 of both **96-well Primer A Plates** is transferred to well B1 and so on). Work carefully to avoid primer cross-contamination. Reseal all plates when finished.

> **Note:** Do not store the primer plates at 4°C. After sealing the plates, store them at -20°C.

e. Thaw the contents of the **96-well Primer A Plate(s)** chosen for use in the library prep. Briefly spin the primer plate(s) in a plate centrifuge to ensure that the contents are at the bottom of the wells. Carefully remove the adhesive seal covering the plate(s). Using a multi-channel pipette, transfer 2 µL of each primer to the empty 96-well plate that was obtained in Step 1c. Ensure that each primer is transferred to the same position from where it originated (i.e., primer in well A1 is transferred to well A1, primer in well B1 is transferred to well B1 and so on). Work carefully to avoid primer cross-contamination. Reseal the **96-well Primer A Plate(s)** with one of the adhesive seals provided with the kit and return it to the freezer. Place the 96-well plate currently in use on ice.

Note: The user can also add primers to the 96-well plate after the addition of A reaction master mix and DNA (see subsequent steps). This will reduce the likelihood of primer barcode cross-contamination during plate preparation but the user loses the ability to prepare, ahead of time, multiple plates with pre-aliquoted primers for future use.

f. Prepare an A reaction master mix for 100 reactions (96 reactions for the 96-well plate + 4 extra reactions) by combining the following reagents on ice (preferably in an Eppendorf DNA LoBind microcentrifuge tube):

200 µL	**dNTP Mix I**
100 µL	**10X Enzyme I Buffer**
100 µL	**Enzyme I**
y µL	**Nuclease-Free Water**

Where y = 400 µL – (volume of DNA to be added to a single well x 100)

g. Pipette the A reaction master mix into each well of the 96-well plate containing primer. Make certain to change pipette tips between wells. The volume of A reaction master mix added to each well is 8 µL minus the volume of DNA to be added to the well. Keep the plate on ice during this and subsequent steps.

h. Incubate the prepared DNA samples (review Steps 1a & 1b) at 98°C for 1 minute in a thermal cycler or heat block. Transfer the samples to ice immediately thereafter and maintain them on ice for 2 – 3 minutes.

Note: (1) If the sample volume is greater than 25 µl, heat the sample for 2 minutes at 98°C; (2) heat denaturation is required for samples with high GC content (>50% GC) but is not essential for samples of low GC content (≤50% GC). If the user is working exclusively with DNA of low GC content, this step may be skipped. If the user would like to heat only those samples with high GC content but not those with low GC content, it may be done without any detrimental effect to the library.

i. Briefly spin the DNA samples in a microcentrifuge or plate centrifuge to collect the contents at the bottom of each tube or well.

j. Carefully pipette the DNA samples intended for sequencing into the wells of the 96-well plate. Mix well after pipetting into each well. Note which sample is in which well (preparing a chart or spreadsheet is useful in this regard). Verify that the DNA input is at least 50 ng per well and it is consistent across wells. After the addition of DNA, each well should contain a volume of 10 µL.

k. Seal the top of the plate with your seal of choice. Using a plate centrifuge, briefly spin the 96-well plate to ensure that the contents of the plate are at the bottom of each well.

l. Place the plate on the heating block of the thermal cycler. Close the lid of the instrument.

m. Input the following program into the thermal cycler:

1. 92°C for 3 minutes
2. 16°C for 5 minutes
3. Slow ramp (0.1°C/sec) to 68°C
4. 68°C for 15 minutes (after slow ramp step)
5. Hold at 4°C

n. While the program is running, remove the **SPRI Beads I** tube from the refrigerator, place it on the laboratory bench and allow the contents to equilibrate to room temperature (20 - 30 minutes is sufficient). When ready to use (see Step 1q), shake the tube thoroughly to create a homogeneous solution.

o. After the program is complete, briefly centrifuge the plate to ensure that the contents of the plate are at the bottom of each well. Place the plate on ice. Carefully remove the plate seal or lid. Obtain a 1.5 mL or 2 mL tube (preferably an Eppendorf DNA LoBind microcentrifuge tube) and place it on ice. Add a volume (in µL) of 150 mM EDTA to the tube equal to the number of samples present in the 96-well plate (e.g., add 96 µL of 150 mM EDTA if 96 samples are present in the plate). Harvest the samples by transferring the contents of the wells of the plate to the tube containing EDTA. Mix the pooled A reaction products by inverting the tube or pipetting up and down a few times.

Note: A collection device, such as the VBLOK200 (Clickbio, Reno, NV), may be used to harvest the reaction products from the 96-well plate. The device is attached to the top of the 96-well plate and centrifuged upside down. Liquid is removed from the collection device with a pipette and transferred to a tube. If a collection device is employed, it is recommended that EDTA be added to the device prior to centrifugation.

p. Determine the approximate volume of the sample pool with a pipette.

q. Divide the sample pool equally into two 2 mL tubes or three 1.5 mL tubes (preferably Eppendorf DNA LoBind microcentrifuge tubes). Add 1.8 volumes of well-resuspended, room-temperature **SPRI Beads I** to each of the tubes. Pipette the contents up and down several times to mix and incubate the tubes at room temperature for 10 minutes.

r. Place the tubes on a magnetic stand. Allow the solutions to clear (5 – 10 minutes) and discard the supernatants without disturbing the beads.

s. Add 1300 µL of freshly prepared 80% ethanol to each tube. Verify that the beads are fully covered by ethanol. If not, add more ethanol. Wait 30 seconds, then remove and discard the ethanol. It is unnecessary to remove the tubes from the magnetic stand during this step.

t. Repeat the wash step with another 1300 µL of 80% ethanol. Ensure that all residual ethanol is removed after this wash.

u. Open the caps of the tubes while they are on the magnetic stand and allow the beads to air dry for 10 minutes.

v. Add 50 µL of room-temperature **10 mM Tris-HCl (pH 8.0)** to the beads in each of the two 2 mL tubes or 33 µL of **10 mM Tris-HCl (pH 8.0)** to the beads in each of the three 1.5 mL tubes. Remove the tubes from the magnetic stand and resuspend the beads in the liquid by pipetting up and down until the solution is homogeneous. Allow the tubes to sit at room temperature for 10 minutes.

w. Place the tubes on the magnetic stand, allow the solutions to clear and transfer the supernatants containing the eluted DNA to a single new 0.5 mL tube (preferably an Eppendorf DNA LoBind microcentrifuge tube). The volume in the tube should be approximately 100 µL. Discard the spent beads.

SAFE STOPPING POINT

At this time, the A reaction sample may be stored temporarily at 4°C or on ice, or frozen at -20°C for long-term storage. Shortly before Step 2d, heat the sample to 95°C for 3 minutes and transfer it to ice. Centrifuge the sample briefly before continuing with Step 2d.

2. DNA Capture and Library Conversion

Ensure that the **HS Buffer** and **Bead Wash Buffer** are at room temperature before continuing with the protocol.

a. Shake the **Capture Beads** tube thoroughly to resuspend the beads and transfer 40 µL of the beads to a new 0.5 mL Eppendorf DNA LoBind tube. Place the tube on a magnetic stand and wait for the solution to clear (0.5 – 1 minute). Carefully remove the supernatant with a pipette and discard it.

b. Remove the tube from the magnetic stand and add 200 µL of room-temperature **HS Buffer** to the beads. Pipette the sample up and down to mix the components and return the tube to the magnetic stand. Wait for the solution to clear. Carefully remove and discard the supernatant.

c. Remove the tube from the magnetic stand and resuspend the beads in 40 µL of **HS Buffer** by pipetting up and down a few times.

d. Before proceeding with this step, ensure that the sample from the A reaction (from Step 1w) has been heated at 95°C for 3 minutes. Review Step 1w again if this has yet to be done. Add the heat-denatured sample from the A reaction to the **Capture Beads** (from the previous step), mix and incubate the sample at room temperature for 10 minutes. Mix the sample again by pipetting up and down a few times and incubate for another 10 minutes at room temperature.

e. Place the tube on the magnetic stand and wait for the solution to clear. Carefully remove and discard the supernatant. Avoid disturbing the beads.

f. Remove the tube from the magnetic stand and resuspend the beads in 100 µL of room-temperature 0.1 N sodium hydroxide by pipetting up and down a few times. Leave the tube on the laboratory bench for 3 - 4 minutes. Return the tube to the magnetic stand, allow the solution to clear and discard the supernatant.

g. Remove the tube from the magnetic stand and resuspend the beads in 200 µL of room-temperature **Bead Wash Buffer** by pipetting up and down a few times. Return the tube to the magnetic stand, allow the solution to clear and discard the supernatant.

h. Repeat the wash step (previous step) two additional times. Carefully remove any remaining liquid after the final wash.

i. Add the reagents listed below to the tube containing the **Capture Beads** to prepare the "B" reaction:

8 µL	**5X Enzyme II Buffer**
3 µL	**dNTP Mix II**
4 µL	**Primer B**
24 µL	**Nuclease-Free Water**
39 µL	Total

If working with multiple samples (i.e., the products of multiple 96-well plates), it is recommended that you prepare a master mix with an additional 10% to compensate for loss during pipetting.

j. Pipette the liquid in the tube up and down several times to resuspend the **Capture Beads**. Place the tube on ice.

k. Add 1 µL of **Enzyme II** to the reaction. Mix gently.

l. Incubate the B reaction for 20 minutes at 24°C (preferably in a thermal cycler or heating block), then transfer it back to ice for 2 – 3 minutes.

m. Place the tube on the magnetic stand. Allow the solution to clear and discard the supernatant without disturbing the beads. If the solution does not clear, use a pipette to resuspend the beads in the liquid while the tube is on the magnetic stand. The solution should clear more effectively now.

n. Remove the tube from the magnetic stand and resuspend the beads in 200 µL of room-temperature **Bead Wash Buffer** by pipetting up and down a few times. Return the tube to the magnetic stand, allow the solution to clear and discard the supernatant.

o. Repeat the wash step (previous step) two additional times. Carefully remove any remaining liquid after the final wash.

3. Amplification

a. Use a pipette to resuspend the beads from Step 2o in 42 µL of **Nuclease-Free Water**. Transfer the beads to a thin-walled PCR tube. Add the following components:

4 µL	**Universal PCR Primer**
4 µL	**Index PCR Primer (barcodes 1 - 12)** (choose one barcoded primer)
50 µL	**2X PCR Amplification Mix**
100 µL	Total

Input the following parameters into a thermal cycler and perform a PCR:

1 cycle:	98°C, 2 minutes
8 cycles:	98°C, 20 seconds 60°C, 30 seconds 72°C, 30 seconds
1 cycle:	72°C, 5 minutes 4°C, hold

The sample can be left in the thermal cycler at 4°C overnight.

SAFE STOPPING POINT

b. Briefly spin the PCR tube in a microcentrifuge to pellet the beads. Place the tube on a magnetic stand, wait for the solution to clear and transfer the supernatant to a new 0.5 mL Eppendorf DNA LoBind microcentrifuge tube. Discard the PCR tube containing the **Capture Beads**.

4. Size Selection

This protocol includes options for five different post-PCR library size selection procedures. Option 1 is for those who would like to use a gel-based size selection procedure to prepare the library for sequencing. In this case, the library is treated with 1.4 volumes of SPRI beads to remove short fragments, after which the library undergoes gel-based selection at the discretion of the user (a few recommendations for gel-based size selection are provided in the "Subsequent Steps" section of this protocol). Options 2, 3, 4 and 5 are for those who would like to follow a two-step SPRI bead size selection procedure to prepare the DNA library for sequencing without a gel-based purification step.

Option 1: 1.4 volumes of **SPRI Beads II**. This will produce a library with fragments in the range of 200 bp – 2500 bp.

Option 2: 0.8 volumes of **SPRI Beads II** followed by 0.4 volumes of the same beads (both volumes are relative to the initial sample volume of 100 µL). This will generate fragments appropriate for a 2 x 75 or 2 x 100 Illumina paired-end sequencing run.

Option 3: 0.7 volumes of **SPRI Beads II** followed by 0.3 volumes of the same beads (both volumes are relative to the initial sample volume of 100 µL). This will generate fragments appropriate for a 2 x 100 or 2 x 150 Illumina paired-end sequencing run.

Option 4: 0.65 volumes of **SPRI Beads II** followed by 0.25 volumes of the same beads (both volumes are relative to the initial sample volume of 100 µL). This will generate fragments appropriate for a 2 x 200 Illumina paired-end sequencing run.

Option 5: 0.65 volumes of **SPRI Beads II** followed by 0.1 volumes of the same beads (both volumes are relative to the initial sample volume of 100 µL). This will generate fragments appropriate for a 2 x 250 or 2 x 300 Illumina paired-end sequencing run.

See Table 1 for a summary of the options for two-step SPRI bead size selection.

Option 1:

a. Add 140 µL of well-resuspended, room-temperature **SPRI Beads II** to the supernatant from Step 3b, pipette several times to mix and incubate the tube at room temperature for 10 minutes (take the beads out of the refrigerator 20 - 30 minutes prior to use to bring them to room temperature). Mix the sample halfway through the incubation by pipetting up and down a few times.

b. Place the tube on the magnetic stand. Allow the solution to clear (3 – 5 minutes) and discard the supernatant without disturbing the beads.

c. Add 200 µL of freshly prepared 80% ethanol to the tube. Wait 30 seconds, then remove and discard the ethanol. It is unnecessary to remove the tube from the magnetic stand during this step.

d. Repeat the wash step with another 200 µL of 80% ethanol. Ensure that all residual ethanol is removed after this wash.

e. Open the cap of the tube while it is on the magnetic stand and allow the beads to air dry for 10 minutes.

f. Add 25 µL of room-temperature **10 mM Tris-HCl (pH 8.0)** to the beads. Remove the tube from the magnetic stand. Mix the contents by pipetting up and down to fully resuspend the beads in the liquid. Allow the tube to sit at room temperature for 10 minutes.

g. Place the tube on the magnetic stand. Allow the solution to clear and transfer the supernatant containing the eluted DNA to a new Eppendorf DNA LoBind microcentrifuge tube. Avoid transferring any beads along with the supernatant.

Options 2, 3, 4 and 5:

a. Obtain the supernatant from Step 3b. Add x µL (x = 80 µL, 70 µL or 65 µL beads, depending on the library fragment sizes desired; see Table 1 to choose the volume best suited to your purpose) of well-resuspended, room-temperature **SPRI Beads II** to the supernatant, pipette several times to mix and incubate the tube at room temperature for 10 minutes (take the beads out of the refrigerator 20 - 30 minutes prior to use to bring them to room temperature). Mix the sample halfway through the incubation by pipetting up and down a few times.

Table 1: Two-step SPRI bead size selection options

Option	SPRI Bead II volumes	Library fragment size peak* (bp)	Median insert size** (bp)	Recommended Illumina sequencing read length (nucleotides)
2	80 µL followed by 40 µL	~450	~200	2 x 75, 2 x 100
3	70 µL followed by 30 µL	~600	~300	2 x 100, 2 x 150
4	65 µL followed by 25 µL	~750	~400	2 x 200
5	65 µL followed by 10 µL	~800	~500	2 x 250, 2 x 300

* Based on Agilent Bioanalyzer 2100 instrument electropherogram data

** Based on post-sequencing analysis of read alignment data (for any particular DNA fragment, insert size = fragment length - 146 nucleotides of adapter sequence)

b. Place the tube on the magnetic stand. Allow the solution to clear (3 – 5 minutes). Use a pipette to transfer the supernatant to a new 0.5 mL Eppendorf DNA LoBind microcentrifuge tube. Discard the tube with beads.

c. Add y µL (y = 40 µL, 30 µL, 25 µL or 10 µL beads, depending on the library fragment sizes desired; see Table 1 to choose the volume best suited to your purpose) of well-resuspended, room-temperature **SPRI Beads II** to the supernatant, pipette several times to mix and incubate the tube at room temperature for 10 minutes. Mix the sample halfway through the incubation by pipetting up and down a few times.

d. Place the tube on the magnetic stand. Allow the solution to clear (3 – 5 minutes) and discard the supernatant without disturbing the beads.

e. Add 200 µL of freshly prepared 80% ethanol to the tube. Wait 30 seconds, then remove and discard the ethanol. It is unnecessary to remove the tube from the magnetic stand during this step.

f. Repeat the wash step with another 200 µL of 80% ethanol. Ensure that all residual ethanol is removed after this wash.

g. Open the cap of the tube while it is on the magnetic stand and allow the beads to air dry for 5 - 7 minutes.

h. Add 25 µL of room-temperature **10 mM Tris-HCl (pH 8.0)** to the beads. Remove the tube from the magnetic stand. Mix the contents by pipetting up and down to fully resuspend the beads in the liquid. Allow the tube to sit at room temperature for 10 minutes.

i. Place the tube on the magnetic stand. Allow the solution to clear and transfer the supernatant containing the eluted DNA to a new Eppendorf DNA LoBind microcentrifuge tube. Avoid transferring any beads along with the supernatant.

5. Subsequent Steps

It is recommended that all libraries, regardless of selection method, be evaluated and quantitated on an Agilent Bioanalyzer or TapeStation instrument (or other equivalent instrument) prior to sequencing.

Any library undergoing a single SPRI Bead II-based purification step at the end of the library prep (size selection option 1) should be size-selected again using an agarose gel-based method. The library should be loaded on a gel and electrophoresed, and fragments of interest extracted and purified for sequencing. The following fragment size ranges are recommended for gel extraction and sequencing:

350 bp – 800 bp for 2 x 75 bp sequencing

450 bp – 900 bp for 2 x 150 bp sequencing

550 bp – 1000 bp for 2 x 300 bp sequencing

Two-step SPRI Bead II-selected RipTide libraries (i.e., libraries undergoing size selection options 2, 3, 4 & 5) can be loaded directly on an Illumina sequencer. Illumina-suggested DNA loading concentrations are recommended, however, the loading concentration should be adjusted to take into account average library size.

Note: Items highlighted in bold throughout the text are provided with the kit.

X. Materials

Reagents are sufficient for ten 96-sample RipTide libraries.

-20°C Reagent Box

- 1 vial: 10X Enzyme I Buffer
- 2 vials: dNTP Mix I
- 1 vial: Enzyme I
- 1 vial: Primer B
- 1 vial: 5X Enzyme II Buffer
- 1 vial: dNTP Mix II
- 1 vial: Enzyme II
- 1 vial: Nuclease-Free Water
- 2 vials: 2X PCR Amplification Mix

4°C Reagent Box

1 vial: Capture Beads

1 vial: HS Buffer (store at room temperature)

1 vial: Bead Wash Buffer (store at room temperature)

1 vial: SPRI Beads I

1 vial: SPRI Beads II

1 vial: 10 mM Tris-HCl (pH 8.0) (store at room temperature)

-20°C Primer Box

- 1 plate: 96-well Low GC Primer A Plate
- 1 plate: 96-well High GC Primer A Plate
- 1 vial: Universal PCR primer
- 1 vial: Index PCR Primer bc #1
- 1 vial: Index PCR Primer bc #2
- 1 vial: Index PCR Primer bc #3
- 1 vial: Index PCR Primer bc #4
- 1 vial: Index PCR Primer bc #5
- 1 vial: Index PCR Primer bc #6
- 1 vial: Index PCR Primer bc #7
- 1 vial: Index PCR Primer bc #8
- 1 vial: Index PCR Primer bc #9
- 1 vial: Index PCR Primer bc #10
- 1 vial: Index PCR Primer bc #11
- 1 vial: Index PCR Primer bc #12

bc = barcode; barcodes are identical to Illumina six nucleotide small RNA TruSeq barcodes 1 - 12.

See Appendix 1 for barcode sequences.

User supplied items:

0.1 N sodium hydroxide

0.5 M EDTA

80% ethanol

Eppendorf LoBind tubes

PCR tubes

96-well plates

Magnetic stand

XI. Appendix 1: Adapter sequences and library barcodes

The RipTide library barcode (also known as the RipTide plate barcode) is incorporated into the library during PCR. Twelve such barcodes or indices are provided with the RipTide kit. The barcodes are identical to Illumina TruSeq small RNA indices.

The table below lists the RipTide adapter sequences and corresponding expected i7 index reads.

Index PCR Primer	Index PCR Primer Sequence	i7 Index Read
1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	ATCACG
2	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	CGATGT
3	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	TTAGGC
4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	TGACCA
5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	ACAGTG
6	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	GCCAAT
7	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	CAGATC
8	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	ACTTGA
9	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	GATCAG
10	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	TAGCTT
11	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	GGCTAC
12	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	CTTGTA

Universal PCR Primer sequence:
AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA