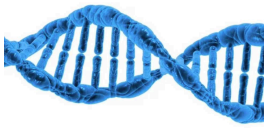
Jun 13, 2025

Library Construction Protocol

DOI
dx.doi.org/10.17504/protocols.io.4r3l28ndql1y/v1



Dakota Betz[1]

[1]ucsd

Rouse Lab

Marina McCowin
UCSD- Scripps Institution of Oceanography

DOI: dx.doi.org/10.17504/protocols.io.4r3l28ndql1y/v1

Protocol Citation: Dakota Betz 2025. Library Construction Protocol. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.4r3l28ndql1y/v1**

Protocol status: Working
We use this protocol and it's working

Created: March 08, 2020

Last Modified: June 13, 2025

Protocol Integer ID: 33943

Keywords: DNA fragmentation, library construction, end repair,

Abstract

Library construction workflows must be tailored and optimized to accommodate specific experimental designs, sample characteristics, sequencing applications and equipment. The protocol provided in this document is generic, and reaction parameters may be adjusted as required to optimize performance, efficiency and cost-effectiveness.

Attachments

PDF

bzv7bbujp.pdf

704KB

Guidelines

In addition to the information in this section, please consult the **KAPA NGS Library Preparation Technical Guide (available on request from Technical Support at sequencing.roche.com/support)** for further guidelines when designing or optimizing your library construction workflow.

The workflow combines enzymatic steps and employs minimal bead-based cleanups, thereby reducing sample handling and overall library preparation time to 1.5 – 3 hrs. The kit contains all of the enzymes and reaction buffers required for:
1. enzymatic fragmentation to produce dsDNA fragments;
2. end repair and A-tailing to produce end-repaired,
5'-phosphorylated, 3'-dA-tailed dsDNA fragments;
3. adapter ligation, during which dsDNA adapters with 3'-dTMP overhangs are ligated to 3'-dA-tailed molecules; and
4. library amplification (optional), which employs highfidelity, low-bias PCR to amplify library fragments carrying appropriate adapter sequences on both ends.

Materials

MATERIALS

KAPA mRNA HyperPrep Kit **Kapa Biosystems Catalog #**KK8514

Safety warnings

Safe Stopping Points

The library construction process, from enzymatic fragmentation to final library, can be performed in 1.5 to 3 hrs—depending on experience, the number of samples being processed, and whether or not library
amplification is performed. If necessary, the protocol may be paused safely after completion of the Post-ligation
Cleanup (step 4.17; the end of the protocol for PCR-free workflows). Purified, adapter-ligated library DNA may be stored at 2°C to 8°C for 1 – 2 weeks, or at -15°C to -25°C for ≤1 month before amplification, target capture and/or sequencing.
To avoid degradation, always store DNA in a buffered solution (10 mM Tris-HCl, pH 8.0 – 8.5) when possible,
and minimize the number of freeze-thaw cycles.

Notes:

- First-time users should refer to Appendix 2: Optimization of Fragmentation Parameters (p. 16)

before trying this kit, as standard fragmentation parameters may not result in the optimal size distribution for libraries prepared from your specific DNA samples. Precious samples should not be used when evaluating
this kit. Instead, parameters should be optimized with a non-precious, bulk DNA sample that is representative
of the actual samples to be processed.

- If your DNA samples contain EDTA, please consult the Appendix 2: Handling of DNA Samples Containing EDTA (p. 16), as well as Important Parameters: Input DNA (p. 4) before starting this protocol.
- This protocol does not include size selection. Please refer to Appendix 1 (p. 15) for a detailed double-sided

size selection protocol that may be included after ligation or after amplification.

- Always ensure that KAPA cleanup beads are fully equilibrated to room temperature and fully resuspended

before use.

Before start

Shipping and Storage

The enzymes provided in this kit are temperature sensitive, and appropriate care should be taken during shipping and storage. KAPA HyperPlus Kits are shipped on dry ice or ice packs, depending upon country of destination. Upon receipt, immediately store enzymes and reaction buffers at -15°C to -25°C in a constant-temperature freezer. When stored under these conditions and handled correctly, the kit components will retain full activity until the expiry date indicated on the kit label.

Handling

Always ensure that KAPA HyperPlus Kit components have been fully thawed and thoroughly mixed before use. The End Repair & A-Tailing Buffer and Ligation Buffer may contain precipitates when thawed at 2°C to 8°C. These buffers must be thawed at room temperature and vortexed thoroughly before use. KAPA HiFi HotStart ReadyMix (2X) contains isostabilizers and may not freeze completely, even when stored at -15°C to -25°C. Nevertheless, always ensure that the ReadyMix is fully thawed and thoroughly mixed before use. Reaction master mixes prepared from the enzymes and buffers for fragmentation, end repair and A-tailing, as well as for ligation, are very viscous and require special attention during pipetting. Keep all enzyme components and master mixes on ice as long as possible during handling and preparation.

Quality Control

All kit components are subjected to stringent functional quality control, are free of detectable contaminating exoand endonuclease activities, and meet strict requirements with respect to DNA contamination.

Enzymatic Fragmentation

1

Note

If the DNA samples contain EDTA, perform a 3X bead-based cleanup with KAPA cleanup beads to remove EDTA prior to fragmentation. Please refer to the relevant Technical Data Sheet
(KR1705 or KR1245) for a detailed DNA cleanup protocol. Alternatively, prepare a sufficient volume of appropriately diluted Conditioning Solution (5 µL per DNA sample, plus excess). Refer to
Table 2 (p. 4) for guidelines on the dilution of the Conditioning Solution.

Dilute the amount of dsDNA to be used for library construction as follows:

- If the DNA preparation does not contain EDTA, dilute in [M] 10 millimolar (mM) Tris-HCl (pH 8.0 – 8.5) in a total of 35 µL
- If the DNA preparation does contain EDTA, dilute in the EDTA-containing buffer in which samples are currently suspended, in a total of 30 µL. To each reaction with 30 µL of EDTA-containing DNA, add 5 µL of diluted Conditioning Solution.

2 Mix by gentle vortexing or pipetting up and down.

3 Assemble each fragmentation reaction on ice by adding the components in this order:

	A	B
	Component	**Volume**
	Double-stranded DNA (with Conditioning Solution, if needed)	35 µL
	KAPA Frag Buffer (10X)*	5 µL
	KAPA Frag Enzyme*	10 µL
	Total volume:	50 µL

* The KAPA Frag Buffer and Enzyme may be pre-mixed and kept on ice prior to reaction setup, and dispensed as a single solution. Please note the volume of buffer is less than the volume of enzyme in this reaction

4 Vortex gently and spin down briefly. Return the plate/tube(s) to ice. Proceed immediately to the
next step.

5 Incubate in a thermocycler, pre-cooled to 4 °C and programmed as outlined below. A heated lid is not
required for this step. If used, set the temperature of the heated lid to ≤ 50 °C .

	A	B	C
	Step Temp Time	**Step Temp Time**	**Step Temp Time**
	Pre-cool block 4°C N/A	Pre-cool block 4°C N/A	Pre-cool block 4°C N/A
	Fragmentation 37°C See table below	Fragmentation 37°C See table below	Fragmentation 37°C See table below
	HOLD 4°C ∞	HOLD 4°C ∞	HOLD 4°C ∞

6 Transfer reactions to ice, and proceed immediately to End Repair and A-tailing (next section).

End Repair & A-tailing

7

Note

Two End Repair & A-Tailing Enzyme Mixes are provided for the End Repair and A-tailing step —see Important Parameters for further guidelines.

8 In the same plate/tube(s) in which enzymatic fragmentation was performed, assemble each End Repair and A-tailing reaction as follows:

	A	B
	Component	**Volume**
	Fragmented, double-stranded DNA	50 µL
	End Repair & A-Tailing Buffer*	7 µL

	A	B
	HyperPrep/HyperPlus ERAT Enzyme Mix**	3 µL
	Total volume:	60 µL

* The buffer and enzyme mix should preferably be pre-mixed and added in a single pipetting step. Premixes are stable for ≤24 hrs at room temperature, for ≤3 days at 2°C to 8°C, and for ≤4 weeks at -15°C to -25°C.
**Use either the HyperPrep ERAT Enzyme Mix (existing chemisty) or the HyperPlus ERAT Enzyme Mix (enhanced chemistry)

9 Vortex gently and spin down briefly. Return the reaction plate/tube(s) to ice. Proceed immediately
to the next step.

10 Incubate in a thermocycler programmed as outlined below. A heated lid is required for this
step. If possible, set the temperature of the heated lid to ~ 85 °C (instead of the usual 105 °C).

	A	B	C
	Step	**Temp**	**Time**
	End repair and A-tailing	65°C*	30 min
	HOLD	4°C**	∞

*Both the fragmentation and end repair enzymes are inactivated at 65°C. When reactions are set up according to recommendations, additional fragmentation should be negligible. The brief period of end repair is sufficient for enzymatically fragmented DNA.
** If proceeding to the adapter ligation reaction setup without any delay, the reaction may be cooled to 20°C instead of 4°C.

11 Proceed immediately to Adapter Ligation (next section).

Adapter Ligation

12 Dilute adapter stocks to the appropriate concentration, as outlined in Table 4 (see below).
Table 4. Recommended adapter concentrations for libraries constructed from 1 ng – 1 µg input DNA*

	A	B	C	D	E	F
	Input DNA	**Adapter stock concentration**	**Adapter: insert molar ratio**	**Input DNA**	**Adapter stock concentration**	**Adapter: insert molar ratio**
	1 µg	15 µM	10:1	25 ng	7.5 µM	200:1
	500 ng	15 µM	20:1	10 ng	3 µM	200:1
	250 ng	15 µM	40:1	5 ng	1.5 µM	200:1
	100 ng	15 µM	100:1	2.5 ng	750 nM	200:1
	50 ng	15 µM	200:1	1 ng	300 nM	200:1

* Adapter:insert molar ratio calculations are based on a mode DNA fragment length of 200 bp, and will be higher for longer DNA fragments, or slightly lower for DNA fragmented to a mode size <200 bp. The lower adapter:insert molar ratios recommended for inputs >100 ng represent a fair compromise between library construction efficiency and cost; higher library yields will be achieved if a higher adapter concentration is used.

13 In the same plate/tube(s) in which end repair and A-tailing was performed, assemble each adapter
ligation reaction as follows:

	A	B
	Component	**Volume**
	End repair and A-tailing reaction product	60 µL
	Adapter stock (diluted as per Table 4 on p. 5)	5 µL
	PCR-grade water*	5 µL
	Ligation Buffer*	30 µL
	DNA Ligase*	10 µL
	Total volume:	110 µL

* The water, buffer and ligase enzyme should preferably be premixed and added in a single pipetting step. Premixes are stable for ≤24 hrs at room temperature, for ≤3 days at 2°C to 8°C, and for ≤4 weeks at -15°C to -25°C.

14 Mix thoroughly and centrifuge briefly.

15 Incubate at 20 °C for 00:15:00 .

Note

Note: to achieve higher conversion rates and library yields, particularly for low-input samples, consider increasing the ligation time to a maximum of 04:00:00 at 20 °C or Overnight at 2 °C to 8 °C. Please note that longer ligation times may lead to increased levels of adapter-dimer. Adapter concentrations may have to be optimized if ligation times are extended significantly.

16 Proceed immediately to Post-ligation Cleanup

Post-ligation Cleanup

17 In the same plate/tube(s), perform a 0.8X beadbased cleanup by combining the following:

	A	B
	Component	**Volume**
	Adapter ligation reaction product	110 µL
	KAPA cleanup beads	88 µL
	Total volume:	198 µL

18 Mix thoroughly by vortexing and/or pipetting up and down multiple times.

19 Incubate the plate/tube(s) at room temperature for 00:05:00 – 00:15:00 to bind DNA to the beads.

20 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

21 Carefully remove and discard the supernatant.

22 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

23 Incubate the plate/tube(s) on the magnet at room temperature for ≥ 00:00:30 .

24 Carefully remove and discard the ethanol.

25 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

26 Incubate the plate/tube(s) on the magnet at room temperature for ≥ 00:00:30 .

27 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

28 Dry the beads at room temperature for 00:03:00 – 00:05:00 , or until all of the ethanol has evaporated. Caution: over-drying the beads may result in reduced yield.

29 Remove the plate/tube(s) from the magnet.

30 Resuspend the beads:
• in 25 µL of elution buffer ([M] 10 millimolar (mM) Tris-HCl, pH 8.0 – 8.5) to proceed with Library Amplification (step 5), or

• in 55 µL of elution buffer ([M] 10 millimolar (mM) Tris-HCl, pH 8.0 – 8.5) to proceed with double-sided
size selection (Appendix 1).

31 Incubate the plate/tube(s) at room temperature for 00:02:00 to elute DNA off the beads.

32 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

33 Transfer the clear supernatant to a new plate/tube(s):
• to proceed with Library Amplification (step 5), transfer 20 µL of supernatant, or
• to proceed with double-sided size selection (Appendix 1), transfer 50 µL of supernatant.

Library Amplificiation

34

Note

Note: Please refer to Important Parameters: Library Amplification (p. 7) and the KAPA NGS Library Preparation Technical Guide (available on request from Technical Support at sequencing.roche.com/support) for more information on optimizing library amplification.

35 Assemble each library amplification reaction as follows:

	A	B
	Component	**Volume**
	KAPA HiFi HotStart ReadyMix (2X)	25 µL
	Library Amplification Primer Mix (10X)*	5 µL
	Adapter-ligated library	20 µL
	Total volume:	50 µL

* Or other, suitable 10X library amplification primer mix.The recommended final concentration of each primer in the library amplification reaction is 0.5 – 4 µM. Also refer to Important Parameters: Library Amplification (p. 7).

36 Mix thoroughly and centrifuge briefly.

37 Amplify using the following cycling protocol:

	A	B	C	D
	Step	**Temp**	**Duration**	**Cycle**
	Initial denaturation	98°C	45 sec	1
	Denaturation	98°C	15 sec	Minimum number required for optimal amplification (Table 5 or 6)
	Annealing*	60°C	30 sec	
	Extension	72°C	30 sec	

	A	B	C	D
	Final extension	72°C	1 min	
	HOLD	4°C	∞	1
				1

* Optimization of the annealing temperature may be required for nonstandard (i.e., other than Illumina TruSeq) adapter/primer combinations.

38 Proceed directly to Post-amplification Cleanup

Post-amplification Cleanup

39 In the library amplification plate/tube(s), perform a 1X bead-based cleanup by combining the following:

	A	B
	Component	**Volume**
	Library amplification reaction product	50 µL
	KAPA cleanup beads	50 µL
	Total volume:	100 µL

40 Mix thoroughly by vortexing and/or pipetting up and down multiple times.

41 Incubate the plate/tube(s) at room temperature for 00:05:00 – 00:15:00 to bind DNA to the beads.

42 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

43 Carefully remove and discard the supernatant.

44 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

45 Incubate the plate/tube(s) on the magnet at room temperature for ≥ 00:00:30 .

46 Carefully remove and discard the ethanol.

47 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

48 Incubate the plate/tube(s) on the magnet at room temperature for ≥ 00:00:30 .

49 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

50 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

51 Incubate the plate/tube(s) on the magnet at room temperature for ≥ 00:00:30 .

52 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

53 Dry the beads at room temperature for 00:03:00 – 00:05:00 , or until all of the ethanol has evaporated. Caution: over-drying the beads may result in reduced yield.

54 Remove the plate/tube(s) from the magnet.

55 Thoroughly resuspend the beads in an appropriate volume of elution buffer ([M] 10 millimolar (mM) Tris-HCl, pH 8.0 – 8.5) or PCR-grade water.

Note

Note: If proceeding with a second post amplification cleanup, or double-sided size selection (Appendix 1), resuspend the beads in 55 µL of elution buffer.

56 Incubate the plate/tube(s) at room temperature for 00:02:00 to elute DNA off the beads.

57 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

58 Transfer the clear supernatant to a new plate/tube(s) and proceed with size selection (refer to Appendix 1), library QC, target capture or sequencing, as appropriate. Store purified, amplified libraries at 2 °C to 8 °C for 1 – 2 weeks, or at -15 °C to -25 °C.

Appendix 1: Size Selection

59

Note

The double-sided size selection protocol outlined in this appendix is designed for the selection of library molecules (inclusive of adapter) in the range of 250 – 450 bp. To obtain a population of shorter or longer molecules, the protocol may be modified as follows:

	A	B	C	D
	Upper size limit	**Modification**	**Lower size limit**	**Modification**
	Increase	Decrease the ratio of the first cut	Increase	Decrease the ratio of the second cut*
	Decrease	Increase the ratio of the first cut	Decrease	Increase the ratio of the second cut*

* The second size cut should be performed with at least 0.2 volumes of KAPA cleanup beads reagent. Please note that the volume of KAPA cleanup beads needed for the second cut is

calculated relative to the volume of the DNA at the start of the size selection procedure, not the volume of the DNA-containing supernatant transferred after the first cut. DNA recovery is dramatically reduced if the difference between first and second cuts is less than ~0.2 volumes. To increase the amount of DNA recovered, >0.2 volumes of KAPA cleanup beads may be used for the second cut, but note that this may result in the recovery of smaller library fragments and/or a broader size distribution. For more information on double-sided size selection, please refer to the KAPA NGS Library Preparation Technical Guide (available on request from Technical Support at sequencing.roche.com/support).

60 Perform the first (0.7X) size cut (to exclude library molecules larger than ~450 bp) by combining the
following:

	A	B
	Component	**Volume**
	DNA to be size selected	50 µL
	KAPA cleanup beads	35 µL
	Total volume per well/tube:	85 µL

61 Mix thoroughly by vortexing and/or pipetting up and down multiple times.

62 Incubate the plate/tube(s) at room temperature for 00:05:00 – 00:15:00 in to bind library molecules larger than ~450 bp to the beads.

63 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

64 Carefully transfer ~ 80 µL of supernatant containing library molecules smaller than ~450 bp to a new plate/tube. It is critical that no beads are transferred with the supernatant. Discard the plate/tube(s) with the beads to which library molecules larger than ~450 bp were bound.

65 Perform the second size cut (0.9X), to retain library molecules >250 bp) by combining the following:

	A	B
	Component	**Volume**
	Supernatant from first size cut	80 µL

	A	B
	KAPA cleanup beads	10 µL
	Total volume per well/tube:	90 µL

66 Mix thoroughly by vortexing and/or pipetting up and down multiple times.

67 Incubate the plate/tube(s) at room temperature for 00:05:00 – 00:15:00 n to bind library molecules larger than ~250 bp to the beads.

68 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

69 Carefully remove and discard the supernatant, which contains library molecules smaller than ~250 bp.

70 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

71 Incubate the plate/tube(s) on the magnet at room temperature for ≥ 00:00:30 .

72 Carefully remove and discard the ethanol.

73 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

74 Incubate the plate/tube(s) on the magnet at room temperature for ≥ 00:00:30 .

75 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

76 Dry the beads for 00:03:00 – 00:05:00 at Room temperature , or until all of the ethanol has evaporated. Caution: over-drying the beads may result in reduced yield.

77 Remove the plate/tube(s) from the magnet.

78 Thoroughly resuspend the beads in the required volume of elution buffer ([M] 10 millimolar (mM) Tris-HCl, pH 8.0 – 8.5).

79 Incubate the plate/tube(s) at room temperature for 00:02:00 to elute DNA off the beads.

80 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

81 Transfer the clear supernatant with size-selected DNA to a new plate/tube(s) and proceed with the next step in your workflow, or store DNA at 2 °C to 8 °C for 1 – 2 weeks, or at -15 °C to -25 °C .

Appendix 2: Optimization of Fragmentation Parameters

82

Note

Fragmentation guidelines provided in the Library Construction Protocol: Enzymatic Fragmentation (step 1) may not result in the optimal library size distribution for your specific DNA samples. For this reason, precious samples should not be used when evaluating the KAPA HyperPlus Kit for the first time. Instead, fragmentation parameters should be optimized in the context of the KAPA HyperPlus workflow, using a non-precious, bulk DNA sample that is representative of the actual samples to be processed.

The information in this Appendix should be considered during the experimental design for your evaluation of the KAPA HyperPlus Kit.

83 **Quantification of Input DNA**

The Qubit fluorometer is recommended for the quantification of high-quality DNA, whereas the KAPA hgDNA Quantification and QC Kit provides both concentration and quality information for FFPE DNA.

84 **Handling of DNA Samples Containing EDTA**

Note

If the DNA samples contain EDTA, perform a 3X beadbased cleanup with KAPA cleanup beads to remove EDTA prior to fragmentation. Please refer to the relevant Technical Data Sheet (KR1705 or KR1245) for a detailed DNA cleanup protocol.

85 **For example:**

• If your DNA samples are in TE buffer, and your input into library construction is 100 ng, dilute 100 ng of each sample into a final volume of 30 µL (i.e., to 3.33 ng/µL) using TE buffer.

• All samples will now contain the same final EDTA concentration once diluted to 50 µL for fragmentation.

This concentration is: EDTA concentration in TE buffer x (30 µL/50 µL) = 1 mM x (30 µL/50 µL) = [M] 0.6 millimolar (mM)

• Make a 2.2-fold dilution of the Conditioning Solution (as per Table 3 on p. 4), and follow the Library
Construction Protocol (second half of step 1).

86

Note

If you are unsure about the presence or concentration of EDTA in your DNA samples, remove the EDTA by performing a column- or bead-based purification or buffer exchange prior to enzymatic fragmentation, or follow the procedure outlined below:

86.1 Set up a series of test reactions with the appropriate amount of input DNA, and different final concentrations
of Conditioning Solution.

86.2 Include at least one reaction with the same input of control DNA known to be EDTA-free. The control DNA
should preferably be of the same type and quality as the test samples.

86.3 Fragment the DNA using the appropriate parameters, as outlined in the Library Construction Protocol:
Enzymatic Fragmentation (step 1). Complete the library construction process, and compare library size

distributions for the test and control samples using an electrophoretic system (see Important Parameters:
Evaluating the Success of Library Construction, p. 8).

86.4 Titrate the final concentration of Conditioning Solution in the reaction until the test samples yield similar
fragmentation profiles as the EDTA-free control sample, or until the desired library size distribution has been
achieved.

86.5 A two-step strategy may be the best. Start with 3 – 4 test samples covering a broad range of final Conditioning
Solution concentrations, then perform a finer titration over a narrower concentration range.