Manual

WB 9224
WB 9296

Westburg NGS NoFrag Library Prep Kit

Westburg

INTRODUCTION

In the constant evolution of Next Generation Sequencing technologies, free-circulating nucleic acids are becoming a fast growing source of input DNA in areas like cancer research and non-invasive prenatal testing (NIPT). Typically, this DNA is present in plasma as short fragments (<500bp), making it unnecessary to further fragment the DNA prior to Library Preparation. Furthermore its concentration is very low, challenging classical Library Prep methods.

Despite the tremendous advances in Next Generation Sequencing (NGS) technology, library preparation, starting from small quantities of cfDNA is a major bottleneck in the NGS workflow. This is mainly due to the suboptimal adapter ligation and clean-up steps in existing methods, which cause a substantial loss of time and sample and limit the possibilities to automate the workflow.

The Westburg NGS NoFrag Library Prep Kit combines a highly efficient adapter ligation and the reduction of the number of clean-up steps, saving cost and maximizing library yield, starting from input levels as low as 250pg fragmented DNA. The complete procedure is carried out in a single tube, which facilitates automation.

Outline of the procedure

In the first step, DNA fragments are end-repaired and A-tailed during the same incubation. Without intermediate clean-up the sample is now ready for ligation of adapters specific for your Illumina sequencing platform (see appendix I).

When working with DNA samples of 100 ng or less it is recommended to perform library amplification with the Westburg NGS DNA Library Amplification Mix. This is a high-fidelity PCR master mix with a robust amplification performance across a wide range of GC percentages, minimizing sequencing bias.

After library prep and optional amplification, reaction clean-up and removal of adapter-dimers is achieved with magnetic beads. Also this step can be easily automated.

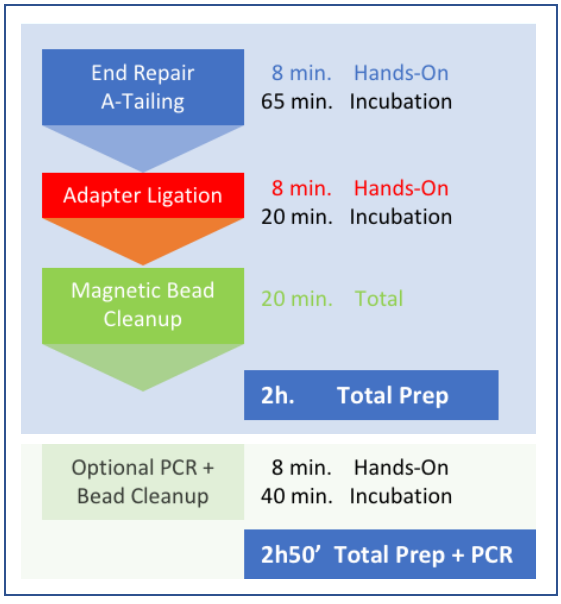

Figure 1. Protocol outline

INTRODUCTION

Kit components

WB 9224 Westburg NGS DNA Library Prep Kit - 24 library preparations

	Volume
ER/A-Tailing Enzyme Mix (5x)	240 µl
ER/A-Tailing Buffer (10X)	250 µl
DNA Ligase	240 µl
Rapid Ligation Buffer (5X)	1.500 µl

WB 9296 Westburg NGS DNA Library Prep Kit - 96 library preparations

	Volume
ER/A-Tailing Enzyme Mix (5x)	1.100 µl
ER/A-Tailing Buffer (10X)	1.000 µl
DNA Ligase	1.100 µl
Rapid Ligation Buffer (5X)	2.200 µl

IMPORTANT: Enzyme mixes and their corresponding reaction buffer are paired per kit for optimal performance. Do not mix between different kits.

WB 9100 Westburg NGS DNA Library Amplification Mix - 100 reactions
Optional, to be ordered separately

	Volume
Amplification Master Mix (2X)	240 µl
Primer Mix (10 µM)	250 µl

Storage

All reagents must be stored at -15°C to -25°C.

To be supplied by the user

- Agencourt® AMPure® XP Beads (cat. no. A63880 or A63881) for bead-based library purification
- 80% ethanol (ACS grade), freshly prepared in nuclease free water
- Nuclease-free water
- 10mM Tris-HCl buffer pH 8.0
- Barcoded adapters
- PCR tubes or plates
- Low DNA-binding pipet filter tips
- Low DNA-binding tubes

INTRODUCTION

To be supplied by the user (continued)

- Thermocycler
- Magnetic rack for magnetic bead separation
- Capillary electrophoresis device (such as Agilent® Bioanalyzer, or similar device) to evaluate the DNA fragmentation profile (optional)

Important considerations before you start

- In order to achieve optimal results, it is important to accurately follow the protocol.
- Plastics must be certified sterile and nuclease-free. Clean the bench surface and pipettes with RNase Away® (ThermoFisher Scientific) to eliminate nuclease and DNA.
- The first time, read the entire protocol before you start so you are well prepared and have all required reagents and materials ready for use.
- After thawing the reagents keep them on ice during the entire protocol. Master mixes should also be prepared and kept on ice
- **Do not vortex the 5x ER/A-Tailing Enzyme mix or the DNA ligase**; mix by tapping the tube gently with your finger. Other components can be mixed by briefly vortexing.

Input DNA requirements

- It is essential to use only high-purity DNA samples that are free of residual salts, proteins, detergents or other contaminants. Use a commercially available purification kit that delivers this DNA purity.
- Use 250 pg - 1000 ng purified DNA as input
- It is very important that the input DNA concentration is accurately determined, especially when it is less than 100ng DNA. Fluorometric methods (Qubit, Picogreen) are recommended.
- The DNA sample has to be in water, 10 mM Tris, 0.1X TE or Buffer EB (QIAGEN elution buffer)

PROTOCOL

A. End repair and dA tailing

1. Set up the following program in a thermocycler.

Step	Temp.	Time
1	4°C	1 min
2	20°C	30 min
3	65°C	30 min
4	4°C	Hold

Use the heated lid of the cycler; if possible, set the temperature of the heated lid to ~70°C. When the cycler block reaches 4°C, pause the program.

2. It is important to follow the procedure described below, in order to achieve optimal results. Prepare a reaction mix in a new thin-walled PCR tube on ice by combining ER/A-tailing Buffer, DNA sample, and nuclease- free water as indicated in the table (per DNA sample). Mix well by gently pipetting.

The Final Reaction Volume is 50µl

	1 reaction (µl)
10X ER/A-tailing Buffer	5
DNA sample	X
Nuclease-free H_2O	(35 - X)
Total	40

3. Add 10 µl of 5X ER/A-Tailing Enzyme Mix to each reaction and gently mix well by mild vortexing. It is recommended to keep the PCR tube on ice for the whole time during reaction setup.

4. Briefly spin the sample tube and immediately transfer it to the pre-chilled thermocycler (4°C). Resume the cycling program.

5. When the program is complete and cycler block has returned to 4°C, remove the samples from the block and place on ice. *Immediately continue to the ligation step*.

PROTOCOL

B. Ligation

6. Equilibrate Agencourt® AMPure® XP beads to room temperature for 20–30 minutes for use in step 11.

7. Thaw adapters, mix briefly by tapping or vortexing and pulse-spin to collect the liquid at the bottom of the tube.

8. Add the required volume of DNA adapter* (subsequently designated as "Y") to the PCR tube containing 50 µl of A-tailed fragmented DNA from step 6. Mix gently by pipetting and cool on ice.

   * DNA adapters are not included. Follow the supplier's recommendation for adapter concentration and usage. For optimal ligation use a *molar* ratio of adapter to insert between 25:1 and 200:1 based on the amount of input DNA and the mean size of the fragmented DNA. See also Appendix I (page 11) for additional guidelines on the adapters.

9. Prepare the following ligation master mix in a separate tube on ice and mix well by gentle vortexing or pipetting up and down 6-8 times. Y is the volume of adapter used in step 8. For multiple samples calculate 10% extra volume to compensate for pipetting loss.

	Per sample
5X Ligation Buffer	20 µl
DNA Ligase	10 µl
Nuclease-free H_2O	(20-Y) µl
Total	(50-Y) µl

10. Add (50 – Y) µl of the ligation master mix to the sample and mix well by pipetting. Incubate the ligation reaction at 20°C for 15 min.
    IMPORTANT: Do not use a thermocycler with a heated lid for this incubation.

11. Proceed immediately to adapter ligation cleanup using 0.8X AMPure® XP beads.

    - Add 80 µl of thoroughly vortexed room-temperature equilibrated AMPure® XP beads slurry to the ligation sample from step 10 in a fresh 1.5ml centrifuge tube and mix well by gently vortexing or pipetting gently up and down 6-8 times.
    - Incubate the mixture for 5 min at room temperature. Pellet the beads on a magnetic stand and carefully discard the supernatant.
    - Wash the beads with 200 µl of 80% ethanol. Pellet the beads on the magnetic stand and discard the supernatant. Repeat the wash once.
    - Air-dry the beads on the magnetic stand for 5 min or until the beads are dry. Over-drying of beads may result in lower DNA recovery.

PROTOCOL

- Resuspend the dried beads in 28 µl of 10 mM Tris-HCl, pH 8.0. Pellet the beads on the magnetic stand. Carefully transfer 25 µl of supernatant into a new tube.

12. At this point, if you are not proceeding immediately, store the sample at -20°C.

If size selection is required, please use your method of choice and follow the corresponding protocols.

In case the starting amount of DNA was < 100ng, or when large quantities of library are needed, proceed to library amplification (protocol C, next page).

If library amplification is not required, please proceed to library quantification (not included in this kit).

PROTOCOL

C. Library amplification (optional)

To ensure maximum yields from small amounts of starting material, typically <100 ng, an additional high-fidelity amplification step can be performed with the Westburg NGS DNA Library Amplification Mix (Ref. No. WB 9100). This High-Fidelity PCR Master Mix can evenly amplify DNA regions with different GC contents, minimizing sequencing bias caused by PCR.

The included primer mix contains both forward and reverse primers compatible with libraries flanked by the standard P5 and P7 adapter sequences.

13. Before you start prepare fresh 80% ethanol.

14. Enter the following program into a thermocycler. The cycler's heated lid must be used (105°C).

Step	Temperature	Incubation time	Cycles
1	98°C	2 min	1
2	98°C	20 sec	6- 12*
3	60°C	30 sec	
4	72°C	30 sec	
5	72°C	1 min	1
6	4°C	Hold	1

* As a general guideline use 6 cycles for 100 ng, 10 cycles for 10 ng, and 12 cycles for 1 ng input DNA.

15. Prepare the following PCR reaction master mix in a separate tube on ice and mix well by pipetting. For multiple samples multiply the volumes by the number of DNA samples and add 10% extra volume to compensate for any pipetting loss.

	per reaction
Library Amplification Mix	**25 µl**
Primer Mix	**1.5 µl**
Total	**26.5 µl**

16. Transfer 23.5 µl of adapter-ligated and purified DNA to a new thin-walled PCR tube. Add 26.5 µl of the master mix from step 15 and gently mix well by pipetting up-and-down 6-8 times. It is important to keep the PCR tube on ice during reaction setup.

17. Pulse-spin the sample tube and immediately transfer to the thermal cycler and start the cycling program.

PROTOCOL

18. During the PCR run, equilibrate AMPure® XP beads to room temperature for 20-30 min.

19. When the PCR is complete and sample block has returned to 4°C, remove samples from block and immediately proceed to DNA library cleanup using 1X AMPure® XP beads.

    - Thoroughly vortex the AMPure® XP bead suspension and add 50 µl of to the PCR mix in a fresh 1.5ml centrifuge tube. Mix well by pipetting.
    - Incubate the mixture for 5 min at RT. Pellet the beads on a magnetic stand and carefully discard the supernatant.
    - Wash the beads with 200 µl of 80% ethanol. Pellet the beads on the magnetic stand and discard the supernatant. Repeat the wash once.
    - Air-dry the beads on the magnetic stand for 2-5 min or until the beads are dry. Over-drying of beads may result in lower DNA recovery.
    - Resuspend the dried beads in 32.5 µl of 10 mM Tris-HCl, pH 8.0.
    - Incubate 3 min at RT with open lid.
    - Pellet the beads on the magnetic stand. Carefully transfer 30 µl of supernatant into a new tube.

20. Determine library quality using an Agilent BioAnalyzer or similar capillary electrophoresis device. Check for the intended size distribution of library fragments and for the absence of a peak of adapters or adapter-dimers around 120bp. Take into account that adapter ligation adds 120 bp to the library fragment size.

21. Store the library at -20°C in a low DNA-binding tube.

TROUBLESHOOTING

If you encounter any issues, the table below may be helpful to find the cause and a solution.
If you need further assistance, do not hesitate to contact our Scientific Support (scientific.support@westburg.eu). Please provide a detailed description of the problem and include data and/or pictures if possible.

Low Library Yield

Poor DNA quality	Use only high-purity DNA as input. Contaminants may interfere with the activity of the enzymes in the kit reducing library yield.
RNA contamination	RNA may have been co-purified with genomic DNA. This contaminating RNA affects the accuracy of DNA quantification. Use a high-quality DNA isolation kit that sufficiently removes RNA or treat the DNA sample with RNase A.
Too little input DNA for sequencing without amplification	For direct sequencing without amplification, you need to start with 100ng DNA or more to generate enough library yield. When starting with less than 100 ng: after the adapter ligation step perform library amplification with the Westburg NGS DNA Library Amplification Mix (Cat. no. WB 9100)

Unwanted peaks seen after capillary electrophoresis

Short peaks at 60 - 120 bp	These are adapter and adapter-dimer peaks caused by incomplete adapter depletion after library prep. Adapter-dimers will reduce the capacity of the flow cell for the library fragments. Use Agencourt® AMPure® XP Beads to remove free adapters and adapter-dimers. A small contamination of the library with adapter-dimers is acceptable.

APPENDIX I. ADAPTER GUIDELINES

The Westburg NGS NoFrag Library Prep kit is not provided with indexed adapters, necessary to be able to sequence the libraries on Illumina Sequencers. However, the kit is compatible with all Illumina indexed adapters.

We recommend to use the TruSeq® LT or HT adapters, which can be ordered either directly from Illumina or as custom oligos for NGS usage from Integrated DNA Technologies (IDT). These adapter sequences are listed below.

TruSeq® HT indexed adapters (Dual indexing):

D501–D508 Adapters

AATGATACGGCGACCACCGAGATCTACAC**[i5]**ACACTCTTTCCCTACACGACGCTCTTCCGATCT

i5 Index Name	i5 Bases for Sample Sheet MiSeq, HiSeq 2000/2500	i5 Bases for Sample Sheet MiniSeq, NextSeq, HiSeq 3000/4000
D501	TATAGCCT	AGGCTATA
D502	ATAGAGGC	GCCTCTAT
D503	CCTATCCT	AGGATAGG
D504	GGCTCTGA	TCAGAGCC
D505	AGGCGAAG	CTTCGCCT
D506	TAATCTTA	TAAGATTA
D507	CAGGACGT	ACGTCCTG
D508	GTACTGAC	GTCAGTAC

D701–D712 Adapters

GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**[i7]**ATCTCGTATGCCGTCTTCTGCTTG

i7 Index	i7 Bases for Sample Sheet
D701	ATTACTCG
D702	TCCGGAGA
D703	CGCTCATT
D704	GAGATTCC
D705	ATTCAGAA
D706	GAATTCGT
D707	CTGAAGCT
D708	TAATGCGC
D709	CGGCTATG
D710	TCCGCGAA
D711	TCTCGCGC
D712	AGCGATAG

APPENDIX I. ADAPTER GUIDELINES (continued)

TruSeq® LT Indexed adapters (Single Indexing):

TruSeq Universal Adapter
5’ AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

TruSeq Index Adapters (Index 1–27)
Index numbers 17, 24, and 26 are reserved.
Index sequences are 6 bases as underlined.

TruSeq Adapter, Index 1
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACATCACGATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 2
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACCGATGTATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 3
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACTTAGGCATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 4
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACTGACCAATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 5
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACACAGTGATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 6
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCAATATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 7
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACCAGATCATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 8
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACACTTGAATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 9
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACGATCAGATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 10
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACTAGCTTATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 11
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACGGCTACATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 12
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACCTTGTAATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 13
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACAGTCAACAATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 14
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACAGTTCCGTATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 15
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACATGTCAGAATCTCGTATGCCGTCTTCTGCTTG

APPENDIX I. ADAPTER GUIDELINES (continued)

TruSeq Adapter, Index 16
5' GATCGGAAGAGCACACGTCTGAACTCCAGTCACCCGTCCCGATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 18
5' GATCGGAAGAGCACACGTCTGAACTCCAGTCACGTCCGCACATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 19
5' GATCGGAAGAGCACACGTCTGAACTCCAGTCACGTGAAACGATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 20
5' GATCGGAAGAGCACACGTCTGAACTCCAGTCACGTGGCCTTATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 21
5' GATCGGAAGAGCACACGTCTGAACTCCAGTCACGTTTCGGAATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 22
5' GATCGGAAGAGCACACGTCTGAACTCCAGTCACCGTACGTAATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 23
5' GATCGGAAGAGCACACGTCTGAACTCCAGTCACGAGTGGATATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 25
5' GATCGGAAGAGCACACGTCTGAACTCCAGTCACACTGATATATCTCGTATGCCGTCTTCTGCTTG

TruSeq Adapter, Index 27
5' GATCGGAAGAGCACACGTCTGAACTCCAGTCACATTCCTTTATCTCGTATGCCGTCTTCTGCTTG

Adapter ligation guidelines

- Per Library, 2 adapters are needed.
  - When working with the TruSeq® LT adapters, use the Universal Forward adapter and an Indexed adapter of choice.
  - When working with the TruSeq® HT adapters, any combination of a D50x and a D7xx adapter can be used
- In general, the concentration of the adapter stock solutions is 15µM.
- For libraries prepared from >100ng input, use 2.5µl of each adapter (5µl adapter mix in total)
- For libraries prepared from 1-100ng input, use 2.5µl of a 1:10 dilution of each adapter (5µl 1:10 diluted adapter mix in total).

Alternatively, when the adapter concentration is unknown, please use the following recommendation: Based on a fragment size of 200bp, the adapter-to-insert ratio can be optimised as follows:

Input amount	1µg ----------> 1ng
Adapter/insert ratio	25:1----------> 200:1

Legal information about adapter sequences

APPENDIX II INPUT DNA CLEANUP: REMOVAL OF EDTA AND DIVALENT CATIONS

If the DNA sample is not in one of the recommended buffers (water, 10 mM Tris, 0.1X TE or Buffer EB) you should first purify the DNA using AMPure® XP beads, as described below.

- Add nuclease-free water to the sample up to 50 µl if the volume is below 50 µl.
- Add 90 µl of thoroughly vortexed AMPure® XP beads slurry to the 50 µl sample and mix well by pipetting. If the DNA sample is more than 50 µl, scale up the volume of AMPure® XP beads accordingly to achieve a 1.8X ratio of beads to DNA.
- Incubate 5 min at room temperature. Pellet the beads on a magnetic stand for 2-4 min and carefully remove the supernatant without disturbing the beads.
- Wash the beads with 200 µl of 80% ethanol. Pellet the beads on the magnetic stand and discard the supernatant. Repeat the wash once.
- Air-dry the beads on the magnetic stand for 10 minutes or until the beads are dry.
- Thoroughly resuspend the dried beads in 45 µl of 10 mM Tris-HCl, pH 8.0. Pellet the beads on the magnetic stand for 2 min. Carefully transfer 42.5 µl of supernatant into a new tube.
- Determine the concentration of the purified DNA using a fluorometric method such as Qubit or Picogreen.

QUALITY CONTROL

All kit components have been subjected to stringent quality control tests, are free of contaminating exo- and endonuclease activities and meet strict requirements with respect to DNA contamination.

PRODUCT USE LIMITATION

This product is developed, designed and sold exclusively for research purposes and *in vitro* use only. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans. Please contact techsupport@westburg.eu for the Material Safety Data Sheet of the product.

NOTES

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