Chapter 17

Preparation of Next-Generation Sequencing Libraries Using Nextera™ Technology: Simultaneous DNA Fragmentation and Adaptor Tagging by In Vitro Transposition

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Abstract

DNA library preparation is a common entry point and bottleneck for next-generation sequencing. Current methods generally consist of distinct steps that often involve significant sample loss and hands-on time: DNA fragmentation, end-polishing, and adaptor-ligation. In vitro transposition with Nextera™ Transposomes simultaneously fragments and covalently tags the target DNA, thereby combining these three distinct steps into a single reaction.

Platform-specific sequencing adaptors can be added, and the sample can be enriched and bar-coded using limited-cycle PCR to prepare di-tagged DNA fragment libraries. Nextera technology offers a streamlined, efficient, and high-throughput method for generating bar-coded libraries compatible with multiple next-generation sequencing platforms.

Key words: Next-generation sequencing, DNA library preparation, Roche/454, Illumina/Solexa, Nextera

1. Introduction

Next-generation sequencing (variously known as deep sequencing, second-generation sequencing, or high-throughput sequencing) has led to genomic data acquisition on a scale never seen before, enabling "hypothesis-free" studies and changing the approaches used to answer the fundamental scientific questions ((1) and references therein). Currently available sequencing platforms, as well as platforms anticipated in the near future, utilize three basic chemistries to sequence DNA, including pyrosequencing, sequencing by synthesis (both cyclic and real time), and sequencing by ligation (recently reviewed in (2–4)).

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Despite the diversity in sequencing chemistries used for sequencing polony arrays or single molecules, upstream sample preparation is generally very similar and follows a common three-step workflow. In the first step, large DNAs are sheared to appropriately sized fragments. Next, the DNA fragments are "repaired" to have blunt ends or A tails. In the final step, platform-specific adaptors are ligated onto the repaired fragments in order to attach the library to a solid surface in a spatially separated array via a complementary sequence (e.g., a tagged glass slide or bead). Each of these steps consists of multiple and distinct handling, incubation, and clean-up procedures, which result in a complex, multi-step protocol. Thus, current sample preparation methods require microgram amounts of genomic DNA, significant hands-on time, and suffer from limited sample throughput (5). In vitro transposition using Nextera technology can be exploited to perform all of these steps in a single, 5-min reaction.

In a classical transposition reaction, a hyperactive transposase enzyme is used under conditions that catalyze near-random insertion of excised transposons into DNA targets with high efficiency in vitro. When Transposome™ complexes are assembled with free transposon-end DNA instead, the target DNA is simultaneously fragmented and tagged with the transposon-end sequence, thereby generating a tagged DNA fragment library in a single reaction (Fig. 1).

Sequencing libraries are prepared using a simple, two-step process. First, genomic DNA is fragmented and tagged using in vitro transposition. Second, platform-specific adaptors and

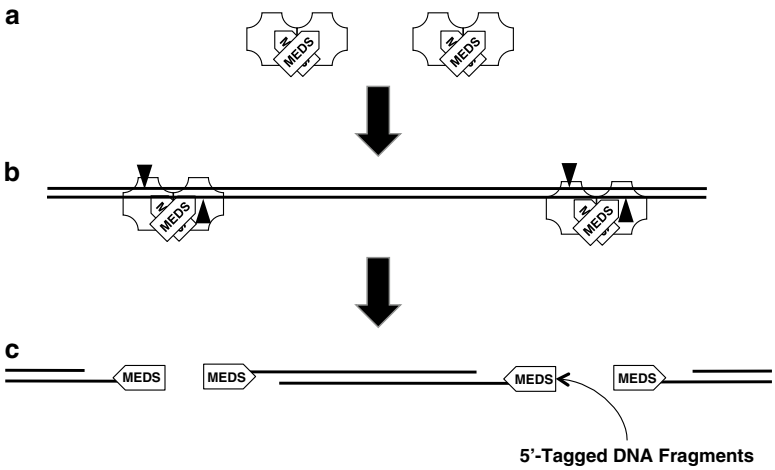

Fig. 1. Simultaneous fragmenting and tagging of DNA by in vitro transposition. Transposomes assembled with free, double-stranded transposon end DNA (**a**) are sufficient to bind and integrate into target DNA (**b**). The resulting tagged fragments (**c**) serve as the foundation for subsequent adaptor addition.

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optional bar codes are added and the library is enriched using limited-cycle PCR.

Methods are described to prepare DNA fragment libraries compatible with the Roche/454 GS FLX and FLX-Titanium platforms and the Illumina/Solexa GAII platform, from 50 ng of genomic DNA in less than 2 h. These methods can be readily adapted to produce DNA fragment libraries that are compatible with other sequencing platforms.

2. Materials

2.1. Roche/454-Compatible Library Production

1. Target DNA – in water or TE, free of contaminating proteins and salts (see Note 1).
2. 12.5 μM Nextera™ Enzyme Mix, Roche FLX or Ti-compatible (EZ-Tn5™ Transposase bound to double-stranded transposon-end DNA, EPICENTRE Biotechnologies, Madison, WI).

   `"METS" 5'-AGATGTGTATAAGAGACAG-3'`

   `"pMENTS" 3'-TCTACACATATTCTCTGTC-PO4-5'`
3. 5× Tagmentation reaction buffer [50 mM Tris-OAc pH 8.0, 25 mM $Mg(OAc)_2$].
4. ZYMO DNA Clean & Concentrator™-5 (ZYMO Research, Cat. No. D4013 or equivalent).
5. ZYMO DNA-Binding Buffer (ZYMO Research Cat. No. D4003-1-L or equivalent).
6. ZYMO Wash Buffer (ZYMO Research Cat. No. D4003-2-6 or equivalent).
7. Nuclease-free water.
8. 2× Nextera™ PCR Buffer (EPICENTRE Biotechnologies, Madison, WI).
9. PCR adaptors and primers.

   Standard FLX-Compatible Adaptors and Primers

   - Adaptor 1 (FLX-Compatible Library) – 0.5 μM in water

     `5'-GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAG-3'`
   - Adaptor 2 (FLX-Compatible Library) – 0.5 μM in water

     `5'-GCCTTGCCAGCCCGCTCAGAGATGTGTATAAGAGACAG-3'`
   - Primer 1 (FLX-Compatible Library) – 10 μM in water

     `5'-GCCTCCCTCGCGCCATCAG-3'`

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- Primer 2 (FLX-Compatible Library) – 10 μM in water
  `5'-GCCTTGCCAGCCCGCTCAG-3'`

FLX Titanium-Compatible Adaptors and Primers

- Adaptor 1 (Ti-Compatible Library) – 0.5 μM in water
  `5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGAGATGTGTATAAGAGACAG-3'`
- Adaptor 2 (Ti-Compatible Library) – 0.5 μM in water
  `5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGAGATGTGTATAAGAGACAG-3'`
- Primer 1 (Ti-Compatible Library) – 10 μM in water
  `5'-CCATCTCATCCCTGCGTGTCTCCGAC-3'`
- Primer 2 (Ti-Compatible Library) – 10 μM in water
  `5'-CCTATCCCCTGTGTGCCTTGGCAGTC-3'`

10. Nextera™ PCR Enzyme (EPICENTRE Biotechnologies, Madison, WI).

2.2. Illumina/Solexa-Compatible Library Production

1. Target DNA – in water or TE, free of contaminating proteins and salts (see Note 1).
2. 12.5 μM Nextera™ Enzyme Mix, Illumina GAII-compatible (EZ-Tn5™ Transposase bound to appended, double-stranded transposon-end DNA, EPICENTRE Biotechnologies, Madison, WI). Equal ratios of:

   `"A-METS"5'-GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAG-3'`

   `"pMENTS" 3'-TCTACACATATTCTCTGTC-PO4-5'`

   and

   `"B-METS"5'-GCCTTGCCAGCCCGCTCAGAGATGTGTATAAGAGACAG-3'`

   `"pMENTS" 3'-TCTACACATATTCTCTGTC-PO4-5'`
3. 5× Tagmentation reaction buffer [50 mM Tris-OAc pH 8.0, 25 mM $Mg(OAc)_2$].
4. ZYMO DNA Clean & Concentrator™-5 (ZYMO Research Cat. No. D4013 or equivalent).
5. ZYMO DNA-Binding Buffer (ZYMO Research Cat. No. D4003-1-L or equivalent).
6. ZYMO Wash Buffer (ZYMO Research Cat. No. D4003-2-6 or equivalent).
7. Nuclease-free water.
8. 2× Nextera™ PCR Buffer (EPICENTRE Biotechnologies, Madison, WI).

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9. PCR adaptors and primers.

   Illumina GAII-Compatible Adaptors and Primers[1]

   - Adaptor 1 (GAII-Compatible Library) – 0.5 μM in water

     `5'-AATGATACGGCGACCACCGAGATCTACACGCCTCC CTCGCGCCATCAG-3'`
   - Adaptor 2 (GAII-Compatible Library) – 0.5 μM in water

     `5'-CAAGCAGAAGACGGCATACGAGATCGGTCTGCC TTGCCAGCCCGCTCAG-3'`
   - Primer 1 (GAII-Compatible Library) – 10 μM in water

     `5'-AATGATACGGCGACCACCGA-3'`
   - Primer 2 (GAII-Compatible Library) – 10 μM in water

     `5'-CAAGCAGAAGACGGCATACGA-3'`
   - Nextera Read 1 Primer – 100 μM in water

     `5'-GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGA CAG-3'`
   - Nextera Read 2 Primer – 100 μM in water

     `5'-GCCTTGCCAGCCCGCTCAGAGATGTGTATAAGAG ACAG-3'`
   - Nextera Index Read Primer – 100 μM in water

     `5'-CTGTCTCTTATACACATCTCTGAGCGGGCTGGCAA GGCAGACCG-3'`
10. Nextera™ PCR Enzyme (EPICENTRE Biotechnologies, Madison, WI).
11. Illumina paired-end sequencing mixes (HP1 and HP2).
12. Illumina GAII Hybridization Buffer (Illumina Cat. No. GA0084204-HT1).
13. 5× SSC, 0.05% Tween®-20.
14. Illumina single-read sequencing mix (HP4).

3. Methods

Using in vitro transposition to simultaneously fragment and tag DNA in a single-tube reaction is a significant improvement upon current procedures, which generally consist of distinct DNA fragmentation, end-polishing, and adaptor-ligation steps. The following library preparation procedure combines these steps into one,

[1] The sequences of Primer 1 and Primer 2 and portions of Adaptor 1 and Adaptor 2 correspond to Illumina bPCR sequences and are copyrighted by Illumina, Inc. Oligonucleotide sequences© 2006–2010 Illumina, Inc. All rights reserved.

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uses only 50 ng of starting DNA, and allows incorporation of platform-specific adaptors and optional bar codes.

This chapter details methods to prepare libraries for the Roche/454 (GS FLX and FLX-Titanium) and Illumina/Solexa (GAII) sequencing platforms. The basic design can be applied to other adaptor-tagged DNA libraries.

3.1. Roche/454-Compatible Library Preparation

Target DNA is fragmented and tagged with Transposomes containing free transposon ends. Limited-cycle PCR with a four-primer reaction adds Roche/454-compatible adaptor sequences (GS FLX or FLX-Titanium). Optional bar coding is added between the upstream emulsion PCR (emPCR) adaptor and the transposon end (Fig. 2).

The Tagmentation reaction is the same for standard Roche GS FLX or for FLX-Titanium-compatible sequencing libraries. Specific adaptors for GS FLX or FLX-Titanium-compatible libraries are used during the limited-cycle PCR.

Roche/454 Tagmentation Reaction

Tagmentation with Roche/454-compatible Transposomes should predominantly yield fragments approximately 500–2,000 bp

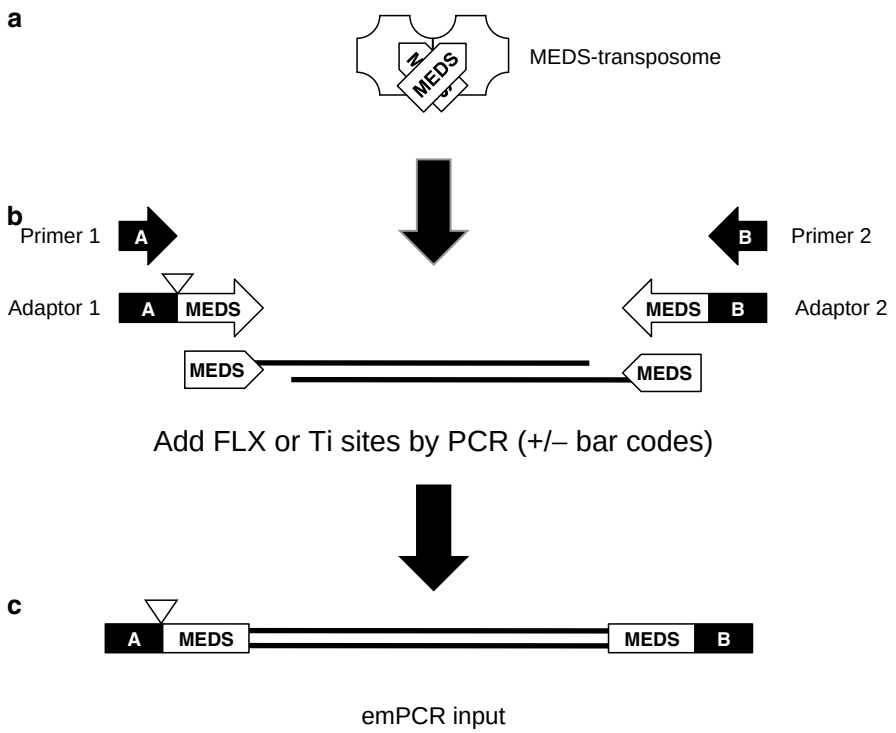

Fig. 2. Schematic representation of Roche/454-compatible sequencing library preparation. Transposomes assembled with free ends (**a**) are used in a tagmentation reaction to produce a 5′-tagged DNA fragment library. Limited-cycle PCR with a four-primer reaction adds Roche/454-compatible adaptor sequences (**b**). Optional bar coding (*triangle*) is incorporated into Adaptor 1 and added between the upstream emPCR adaptor and the transposon end (**c**).

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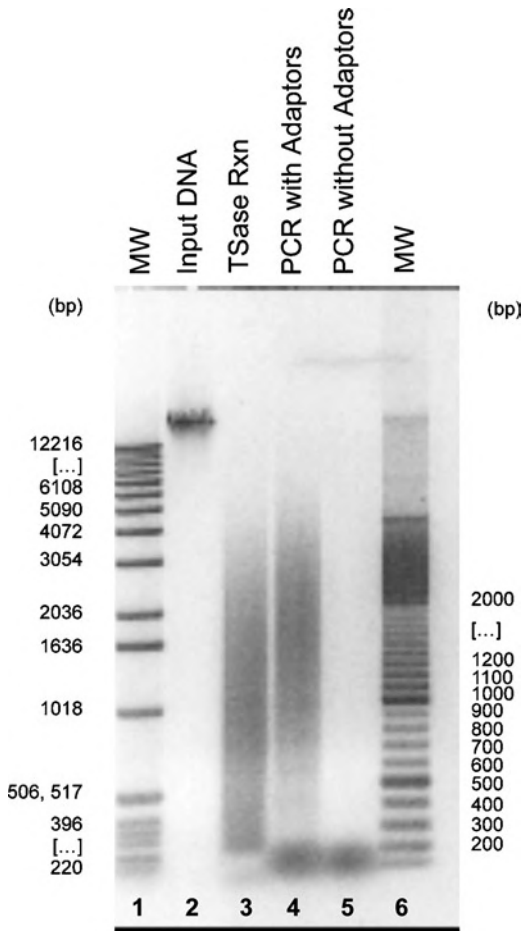

Fig. 3. Preparation of a Roche/454-compatible sequencing library. HeLa DNA (*lane 2*) was tagmented with Transposomes assembled with free ends (*lane 3*). Limited-cycle PCR was used to add Roche/454 GS FLX-compatible adaptors (*lane 4*). The recovered DNA was used directly in emPCR prior to pyrosequencing. Control PCR without Adaptor 1 and Adaptor 2 (including Primer 1 and Primer 2) confirmed reaction specificity (*lane 5*).

(Fig. 3). The actual fragment size distribution will depend on a number of factors, including the quantity and quality of starting DNA.

1. Assemble the following reaction components on ice, in the order listed (see Note 2)

*x*μl	Nuclease-free water
50 ng	Target DNA (see Note 1)
4 μl	5× Tagmentation reaction buffer
1 μl	Nextera Transposomes (Roche FLX or Ti-Compatible)
20 μl	Total reaction volume

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2. Mix briefly by vortexing and incubate at 55°C for 5 min. To prevent evaporation, the reaction should be carried out in a thermocycler with a heated lid or the reaction should be overlaid with mineral oil. Adding DNA-Binding Buffer in step 3 stops the reaction.
3. Purify the tagmented DNA using a Zymo DNA Clean & Concentrator-5 Kit (or equivalent) according to the manufacturer's instructions (see Note 3). A synopsis of the ZYMO protocol is shown in steps 4–13. Perform these steps at room temperature.
4. Add 100 μl of DNA-binding buffer to the 20 μl tagmentation reaction from step 2.
5. Mix briefly by vortexing and transfer the mixture to a Zymo-Spin™ Column in a Collection Tube.
6. Centrifuge at 10,000 × *g* for 60 s. Discard the flow-through.
7. Add 250 μl of wash buffer to the column. Centrifuge at 10,000 × *g* for 60 s. Discard the flow-through.
8. Repeat the wash step once for a total of two washes.
9. Centrifuge the empty column again at 10,000 × *g* for 60 s to dry and to eliminate any residual wash buffer.
10. Transfer the column to a clean and sterile 1.5-ml microcentrifuge tube.
11. Add 11 μl of nuclease-free water directly to the column and incubate at room temperature for 1–2 min. Centrifuge at 10,000 × *g* for 60 s to elute the DNA into the clean microcentrifuge tube.
12. The final eluted volume should be ~10 μl. Use 5 μl of recovered DNA as PCR template in step 14.
13. The recovered DNA may be stored at −20°C.

Addition of emPCR-Compatible Adaptors and Library Enrichment

14. Assemble the following reaction components at room temperature:

15 μl	Nuclease-free water
5 μl	Recovered DNA fragment library (from step 12)
25 μl	2× Nextera PCR buffer
1 μl	0.5 μM Adaptor 1 (FLX or Ti-compatible)
For a bar-coded Roche-compatible library, replace Adaptor 1 with a bar-coded Adaptor 1 with the sequence composition, see Note 4. The other three primers and the cycling conditions remain unchanged.	
1 μl	0.5 μM Adaptor 2 (FLX or Ti-compatible)

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1 μl	10 μM Primer 1 (FLX or Ti-compatible)
1 μl	10 μM Primer 2 (FLX or Ti-compatible)
1 μl	Nextera PCR enzyme (2.5 U/μl)
50 μl	Total reaction volume

15. Incubate the sample in a thermocycler under the following conditions (see Note 5):

    72°C for 3 min, 95°C for 30 s, followed by 15 cycles of (95°C for 10 s, 55°C for 30 s, 72°C for 3 min), Hold at 4°C.

16. Purify the DNA sequencing library using a Zymo DNA Clean & Concentrator-5 kit, or equivalent. The anticipated yield is ~300–500 ng of PCR-amplified DNA (Fig. 3).

17. The recovered DNA is ready to be quantified and used as input for Roche/454 emPCR and sequencing. No changes to the standard Roche/454 protocol are needed (see Note 6).

3.2. Illumina/Solexa-Compatible Library Preparation

Target DNA is fragmented and tagged with Transposomes containing transposon ends appended with sequencing tags. Limited-cycle PCR with a four-primer reaction adds Illumina/Solexa bridge PCR (bPCR)-compatible adaptor sequences. Optional bar coding is added between the upstream bPCR adaptor and the sequencing tag on Adaptor 2 (see Fig. 4).

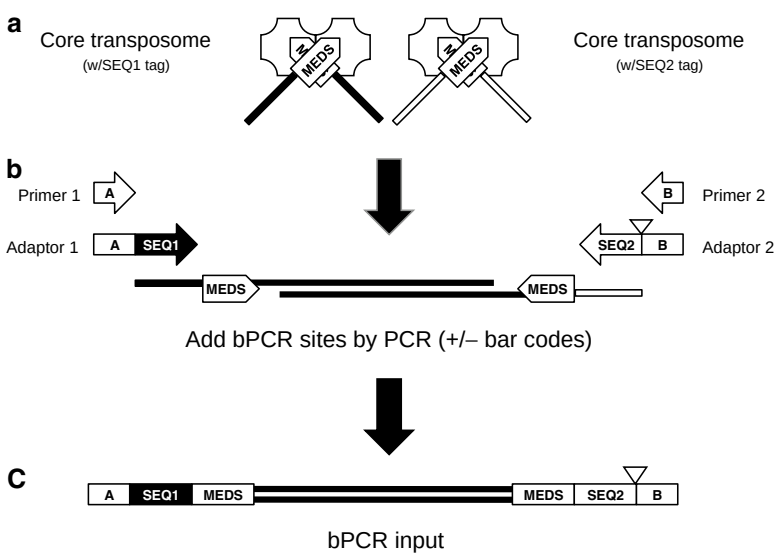

Fig. 4. Schematic representation of Illumina/Solexa-compatible sequencing library preparation. Transposomes assembled with free ends appended with sequencing tags (**a**) are used in a tagmentation reaction to produce a 5′-tagged DNA fragment library. Limited-cycle PCR with a four-primer reaction adds Illumina/Solexa bPCR-compatible adaptor sequences (**b**). Optional bar coding (*triangle*) is incorporated into Adaptor 2 and added between the upstream bPCR adaptor and sequencing tag 2 (**c**).

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Illumina–Solexa Tagmentation Reaction

Tagmentation with Illumina GAII-compatible Transposomes should predominantly yield fragments approximately 250–1,000 bp (Fig. 5). The actual fragment size distribution will depend on a number of factors, including the quantity and quality of starting DNA.

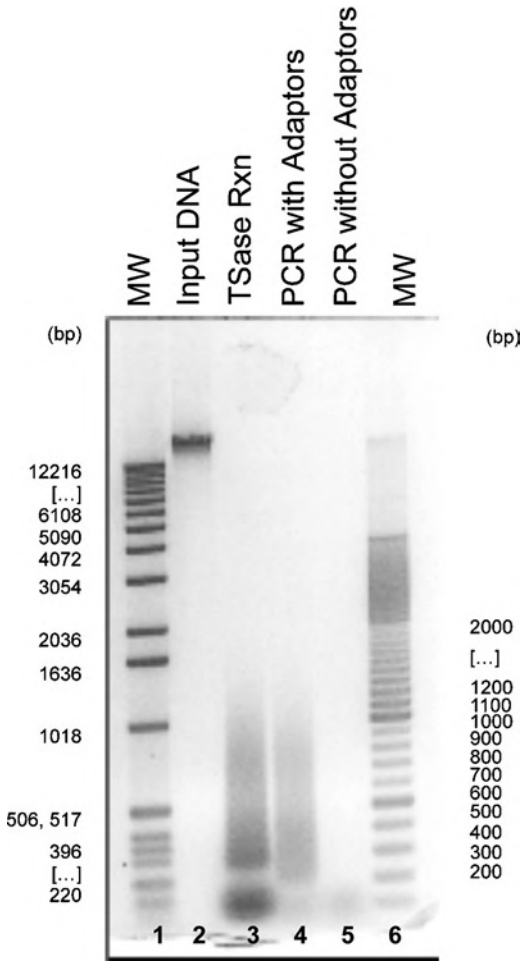

Fig. 5. Preparation of an Illumina GAII-compatible sequencing library. HeLa DNA (*lane 2*) was tagmented with Transposomes assembled with free ends appended with sequencing tags (*lane 3*). Limited-cycle PCR was used to add Illumina/Solexa bPCR-compatible adaptors (*lane 4*). The recovered DNA was used directly in bPCR prior to sequencing on the Illumina GAII instrument. Control PCR without Adaptor 1 and Adaptor 2 (including Primer 1 and Primer 2) confirmed reaction specificity (*lane 5*).

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1. Assemble the following reaction components on ice, in the order listed (see Note 2)

*x*μl	Nuclease-free water
50 ng	Target DNA (see Note 1)
4 μl	5× Tagmentation reaction buffer
1 μl	Nextera Transposomes (Illumina GAII-Compatible)
20 μl	Total reaction volume

2. Mix briefly by vortexing and incubate at 55°C for 5 min. To prevent evaporation, the reaction should be carried out in a thermocycler with a heated lid or the reaction should be overlaid with mineral oil. Adding DNA-Binding Buffer in step 3 stops the reaction.
3. Purify the DNA sequencing library using a Zymo DNA Clean & Concentrator-5 Kit (or equivalent) according to the manufacturer's instructions (see Note 3). A synopsis of the ZYMO protocol is shown in steps 4–13. Perform these steps at room temperature.
4. Add 100 μl of DNA-binding buffer to the 20 μl tagmentation reaction from step 2.
5. Mix briefly by vortexing and transfer the mixture to a Zymo-Spin™ Column in a Collection Tube.
6. Centrifuge at 10,000 × *g* for 60 s. Discard the flow-through.
7. Add 250 μl of wash buffer to the column. Centrifuge at 10,000 × *g* for 60 s. Discard the flow-through.
8. Repeat the wash step once for a total of two washes.
9. Centrifuge the empty column again at 10,000 × *g* for 60 s to dry and to eliminate any residual wash buffer.
10. Transfer the column to a clean and sterile 1.5-ml microcentrifuge tube.
11. Add 11 μl of nuclease-free water directly to the column and incubate at room temperature for 1–2 min. Centrifuge at 10,000 × *g* for 60 s to elute the DNA into the clean microcentrifuge tube.
12. The final eluted volume should be ~10 μl. Use 5 μl of recovered DNA as PCR template in step 14.
13. The recovered DNA may be stored at −20°C.

Addition of bPCR-Compatible Adaptors and Library Enrichment

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14. Assemble the following reaction components at room temperature:

15 μl	Nuclease-free water
5 μl	Recovered DNA Fragment Library (from step 3)
25 μl	2× Nextera PCR Buffer
1 μl	0.5 μM Adaptor 1 (Illumina GAII-Compatible)
1 μl	0.5 μM Adaptor 2 (Illumina GAII-Compatible, see Note 7)
For a bar-coded Illumina-compatible library, replace Adaptor 2 with a bar-coded Adaptor 2 with the sequence composition, see Note 7. The other three primers and the cycling conditions remain unchanged.	
1 μl	10 μM Primer 1 (Illumina GAII-Compatible)
1 μl	10 μM Primer 2 (Illumina GAII-Compatible)
1 μl	Nextera PCR Enzyme (2.5 U/μl)
50 μl	Total reaction volume

15. Incubate the samples in a thermocycler under the following conditions (see Note 5): 72°C for 3 min, 95°C for 30 s, followed by 12 cycles of (95°C for 10 s, 62°C for 30 s, 72°C for 3 min), hold at 4°C.
16. Purify the tagged DNA fragments using a Zymo DNA Clean & Concentrator-5 kit, or equivalent. The anticipated yield is ~300–500 ng of PCR-amplified DNA (Fig. 5).
17. The recovered DNA is ready to be used as input for Illumina GAII bPCR and cluster formation. No changes to the standard Illumina protocol are needed for cluster formation. However, alternate sequencing primers must be included during the sequencing reactions as described below (see Note 8).

    For Paired-End Reads: Illumina paired-end sequencing mixes (HP1 and HP2) contain the Illumina GAII sequencing primers. The Nextera sequencing primers are compatible with the Illumina GAII sequencing primers and can be used together in the same flow cell channel.

    - Paired-End Read 1: Dilute 100 μM Nextera Read 1 Primer 1:200 into Illumina GAII Sequencing Mix HP1.
    - Index Read: Dilute 100 μM Nextera Index Read Primer 1:200 into Illumina GAII Hybridization Buffer.
    - Paired-End Read 2: Dilute 100 μM Nextera Read 2 Primer 1:200 into Sequencing Mix HP2.
    - Alternatively, if it is required to perform sequencing in the presence of only the Nextera primers, the 100 μM Nextera sequencing primers can be diluted 1:200 into Illumina GAII Hybridization Buffer or 5× SSC, 0.05% Tween®-20.

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For Single Reads: Illumina single-read sequencing mix (HP4) does not contain the Illumina sequencing primer.

- Single-Read Sequencing: Dilute Nextera Read 1 Primer 1:200 into Sequencing Mix HP4.
- Index Read: Dilute Nextera Index Read Primer 1:200 into Illumina GAII Hybridization Buffer or 5× SSC, 0.05% Tween®-20.

4. Notes

1. The quality of the starting DNA is critical. Contaminants such as protein and RNA may interfere with accurate quantification and/or inhibit the transposome fragmentation and tagging reaction if present in the DNA preparation. If DNA purity is in question, the DNA should be cleaned using Zymo Genomic DNA Clean & Concentrator or equivalent (ZYMO Research, Cat. No. D4010) prior to the Tagmentation reaction.
2. The tagmentation reaction does occur, although very slowly, at room temperature. Therefore, it is recommended that the components be assembled on ice and immediately incubated at 55°C.
3. The tagmentation reaction results in the simultaneous tagging and fragmentation of the target DNA. However, the transposase remains tightly bound to the ends of the resulting fragment. It is necessary to remove the transposase with chaotropic salts prior to tagging the 3′ ends and adding platform-specific adaptors by PCR. Equivalent kits can also be used; however, care must be taken not to denature the dsDNA fragments.
4. For a bar-coded Roche-compatible library, replace Adaptor 1 with a bar-coded Adaptor 1 with the sequence composition shown below. The other three primers and the cycling conditions remain unchanged.
   - Standard GS FLX-compatible Adaptor 1 with bar code.

     `5'-GCCTCCCTCGCGCCATCAG-[BAR CODE]-AGATGTGTATAAGAGACAG-3'`
   - Standard FLX Titanium-compatible Adaptor 1 with bar code.

     `5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-[BAR CODE]-AGATGTGTATAAGAGACAG-3'`
5. It is critical to perform the 72°C extension step to tag the 3′ ends of the DNA fragments *before* denaturing the PCR

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templates. The 3′ ends are tagged by polymerase extension from the 3′ end of the genomic fragment and displacement of the MENTS oligonucleotide.

6. Nextera-generated and "standard" libraries can be sequenced in the same region of a plate. Perform the sequencing reactions with the standard Roche/454 sequencing primers. Sequencing reads will contain any added bar code sequence and the 19-base transposon-end sequence at the 5′ end of all reads. The bar code sequence can be used to bin the sequence reads. The 19-base transposon-end sequence should be filtered out prior to assembly and analysis (19-base transposon-end sequence: 5′-AGATGTGTATAAGAGACAG-3′).
7. For a bar-coded Illumina-compatible library, replace Adaptor 2 with a bar-coded Adaptor 2 with the sequence composition shown below. The other three primers and the cycling conditions remain unchanged.
   - Illumina GAII-compatible Adaptor 2 with bar code.

     ```
     5'-CAAGCAGAAGACGGCATACGAGA-[BAR CODE]-TC
     GGTCTGCCTTGCCAGCCCGCTCAG-3'
     ```
8. It is *critical* to use the Nextera Read 1, Nextera Read 2, and Index Read primers during cluster sequencing. While the resulting libraries are compatible with Illumina GA II bPCR and cluster formation, the transposon-derived sequencing primer sites are *not compatible with Illumina GAII sequencing primers.* The 19-bp transposon DNA sequence is present at the 5′ end of all Illumina-compatible libraries. However, the 19-bp transposon DNA sequence is NOT sequenced on the Illumina platform. The Nextera Read 1 and Read 2 primers anneal to this sequence so that the first nucleotide sequenced is target DNA.

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Patent Nos. 5,965,443, and 6,437,109; European Patent No. 0927258, and related patents and patent applications, exclusively licensed to EPICENTRE, cover Nextera™ Products.

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