CHAPTER SEVEN

Measuring Genome-Wide Nucleosome Turnover Using CATCH-IT

Sheila S. Teves[*,†], **Roger B. Deal**[*], **Steven Henikoff**[*,‡,1]

[*]Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA
[†]Molecular and Cellular Biology Program, University of Washington, Seattle, Washington, USA
[‡]Howard Hughes Medical Institute, Seattle, Washington, USA
[1]Corresponding author: e-mail address: steveh@fhcrc.org

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Abstract

The dynamic interplay between DNA-binding proteins and nucleosomes underlies essential nuclear processes such as transcription, replication, and DNA repair. Manifestations of this interplay include the assembly, eviction, and replacement of nucleosomes. Hence, measurements of nucleosome turnover kinetics can lead to insights into the regulation of dynamic chromatin processes. In this chapter, we describe a genome-wide method for measuring nucleosome turnover that uses metabolic labeling followed by capture of newly synthesized histones, which we have termed *C*ovalent *A*ttachment of *T*agged *H*istones to *C*apture and *I*dentify *T*urnover (CATCH-IT). Although CATCH-IT can be used with any genome-wide mapping procedure, high-resolution

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profiling is attainable using paired-end sequencing of native chromatin. Our protocol also includes an efficient Solexa DNA sequencing library preparation protocol that can be used for single base-pair resolution mapping of both nucleosome and sub-nucleosomal particles. We not only describe the use of these protocols in the context of a Drosophila cell line but also provide the necessary changes for adaptation to other model systems.

1. INTRODUCTION

Nucleosomes have evolved to tightly package DNA in chromosomes and so must be mobilized to allow DNA-binding proteins to gain access to their binding sites and perform DNA-templated processes. For DNA to be accessible, nucleosomes must be displaced, partially unwrapped, or evicted, and measuring these dynamic events can provide mechanistic insights into the regulation of chromatin-dependent processes. We have recently developed a method that combines kinetic measurement of nucleosome dissociation and replacement (turnover) with genome-wide readout technologies that we termed *C*ovalent *A*ttachment of *T*agged *H*istones to *C*apture and *I*dentify *T*urnover (CATCH-IT) (Deal et al., 2010). In CATCH-IT, a methionine analog containing an azide moiety is incorporated into newly synthesized proteins, which can then be biotinylated through copper-catalyzed cycloaddition reaction with a biotin-alkyne substrate. Nucleosomes containing the newly synthesized biotinylated histones can be isolated using streptavidin-coated beads and the extracted DNA used to measure the degree of nucleosome turnover genome-wide.

The increasing affordability of short-read massively parallel sequencing potentially enables the study of epigenomic events at single base-pair resolution. Paired-end (PE) sequencing using Illumina's Solexa platform (Bentley, 2006) is becoming especially valuable for epigenomic mapping, as it allows precise determination of both fragment lengths and positions. However, current Solexa library preparation protocols were designed for genomics applications, where fragmentation by random shearing and size selection were intended to provide a uniform population of DNA templates for bulk sequencing and so include a gel-based size-selection step to exclude both large and small fragments. However, for many epigenomic mapping applications, random fragmentation and size selection is inappropriate, and the requirement for large amounts of starting material can be limiting. To address these issues, we have developed a modified Solexa library protocol that removes gel-based size selection and streamlines DNA cleanup to

allow for sequencing of as little as ~10 ng of starting material with fewer manipulations (Henikoff, Belsky, Krassovsky, Macalpine, & Henikoff, 2011). Combining this modified protocol with chromatin-probing experiments, such as micrococcal nuclease (MNase) mapping (Henikoff et al., 2011; Kent, Adams, Moorhouse, & Paszkiewicz, 2011), salt fractionation (Henikoff, Henikoff, Sakai, Loeb, & Ahmad, 2009), native chromatin immunoprecipitation (Teves & Henikoff, 2011; Weber, Henikoff, & Henikoff, 2010), and as we discuss in this chapter, CATCH-IT (Deal et al., 2010) allows for single base-pair resolution analyses of chromatin-based processes.

In this chapter, we present detailed protocols and tips to perform CATCH-IT at high resolution using a Drosophila cell line and provide illustrative data of expected results. These protocols should be adaptable to cell lines of other organisms, and we highlight the steps where necessary changes should be made when performing CATCH-IT for other systems.

2. COVALENT ATTACHMENT OF TAGGED HISTONES TO CAPTURE AND IDENTIFY TURNOVER

Below, we provide a detailed protocol for nucleosome turnover analysis through metabolic labeling. The method relies on the depletion of methionine in growth medium followed by incorporation of the methionine analog azidohomoalanine (Aha) into newly synthesized proteins in place of methionine. Following nuclei isolation from Aha-labeled cells, newly synthesized nuclear proteins containing an Aha-azide moiety can be coupled to an alkyne-linked biotin tag through a copper-catalyzed cycloaddition reaction. Chromatin can then be fragmented down to mononucleosomes using MNase, which digests away unprotected DNA fragments. A standard salt extraction step provides the input material for affinity purification of newly synthesized chromatin using streptavidin beads. Because the turnover rate of H2A/H2B dimers is faster than that of the central $(H3/H4)_2$ tetramer (Rufiange, Jacques, Bhat, Robert, & Nourani, 2007; Thiriet & Hayes, 2005), the immobilized chromatin is washed with a urea-containing solution that strips off the H2A/H2B from nucleosomes and also removes virtually all other bound proteins. DNA extracted from the bead-bound material can then be isolated for genome-wide analysis.

2.1. Solutions and materials

1. Drosophila S2 cells maintained in log-phase growth
2. Shields and Sang M3 (SSM3-Met) Insect growth medium without methionine
3. Azidohomoalanine (Anaspec cat # 63669)
4. Methionine (Sigma-Aldrich # M9625)
5. Round-bottom 30 mL Corex tubes
6. Table-top centrifuge
7. 1 × phosphate-buffered saline (PBS)
8. ⋆TM2 buffer (10 m*M* Tris, pH 7.5, 2 m*M* $MgCl_2$)
9. 10% NP-40 (Sigma-Aldrich # 74385)
10. Refrigerated table-top centrifuge
11. ⋆HB125 buffer (0.125 *M* sucrose, 15 m*M* Tris, pH 7.5, 15 m*M* NaCl, 40 m*M* KCl, 0.5 m*M* spermidine, 0.15 m*M* spermine)
12. Copper(II) sulfate pentahydrate (Sigma-Aldrich # C7631)
13. L-Ascorbic acid (Sigma-Aldrich # A5960)
14. Biotin-alkyne (Invitrogen # B10185)
15. End-over-end microcentrifuge tube rotator (ex. Labquake Shaker—Thermo Scientific)
16. 0.5 *M* EDTA
17. 1 *M* $CaCl_2$
18. 37 °C water bath
19. MNase 200 U powder, resuspended to 0.2 U/μL (Sigma-Aldrich # N3755)
20. ⋆CSB 350 buffer (1 × PBS, 213 m*M* NaCl (350 m*M* total), 2 m*M* EDTA, 0.1% Triton X-100)
21. Dynabeads M-280 streptavidin (Invitrogen # 112.05D)
22. Magnetic rack for microcentrifuge tubes
23. ⋆Urea/NaCl wash buffer (4 *M* urea, 0.3 *M* NaCl, 20 m*M* Tris, pH 8, 1 m*M* EDTA)
24. 10% SDS
25. Phenol–chloroform–isoamyl alcohol (25:24:1, v/v/v)
26. Glycogen 20 mg/mL
27. 200 proof ethanol
28. TE, pH 8 (10 m*M* Tris, pH 8, 1 m*M* EDTA)
29. Complete-mini, protease inhibitor cocktail EDTA free (Roche # 1 830 170)

⋆Buffers are supplemented with complete-mini, protease inhibitor cocktail prior to use.

2.2. Methionine-free growth medium

In many systems, growth medium without methionine is commercially available. However, for insect cell culture systems, this is not the case. We therefore prepare methionine-free SSM3-Met insect medium for use in CATCH-IT experiments. Table 7.1 lists the components of M3 medium without methionine for 1 L of medium.

1. Add 1.05 g of Bis–Tris to 650 mL of H_2O and adjust pH to 6.8.
2. Combine all solids (salts, vitamins, amino acids, sugar) and grind into fine powder using a mortar and pestle.
3. Slowly add the ground powder to the Bis–Tris–H_2O while mixing using a magnetic stir bar.
4. After all solids have dissolved, readjust the pH to 6.8 and bring the final volume to 1 L. Filter sterilize, and store at 4 °C.

2.3. Aha labeling and biotin coupling

1. Grow S2 cells in two 75 cm^2 flasks to late log phase in rich medium. Remove the medium and replace with 7 mL of SSM3-Met medium. Place the flasks back in the incubator for 30 min to starve cells of methionine.
2. Remove the medium and replace with 7 mL of SSM3-Met medium supplemented with 4 m*M* Aha for one flask and 4 m*M* Met for the other flask. Place the flasks back in the incubator for the desired amount of time (20 min to several hours).
3. Harvest the cells from each treatment in 30 mL round-bottom Corex tubes. Spin at 1200 × *g* for 3 min. Decant the medium and wash cells with 10 mL of 1 × PBS, spin again, and decant PBS.

Note: The following steps (4–6) are used to isolate nuclei from Drosophila S2 cells. To adapt this method to other systems, the final concentration of NP-40 must be optimized. Alternatively, other standard protocols for nuclei preparation can be substituted.

4. Resuspend the cells in 1 mL of ice-cold TM2 buffer and transfer to 1.5 mL microcentrifuge tubes. Place on ice for 3 min.
5. Add 60 μL of 10% NP-40 and vortex for 5 s at the low-medium setting. Place cells back on ice for 3 min, vortex one more time as before, and spin out nuclei at 100 × *g* for 10 min at 4 °C.
6. Wash nuclei with 1 mL cold TM2 buffer. Pellet nuclei at 100 × *g* for 10 min at 4 °C, decant supernatant, and resuspend gently in 200 μL of cold HB 125 buffer. If performing a time course, leave each successive sample on ice at this point until all are collected and then proceed to step 7 with all samples.

Table 7.1 Components of Methionine-free growth medium 1 L of medium

	(g/L)
Bis–Tris	1.05
$CaCl_2$	0.76
$MgSO_4$	2.15
$NaHCO_3$	0.42
Na_2HPO_4	0.88
Glucose	10
Choline chloride	0.05
Oxalacetic acid	0.25
Amino acids	
L-α-Alanine	1.5
L-β-Alanine	0.25
L-Arginine	0.5
L-Asparagine	0.3
L-Aspartic acid	0.3
L-Cysteine-HCl	0.2
L-Glutamic acid-K	7.88
L-Glutamic acid-Na	6.53
L-Glutamine	0.6
L-Glycine	0.5
L-Histidine	0.55
L-Isoleucine	0.25
L-Leucine	0.4
L-Lysine-HCl	0.85
L-Phenylalanine	0.25
L-Proline	0.4
L-Serine	0.35
L-Threonine	0.5
L-Tryptophan	0.1
L-Tyrosine-2Na	0.3601
L-Valine	0.4

7. Prepare reagents as follows for the cycloaddition coupling reaction:
   a. Weigh out 25 mg of $CuSO_4$ and 88 mg of ascorbic acid into 1.5 mL tubes and dissolve each in 1 mL of H_2O to give solutions of 100 m*M* $CuSO_4$ and 500 m*M* ascorbic acid. Combine 100 μL of $CuSO_4$ with 100 μL of ascorbic acid. The solution turns yellow as Cu^{2+} is reduced to Cu^{+} by ascorbic acid.
   b. Prepare 20 μL of 2 m*M* biotin–PEO–alkyne by 1:10 dilution of 20 m*M* stock solution.
8. Place nuclei suspensions at room temperature and then add the following to each 200-μL sample, mixing well after each addition:
   5 μL of 2 m*M* biotin–PEO–alkyne (50 μ*M* final concentration)
   4 μL of $CuSO_4$/ascorbic acid mixture (final concentration is 1 m*M* $CuSO_4$, 5 m*M* ascorbic acid)
9. Place nuclei suspensions on a microcentrifuge tube rotator at 4 °C for 30 min. Save 2 μL (1% of total) for Western analysis at the end of the procedure.
10. Pellet nuclei at 100 × *g* for 5 min at 4 °C.
11. Remove supernatant thoroughly and resuspend nuclei gently in 200 μL of cold HB 125 buffer. Repeat steps 8 through 10 using freshly prepared cycloaddition reagents.

We tested the labeling and coupling process using total cellular extracts from Met- or Aha-labeled cells. We performed Western blot analysis on aliquots saved from each step of the process and probed for biotin using α-streptavidin antibody (Fig. 7.1). We found that Aha- but not methionine-labeled cells have incorporated biotin into general cellular proteins. This test also showed that a second biotin coupling is required for saturation of all Aha-labeled proteins.

2.4. Chromatin fragmentation and extraction

1. Resuspend nuclei in 250 μL HB 125 with 1 m*M* EDTA and add $CaCl_2$ to 2 m*M* final concentration. Place tubes in a 37 °C water bath for 5 min.
2. Add 2 μL of the MNase solution to each tube in the water bath and mix gently by inversion and flicking several times. Continue digestion for 10 min with mixing each minute. This level of digestion should give mostly mononucleosomes (Fig. 7.2), but this should be optimized for each system by varying the concentration of MNase and/or the length of digestion time.
3. Add EDTA to 2 m*M* to stop the reaction and place the tubes on ice. Spin at 100 × *g* for 10 min at 4 °C.

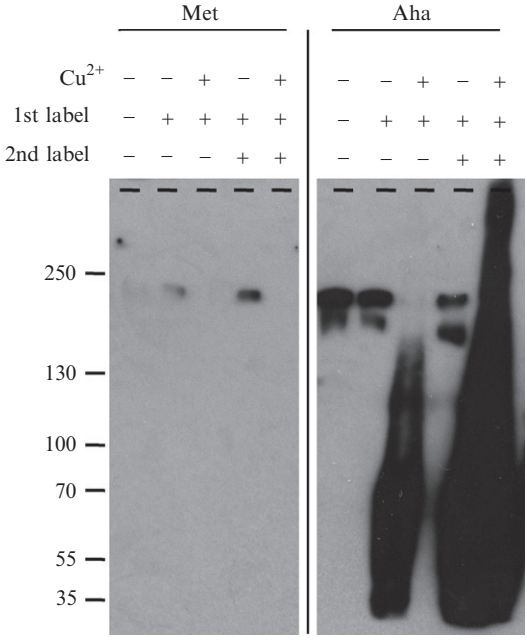

Figure 7.1 Incorporation of Aha into cellular proteins. Cells depleted of methionine were incubated in SSM3-Met medium supplemented with 4 m*M* Met or Aha for 20 min. Cells were then lysed in 1% SDS in 1× PBS, and the resulting total protein extract was subjected to biotin coupling as described either with or without Cu^{+}. Aliquots before treatment, after the first labeling, and after the second labeling were subjected to Western blot analysis using α-streptavidin to visualize biotinylated proteins.

4. Resuspend nuclei in 300 μL of CSB 350 and mix on a microcentrifuge tube rotator at 4 °C for at least 1 h to overnight.
5. Centrifuge nuclei at 100 × *g* for 10 min at 4 °C and save the supernatant (soluble chromatin). Clarify the soluble chromatin by centrifugation at full speed for 5 min and move to a fresh tube.
6. Remove a 10-μL aliquot of the soluble chromatin (Input) for DNA purification and 12 μL for Western analysis, coomassie gel, and protein concentration measurement. Resuspend the pellet in loading buffer and save for Western analysis.

2.5. Streptavidin affinity capture

1. To the soluble chromatin, add 25 μL (beads + buffer, same as original ratio as in stock slurry) of CSB-washed M280 streptavidin-coated Dynabeads. Rotate for 1.5 h at 4 °C.

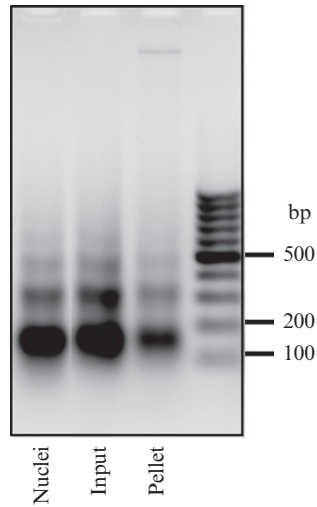

Figure 7.2 Nucleosome laddering of MNase-digested chromatin. After biotin coupling, intact nuclei were digested with 0.4 U MNase for 10 min and subjected to salt extraction. DNA from total nuclei, 350 m*M* salt-extracted chromatin, and pellet fractions were electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide.

2. Place tubes on a Dynal magnet rack for several minutes, and then save the supernatant as "unbound" for Western analysis. Resuspend the beads in 700 μL of urea/NaCl wash buffer and rotate at 4 °C for 5 min to strip the H2A/H2B dimers from the chromatin.
3. Place tubes on the magnet rack, decant, and resuspend in 700 μL of CSB 350. Transfer the beads and buffer to a fresh tube and rotate at 4 °C for 5 min. Collect and decant the beads and proceed with DNA isolation or resuspend the beads in 20 μL CSB 350 and freeze at −20 °C.

Western analysis of total nuclei, input, unbound, and pellet fractions during the streptavidin affinity capture process (Fig. 7.3) shows lack of streptavidin signal in the unbound fraction compared to others, indicating an efficient capture of biotin-coupled, Aha-labeled proteins.

2.6. DNA isolation

1. Bring the volumes of input and beads to 200 μL with CSB 350 and add SDS to 0.5%. Add 1 μL of RNAse A and incubate at 37 °C for 10 min, and then add 1 μL of proteinase K and incubate at 70 °C for 10 min. Mix beads frequently during each enzyme digestion.

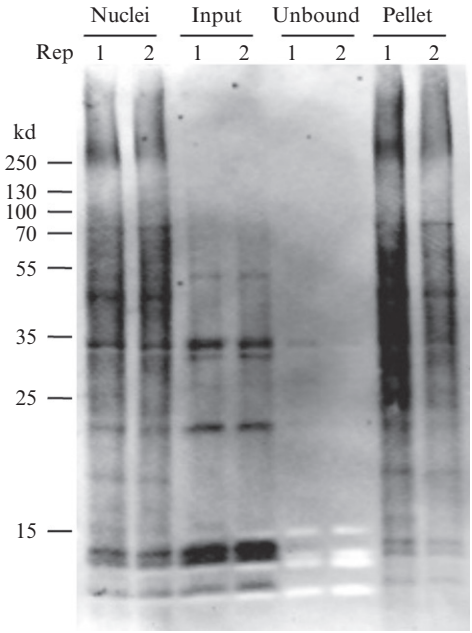

Figure 7.3 Efficient capture of biotinylated proteins. CATCH-IT was performed on Drosophila S2 cells in two replicates. Aliquots of total nuclei, input, unbound, and pellet fractions were subjected to a Western analysis as in Fig. 7.1.

2. Extract the DNA twice with phenol/chloroform and precipitate by adding 2.5 volumes of cold ethanol. Wash with 75% ethanol and resuspend in 20 μL of 0.1 × TE, pH 8.
3. Quantify DNA either by using the PicoGreen fluorescence assay (Invitrogen) or by measuring the OD_{260} with a spectrophotometer such as Nanodrop.

3. MODIFIED SOLEXA LIBRARY PREPARATION

The following protocol for library uses the same enzymological steps as in the established Illumina Paired-End Sample Preparation protocol (Quail et al., 2008), with modifications to the cleanup and purification steps that allow for efficient recovery of fragments as small as 25 bp (Henikoff et al., 2011). In this process, the ends of the starting DNA material are made blunt and 5′-phosphorylated. An "A" nucleotide is added at the 3′-ends to prevent self-ligation and permit specific base-pairing of adapters with a 5′-phosphate and a 3′-T-overhang. The adapted samples are then amplified using primers with 3′-complementarity to the adapters, using a 60 °C

extension step to minimize preferential loss of AT-rich fragments (Lopez-Barragan et al., 2010). In the Illumina protocol, the samples are size selected following the ligation of adapters both to remove unligated adapters and to isolate a specific subset of the samples for sequencing. This step is designed for samples that are randomly sheared or where single-end sequencing leads to fragment size ambiguity. However, if chromatin is enzymatically cleaved and PE sequenced, the fragment size distribution can reveal valuable features of the chromatin landscape. Therefore, we eliminated the gel-based size-selection step. Instead, we use Agencourt AMPure XP beads both to purify the ligated products from unligated primers and for post-PCR cleanup. Using the dilution factor specified by the manufacturer, the AMPure beads result in a strict size cutoff at 90–100 bp. Because the adapters add ~65 bp to the starting material, insert sizes as small as 25 bp will be present in the library. Another modification is that all QIAgen cleanup steps have been replaced with phenol/chloroform extractions to stop the reactions followed by spin column cleanup to purify the DNA. This modification, combined with the use of low-retention (siliconized) microcentrifuge tubes, minimizes the loss of DNA and allows for lower amounts of starting material to be used.

3.1. Solutions and materials

1. Low-retention 1.5 mL microcentrifuge tubes
2. 10× annealing buffer (0.5 *M* NaCl, 0.1 *M* Tris, pH 8, 0.01 *M* EDTA)
3. 10× T4 DNA ligase buffer with 10 m*M* ATP (New England Biolabs (NEB) # B0202S)
4. 40 m*M* dNTP (10 m*M* each)
5. T4 DNA polymerase 5 U/μL (Invitrogen # 100004994)
6. DNA polymerase I, large (Klenow) fragment 50 U/μL (NEB # M0210M)
7. T4 polynucleotide kinase (PNK) 10 U/μL (NEB # M0201L)
8. Phenol/chloroform/isoamyl (25:24:1)
9. Illustra MicroSpin S-300 HR columns (GE Healthcare # 27-5130-01)
10. SpeedVac
11. 10× NEB buffer 2 (NEB # B7002S)
12. 100 m*M* dATP (Invitrogen # 10216018)
13. Klenow fragment (3′→5′-exo-) 50 U/μL (NEB # M0212M)
14. Illumina PE adapter mix or independently synthesized adapter mix
15. T4 DNA ligase (Rapid) and 2× buffer (Enzymatics # L603-HC-L)
16. Agencourt AMPure XP magnetic beads (Agencourt # A63881)
17. Magnetic rack for microcentrifuge tubes

18. 70% ethanol
19. 0.1 × TE (1 m*M* Tris, pH 8, 0.1 m*M* EDTA)
20. Phusion high-fidelity DNA polymerase with 5 × HF buffer (Finnzymes # F-530L)
21. Illumina PE primers or independently synthesized primers
22. 10 m*M* dNTP (2.5 m*M* each)
23. Thermocycler

3.2. Paired-end adapter and primers

We have used both commercially available PE adapter mix and PCR primers from Illumina and custom-made adapter and primer oligos with comparable results. The following synthesized adapters and primers are compatible with the Illumina PE platform. Therefore, the protocol outlined below does not make a distinction between these options. The oligonucleotide sequences are as follows:

PE Adapter1. [Phosphate]GATCGGAAGAGCGGTTCAGCAGGAATGCCGA⋆G

PE Adapter2. ACACTCTTTCCCTACACGACGCTCTTCCGATC⋆T

PE forward primer. AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC⋆T

PE reverse primer. CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC⋆T

These oligonucleotides are PAGE purified. A phosphorothioate linkage before the last nucleotide as denoted in the sequence (⋆) is intended to prevent 3′-exonuclease activity during the ligation step (Quail et al., 2008).

Adapter oligonucleotides are dissolved to 100 μ*M* in water, mixed in equimolar amounts, and annealed by addition of 10 × annealing buffer to a final concentration of 50 m*M* NaCl, 10 m*M* Tris, pH 8, 1 m*M* EDTA. The mixed adapters are annealed by heating to 98 °C for 10 min in a thermocycler and slowly cooling (− 1 °C/min) to 25 °C.

3.3. End repair

1. Measure the starting DNA concentration spectrophotometrically or by fluorescence. A total of 10–500 ng of enzymatically digested DNA can be used to prepare sequencing libraries. Bring the desired starting amount to 20 μL by addition of water or using a SpeedVac without heat to concentrate in a low-retention microcentrifuge tube.

2. Prepare the "End Repair" (ER) master mix by combining the following:
   a. 18.5 μL water
   b. 5 μL 10× T4 DNA ligase buffer
   c. 2 μL 40 m*M* dNTP
   d. 1.5 μL T4 DNA polymerase
   e. 0.5 μL Klenow fragment (diluted to 5 U/μL from 50 U/μL stock)
   f. 2.5 μL T4 PNK
3. If preparing multiple samples, combine a slight excess of each component of the ER master mix to compensate for pipetting losses during transfers. For example, to make libraries from eight different samples, combine 8.2 times each of the component in the master mix.
4. Add 30 μL of ER master mix to 20 μL of DNA (10–500 ng).
5. Incubate the sample in a 20 °C water bath for 30 min. A water bath can be as simple as an ice bucket filled with water that is measured to be 20 °C using a thermometer.

3.4. Phenol extraction and column purification

1. Extract the DNA with 50 μL of phenol/chloroform/isoamyl. Vortex to thoroughly mix, and separate the aqueous layer by centrifugation at maximum speed for 1 min using a table-top centrifuge. Carefully remove the organic layer from the bottom of the tube.
2. To prepare the MicroSpin S-300 HR column, snap off the bottom tip, slightly unscrew the cap, and place in tube holder. Spin buffer off for 1 min at 800 × *g* and replace the column into a new low-retention microcentrifuge tube.
3. Decant the aqueous layer from step 1 and drain the remaining organic layer by touching the pipette tip to the tube wall. Transfer the aqueous layer into the prepared MicroSpin S-300 HR column. Extract the DNA by centrifugation for 2 min at 800 × *g*. The resulting eluate will be about 50–60 μL in volume.

3.5. A-tailing

1. Reduce the eluate volume to 35 μL in a SpeedVac without heat for about 15 min.
2. Prepare the "A-tailing" (A-t) master mix by combining the following:
   a. 5 μL 10× NEB buffer 2
   b. 10 μL 1 m*M* dATP
   c. 0.3 μL Klenow exo- (50 U/μL)

3. Again, if preparing multiple samples, include a slight excess of each component as in the ER master mix procedure.
4. Add 15.3 μL of the A-t master mix to 35 μL of end-repaired DNA sample.
5. Incubate at 37 °C for 30 min.
6. Extract the DNA by phenol extraction and column purification (Section 3.3).

3.6. Adapter ligation and AMPure bead purification

1. Reduce the eluate volume to 18 μL in a SpeedVac without heat for about 30–45 min.
2. Add 1 μL of 1 m*M* PE adapter mix. This amount is best used for small amounts of starting material (10–50 ng of DNA). When using a large amount of starting material, adjust the adapter amount to give an estimated 10:1 adapter:starting DNA molar ratio.
3. Add 25 μL of 2× Rapid DNA ligase buffer (Enzymatics) and 5 μL of Rapid T4 DNA ligase. If preparing multiple samples, combine the buffer and ligase for a master mix and add 30 μL of this to the DNA-adapter mix.
4. Incubate the sample at 20 °C water bath for 15 min to ligate adapters.
5. To extract the DNA and remove excess adapters, add 90 μL of AMPure XP magnetic bead slurry to the sample, mix by pipetting 10 times, and hold at room temperature for 5 min.
6. Place the sample in a magnetic tube holder until the beads are cleared from the slurry, accumulate on the side (~2 min), and aspirate off the solution.
7. While still on the magnet, wash the beads by adding 1 mL 70% ethanol. Aspirate the ethanol after 30 s. Repeat this wash one more time, carefully removing excess ethanol.
8. Allow the beads to dry for no more than 5 min. Remove the tube from the magnet and add 40 μL of 0.1× TE, pH 8. Mix thoroughly and replace the tube on the magnetic tube holder. Transfer the eluate into a new low-retention microcentrifuge tube. This now contains the DNA material with ligated PE adapters.

3.7. PCR amplification and final purification

1. A 20 μL PCR reaction volume is generally sufficient to produce enough products for PE sequencing using the Illumina platform. Prepare a "PCR" master mix by combining the following:

a. 4 μL 5 × Phusion buffer HF
b. 1.6 μL 10 m*M* dNTP (2.5 m*M* each)
c. 0.4 μL forward primer
d. 0.4 μL reverse primer
e. 0.2 μL Phusion HF Polymerase (Finnzymes)
f. 8.4 μL H_2O

2. Add 15 μL of the PCR master mix to 5 μL of adapter-ligated material and proceed with PCR using the following cycling parameters:
   a. 98 °C for 30 s
   b. 12–18 cycles of:
      i. 98 °C for 10 s
      ii. 65 °C for 30 s
      iii. 60 °C for 30 s
   c. 60 °C for 5 min
   d. Hold at 8 °C
3. Clean up the PCR products by adding 36 μL of AMPure XP beads and following the purification method described above. Elute the sample with 40 μL of 0.1 × TE, pH 8. Check the library on a 2% agarose gel

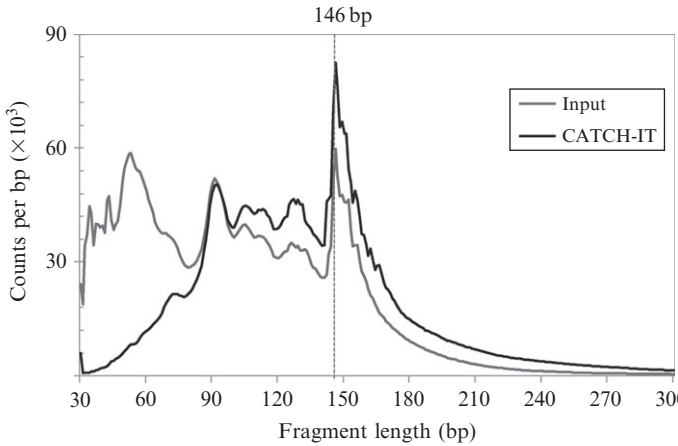

Figure 7.4 Length distribution of sequenced fragments. CATCH-IT was performed on Drosophila S2 cells, and a paired-end Solexa library was generated from the input and CATCH-IT material as described here. Paired-end sequencing for each sample was performed in a single lane of an Illumina Hi-Seq 2000 Instrument by the FHCRC Genomics Shared Resource (http://sharedresources.fhcrc.org/core-facilities/genomics), and fragments were mapped to the Drosophila genome using Novoalign alignment program (http://www.novocraft.com/main/index.php). The length distribution of all mapped fragments is shown here at base-pair resolution. The dominant nucleosomal peak is centered at 146 bp.

with ethidium bromide. The adapter and PCR primers add about 120 bp onto the starting DNA. Therefore, the distribution of the library should be shifted 120 bp higher than the starting DNA (Fig. 7.4).

4. Accurately measure the concentration of the library either by fluorescence-based assays such as the PicoGreen (Invitrogen) or by qPCR as outlined in Illumina protocols and dilute for application to the flow cell (2 μ*M* for the Illumina Hi-Seq 2000).

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