

## ChIP-nexus (version 2019)

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This protocol describes the updated, simplified version of the ChIP-nexus approach introduced by He Q, Johnston J, and Zeitlinger J, in Nature Biotechnology, 2015:  
<http://www.nature.com/nbt/journal/v33/n4/full/nbt.3121.html>

In ChIP-nexus (Chromatin-ImmunoPrecipitation with nucleotide resolution using exonuclease digestion, unique barcode and single ligation) is a ChIP-exo protocol that makes use of a unique barcode to identify duplicate reads and a library preparation strategy that involves self-circularization by cirLigase.

### Overview of updates

- Overall hands-on-time was reduced to ~8 h (when working with 8 samples) by streamlining the protocol and removal of several steps without compromising the quality of the prepared libraries (see below for details).
- Part C: ChIP-exo treatment
  - Strand extension after Nexus adapter ligation is now performed with phi29 DNA polymerase to circumvent excessive 3' end trimming by T4 DNA polymerase.
  - The RecJ digestion and RNase A digestion steps were removed (RNase A digestion is only important when using columns or beads for subsequent DNA purification).
  - All Tris washes are performed with a Tris buffer of the same pH (pH 8.0).
- Part E: ChIP-nexus library preparation
  - The circularized ssDNA fragments are directly used as template for the PCR library amplification. (Enzymatic digestion with the help of cut oligos prior to PCR is not necessary since the polymerase does not have strand displacement activity to produce rolling circles)
- The protocol has been extensively tested and optimized for application on mammalian cells (details for library preparations are included).

An official updated protocol can be found at: <http://research.stowers.org/zeitlingerlab>

## A. Preparation of cross-linked cells

Cross-linking of cultured cells and *Drosophila* embryos should be performed in a timely manner, without interruption of the procedures detailed below.

We recommend using 10 million cultured cells per ChIP.

### Option 1: Cultured cells

#### *Drosophila* cells

Harvest up to ~50 million cells and transfer to a 15 ml conical tube. Adjust volume to 10 ml using standard media.

Add 270  $\mu$ l of 37% formaldehyde and fix for 10 min at room temperature (rotating).

Add 1 ml of 2.5 M glycine in PBS and quench for 1 min at room temperature (rotating).

Pellet fixed cells at 200 x g for 3 min at 4 °C.

Remove media and wash cells twice with 10 ml cold PBS. Pellet cells at 200 x g for 3 min at 4 °C. Remove supernatant.

*Optional: freeze the cross-linked cells in liquid nitrogen and store at -80 °C.*

#### Mammalian cells

*Protocol for up to ~50 million cells in 150 mm cell culture dish.*

Wash cells twice with 10 ml PBS.

Add 15 ml PBS and 400  $\mu$ l of 37% formaldehyde to dish. Fix cells for 10 min at room temperature (with movement, e.g. on orbital shaker).

Add 1.5 ml of 2.5 M glycine in PBS and quench for 5 min at room temperature (with movement, e.g. on orbital shaker).

Wash cells twice with 10 ml cold PBS (with protease inhibitors).

Add 15 ml PBS (with protease inhibitors) and scrape cells of the dish. Transfer to 15 ml conical tube.

Pellet fixed cells at 1000 x g for 3 min at 4 °C. Remove supernatant.

*Optional: freeze the cross-linked cells in liquid nitrogen and store at -80 °C.*

### Option 2: *Drosophila* embryos

Add Clorox bleach (50-100%) to apple plates with embryos for up to 3 min. Use a soft paintbrush to help removing the embryos by scraping the plate surface and pour into a sieve. Use dH<sub>2</sub>O to rinse embryos from plate into sieve.

Wash embryos well with lots of dH<sub>2</sub>O. Remove mesh containing embryos from sieve, transfer embryos to 15 ml conical tube by washing mesh with PBT (PBS with 0.1% Triton X-100).

Let embryos settle down without centrifugation. Discard supernatant and add fixation solution. Fix up to ~1 g embryos in:

- 2.3 ml Fix Buffer (50 mM Hepes, 1 mM EDTA, 0.5 mM EGTA, 100 mM NaCl; total volume with embryos should be ≤ 3 ml)
- 130 µl 37% formaldehyde (final concentration in water phase: 1.8%)
- 7.5 ml n-heptane

Shake vigorously (e.g. on vortexer) for 15 min at room temperature.

Spin for 1 min at 500 x g and 4 °C to pellet embryos and discard supernatant by carefully pipetting off as much fixation solution as possible.

Add ~15 ml PBT-glycine (PBT with 250 mM glycine) to quench fixation. Shake embryos vigorously for 1 min at room temperature and spin for 1 min at 500 x g and 4 °C. Carefully decant the supernatant and remove remaining liquids with a pipette.

Wash twice with ~15 ml PBT. Add PBT, shake 1 min by hand and spin for 1 min at 500 x g and 4 °C. Decant supernatant very carefully (pellet is not as stable as in the step above).

Resuspend embryos in ~2 ml PBT.

*Optional for staging embryos: Transfer ~50 µl embryos with a cut pipette tip into a microcentrifuge tube (continue with DAPI staining after freezing embryos, described below).*

Transfer the remaining embryos into 1.5 ml pre-weighed microcentrifuge tube.

Spin for 30 s at 500 x g and 4 °C. Remove all excess PBT by pipetting. Re-weigh tube and determine weights of embryos.

*Optional: freeze the cross-linked embryos in liquid nitrogen and store at -80 °C.*

#### DAPI staining for embryo staging

Incubate embryos in 1 ml PBT with 10 µl 100x DAPI for 10 min at room temperature (note: DAPI is light sensitive, cover tubes during incubation).

Wash twice with 500 µl PBT for 10 min.

Remove PBT and resuspend embryos in 120 µl 70% glycerol/ 30% PBS. Transfer ~100 µl embryos with a cut pipette tip onto a glass slide. Putting on the coverslip gently and at an angle will help to avoid bubbles. Seal the edges of the coverslip with nail polish to prevent from drying out.

## B. ChIP set up

### Bead preparation

Use 50  $\mu$ l beads (e.g. Dynabeads Protein A and/or G from Invitrogen) per ChIP. Prepare beads in microcentrifuge tubes (e.g. in Eppendorf Safe-Lock to prevent evaporation).

Wash beads 3 times with 1 ml Standard ChIP buffer or ChIP Buffer A2.

Per ChIP, resuspend beads in 500  $\mu$ l Standard ChIP buffer or ChIP Buffer A2 and add 5-10  $\mu$ g antibody.

Incubate on rotator at 4 °C for at least 2 h to overnight.

*Note: antibodies with high background might benefit from pre-incubation with materials that do not contain the epitope (e.g. fixed tissue from mutants or different stage, Western blot without the epitope band).*

### Option 1: Preparation of chromatin extracts for cultured cells

Use Standard ChIP buffer (with protease inhibitors) for chromatin extraction from *Drosophila* cells. Use ChIP Buffer A2 (with protease inhibitors) for mammalian cells. Place buffer on ice.

Resuspend cross-linked cells in Standard ChIP buffer or ChIP Buffer A2 (use 300  $\mu$ l per 10 million cells). Incubate for 10 min on ice.

Sonicate in Bioruptor on HIGH, 30 sec on/30 sec off for 4-6 cycles. *Cycle number and duration depends on the type of bioruptor and may need to be optimized. Desired size distribution of fragments is typically 100-500 bp.*

Spin for 20-30 min at max. speed and 4 °C. Transfer and combine supernatants in a clean tube. This is the input for the ChIP.

### Option 2: Preparation of chromatin extract for *Drosophila* embryos

This protocol is for up to ~1 g embryos. The amount of embryos per ChIP depends on the embryonic stage: 100-300 mg for early stages or 10-15 mg for later stages (> stage 6).

Place cross-linked embryos, Lysis Buffers A1 (with protease inhibitors) and ChIP buffer A2 (with protease inhibitors) on ice.

Resuspend cross-linked embryos in 5 ml Lysis Buffer A1 and transfer to 7 ml Dounce homogenizer. *Work with embryos on ice from now on.*

Dounce with each pestle until homogenized (this may take 5-40 times for each pestle dependent on stage and amount of embryos).

Transfer homogenate to 15 ml tube.

*Optional: Filter the homogenate through 2 layers of Miracloth into 15 ml tube. Rinse Dounce homogenizer with additional 5 ml Lysis Buffer A1.*

Spin tube for 3 min at 650 x g at 4 °C.

Discard supernatant and add 5 ml Lysis Buffer A1. Resuspend pellet by gently pipetting with a wide bore or cut pipette tip. *Do not vortex.* Spin for 3 min at 650 x g at 4 °C. Decant supernatant. Wash pellet two more times with 5 ml Lysis Buffer A1 (three washes total).

Wash sample with 5 ml CHIP Buffer A2 and remove supernatant. Resuspend the sample in CHIP Buffer A2 for sonication (optimized for our conditions: 300 µl per CHIP). Incubate for 10 min on ice.

Sonicate in Bioruptor on HIGH, 30 sec on/30 sec off for 5 cycles. *Cycle number and duration depends on the type of bioruptor and may need to be optimized. Desired size distribution of fragments is typically 100-500 bp.*

Spin for 20-30 min at max. speed and 4 °C. Transfer supernatant to a clean tube. This is the input for the CHIP.

## Incubate CHIP

If not already done, aliquot beads for each CHIP into a separate microcentrifuge tube (e.g. Eppendorf Safe-Lock).

Wash beads 3 times with 1 ml CHIP Buffer A2 or Standard CHIP buffer (with protease inhibitors). Remove supernatant.

Add 300 µl input to each microcentrifuge tube. Add 300 µl CHIP Buffer A2 or Standard CHIP buffer (with protease inhibitors) to each tube to increase total volume to a suitable volume for tube rotation (at least 500 µl in 1.5 ml tube).

Incubate samples over night at 4 °C with rotation.

*Note: These conditions work for a wide variety of antibodies and epitopes but some CHIPs benefit from optimization. For example, high background may be reduced by decreasing the amount of antibody-coated beads (e.g. in case of rare epitopes). Changing the concentration of the input may also improve the signal-to-noise ratio.*

## C. ChIP-exo treatment

*Note: protocol is optimized for 50  $\mu$ l Dynabeads, which have a  $\sim$ 5  $\mu$ l volume after removing liquids. If a different volume is used, water volumes need to be adjusted accordingly.*

### For all wash steps:

- Keep wash buffers A-D and 10 mM Tris (pH 8.0) cold (in fridge, on ice or work in cold room).
- Wash each sample with 1 ml buffer fast and fairly vigorously, i.e. shake with a 90° wrist motion around 10 times within 5 s. All beads should be resuspended after each wash.
- To remove liquid, place samples on a magnetic rack that retains the beads. Let the liquid settle for about 1 min, then remove the beads that are stuck in the lid using 2-5 wrist shakes (repeat if necessary to avoid losing beads). Wait another 1 min and completely remove the liquid, preferably by aspiration (if decanting is used, remove residual liquid by centrifuging and pipetting prior to washing with buffer A and after washing with buffer D and Tris).
- After the last wash in each round of washes, briefly spin tubes to collect the beads at the bottom of the tube, so that they can be resuspended in the master mix of the next enzyme reaction.

**Wash ChIP samples with buffers A, B, C, D, then 10 mM Tris, pH 8.0.**

### End repair

Add master mix:	<u>1x</u>
NEBNext End Repair reaction buffer (10x)	5 $\mu$ l
NEBNext End Repair enzyme mix	1 $\mu$ l
dH <sub>2</sub> O	<u>39 <math>\mu</math>l</u>
	45 $\mu$ l

Incubate at 25 °C for 30 min.

**Wash with buffers A-D, then 10 mM Tris, pH 8.0.**

**dA-tailing**

Add master mix:	<u>1x</u>
NEBNext dA-tailing reaction buffer (10x)	5 $\mu$ l
Klenow Fragment (3' > 5' exo-)	1 $\mu$ l
dH <sub>2</sub> O	<u>39 <math>\mu</math>l</u>
	45 $\mu$ l

Incubate at 37 °C for 30 min.

**Wash with buffers A-D, then 10 mM Tris, pH 8.0.**

**Nexus adapter ligation**

Add master mix:	<u>1x</u>
Quick Ligase buffer (2x)	12.5 $\mu$ l
Nexus adapters (1 $\mu$ M working stock)	1 $\mu$ l
Quick Ligase	3 $\mu$ l
dH <sub>2</sub> O	<u>3.5 <math>\mu</math>l</u>
	20 $\mu$ l

Incubate at 25 °C for 30 min.

**Wash with buffers A-D, then 10 mM Tris, pH 8.0.**

**Barcode extension**

Add master mix:	<u>1x</u>
phi29 reaction buffer (10x)	5 $\mu$ l
dNTPs (10 mM working stock)	1 $\mu$ l
BSA (20 mg/ml)	0.5 $\mu$ l
phi29 DNA polymerase	1 $\mu$ l
dH <sub>2</sub> O	<u>37.5 <math>\mu</math>l</u>
	45 $\mu$ l

Incubate at 30 °C for 30 min.

**Wash with buffers A-D, then 10 mM Tris, pH 8.0.**

### Lambda exonuclease digestion

Add master mix:	1x
Lambda exonuclease buffer (10x)	10 $\mu$ l
Triton X-100 (10% working stock)	1 $\mu$ l
DMSO	5 $\mu$ l
Lambda exonuclease	4 $\mu$ l
dH <sub>2</sub> O	75 $\mu$ l
	95 $\mu$ l

Incubate in thermomixer at 37 °C and 1000 rpm for 60 min.

**Wash 3 times with RIPA buffer.**

## D. Standard DNA purification

### Reverse cross-linking and DNA purification

To each sample, add 300  $\mu$ l elution buffer and 3  $\mu$ l Proteinase K (20 mg/ml). Incubate for 6 h to overnight at 65 °C in thermomixer at 1000 rpm.

Add 300  $\mu$ l Phenol:Chloroform:IAA (25:24:1, v/v) to each sample and mix thoroughly (mix at least 10-times if done by hand inversion). Spin at max. speed for 5 min and room temperature. Transfer upper aqueous phase to a new microcentrifuge tube. Add 3  $\mu$ l glycogen (20 mg/ml), 12  $\mu$ l 5 M NaCl and 750  $\mu$ l cold 100% EtOH. Incubate for 30 min at -80 °C or overnight at -20 °C.

Spin for 30 min at max. speed and 4 °C. Remove supernatant, and wash sample with 500  $\mu$ l cold 70% EtOH. Spin for at least 10 min at max. speed and 4 °C. Air dry and resuspend pellet in 12  $\mu$ l dH<sub>2</sub>O. Transfer sample into PCR tubes.



## E. ChIP-nexus library preparation

### Single-stranded DNA circularization

Denature sample for 5 min at 95 °C, then chill on ice.

Add master mix:	<u>1x</u>
CircLigase Buffer (10x)	1.5 µl
MnCl <sub>2</sub> (50 mM)	0.75 µl
ATP (1 mM)	<u>0.75 µl</u>
	3.0 µl

Add individually per sample:	
CircLigase ssDNA Ligase	0.5 µl

Incubate for 1 h at 60 °C.

After circularization, chill samples on ice and proceed to PCR amplification.

### PCR library amplification

To each sample, add master mix:	<u>1x</u>
Q5 Master Mix (2x)	25 µl
Universal primer (Nex_primer_U, 10 µM)	1 µl
dH <sub>2</sub> O	<u>8 µl</u>
	34 µl

Add individually per sample:	
Index primer (e.g. Nex_primer_01, 10 µM)	1 µl

### PCR program

1x	{	98 °C	2 min
		98 °C	10 s
18x		65 °C	30 s
		72 °C	30 s
1x		72 °C	5 min
		Hold at 4 °C.	

## Library DNA extraction with agarose gel

Prepare a 2% agarose gel (with dye) in 1x TAE buffer. Use ultrapure agarose for gel preparation.

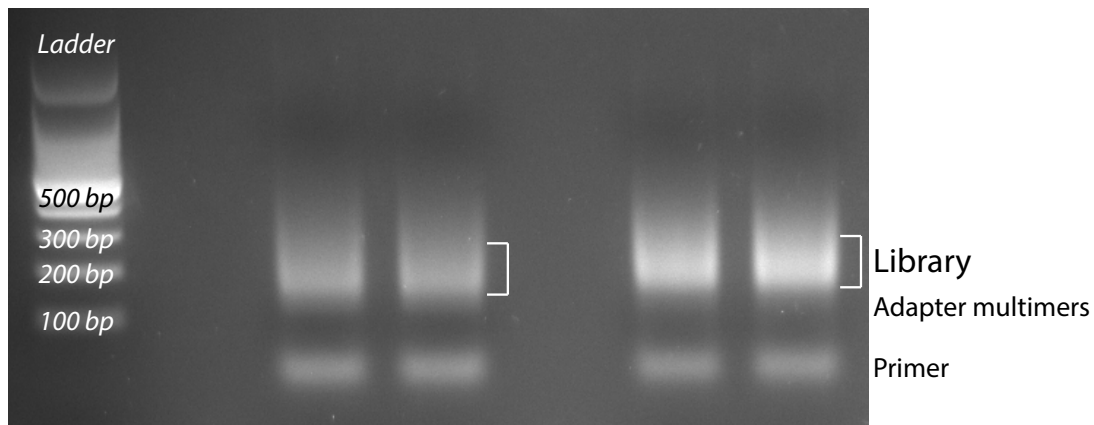
Load 50 bp or 100 bp DNA ladder according to manufacturer's instructions.

Add 8  $\mu$ l of 6x loading dye to 50  $\mu$ l PCR library DNA and load each sample in two lanes of the gel. Leave at least one empty lane between samples.

Run gel for ~50 min (or until samples reach near end of the gel) at 80 V.

View and document gel on a transilluminator (minimize UV exposure). Use a clean, sharp razor blade to precisely excise the band containing the library, avoiding the primer and adaptor dimers. Place gel slice into a new microcentrifuge tube.

### Example of agarose gel (before excision)



### Purification of DNA from gel slice

Purify the DNA from agarose slices with e.g. Monarch DNA Gel Extraction Kit (NEB) according to manufacturer's instructions.

Weigh the gel slices in tube. Add 4 volumes of Gel Dissolving Buffer to 1 volume of gel (100 mg gel = 400  $\mu$ l buffer). Incubate at room temperature to 32 °C in thermomixer (with mixing at 500-1000 rpm) until the gel slices are completely dissolved.

Before proceeding, vortex each sample and spin briefly.

Load sample onto column and spin for 1 min. Discard flow-through and place the column back into the same collection tube.

Wash with 200  $\mu$ l Wash Buffer and spin for 1 min. Repeat washing step.

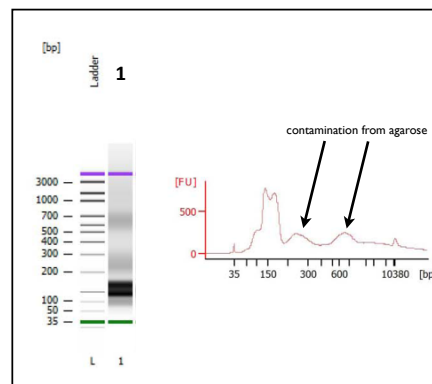
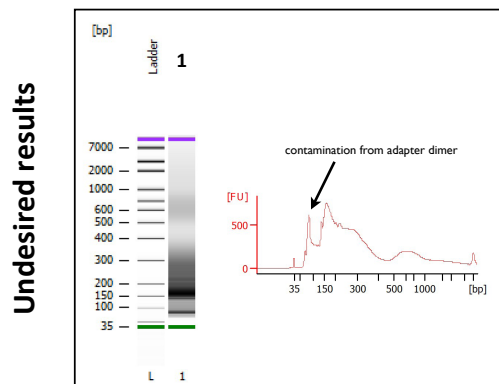
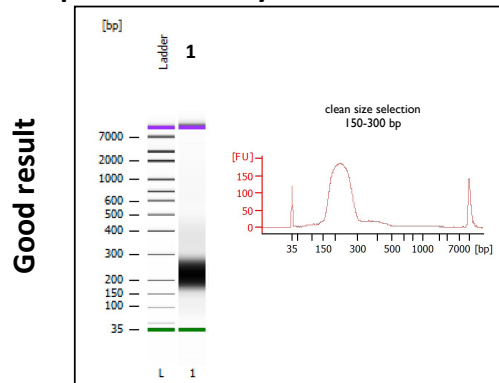
Discard flow-through and spin for 2 min to remove residual Wash Buffer.

To elute, place each column into a clean 1.5 ml microcentrifuge tube. Add 10-20  $\mu$ l water to the center of the column membrane. Let the column stand for 2 min, and spin for 1 min (elution step can be repeated which might improve DNA yield).

### Optional: Bioanalyzer

Run samples on Bioanalyzer (e.g. Agilent, DNA HS Chip) to determine purity and fragment size distribution.

### Examples of Bioanalyzer results



## Sequencing

Sequence DNA samples on an Illumina NextSeq platform with single-end sequencing over 50 cycles of extension according to manufacturer's instructions. Paired-end sequencing is also possible, but not necessary since each mapped read's "start" site will be the only position kept.

For a typical transcription factor in *Drosophila melanogaster* (and organisms with similarly sized genomes), we recommend targeting at least 15 million uniquely alignable reads (~25 million raw sequencing reads). For mammalian cells, we recommend targeting at least 60 million uniquely alignable reads (~100 million raw sequencing reads).

## Data processing

There are two sets of tools recommended for the (1) preprocessing, (2) alignment, and (3) deduplication of aligned ChIP-nexus reads. Each toolset performs the same set of tasks, with varying customizability and compatibilities.

- Original scripts provided in R (Johnston and Weilert)  
<https://github.com/mlweilert/chipnexus-processing-scripts>
  - Scripts are intended to be run in the command-line and provide option parsing.
  - Scripts are intended to be run individually - good starting point if analysis/script customization is required.
  - Includes paired-end sequencing preprocessing scripts.
- C/C++ executable CLI using Nim (Avsec)  
<https://github.com/Avsecz/nimnexus>
  - Highly parallelizable - each job has low memory requirements.
  - Easy to install and use.

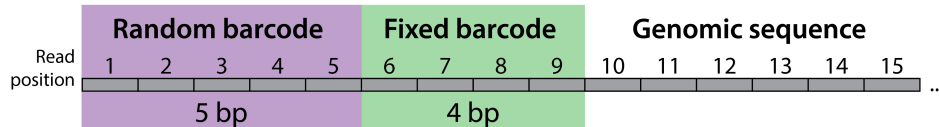
Below is a summary of the steps documented in the above links:

### Format sequencing to FASTQ files (BCL → FASTQ)

If your sequencing returns raw .bcl files, apply bcl2fastq to demultiplex raw .bcl files to .FASTQ files. Keep reads passing the default Illumina quality filter (CASAVA v1.8.2).

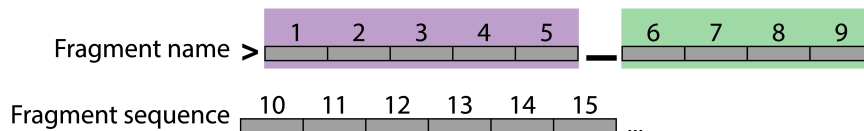
### Formatting ChIP-nexus reads (FASTQ → FASTQ)

ChIP-nexus fragment reads should now have the format:



In order to align ChIP-nexus reads, a few preprocessing steps need to occur:

1. For each read in the .FASTQ file, check for the presence of the reverse complement of the fixed barcodes (ACTG, CTGA, GACT, TGAC) at read position 6. *If you use different sets of fixed barcodes, make sure to adjust your scan accordingly when using the tools provided above.*
2. Remove the fixed barcode sequence (read positions 6-9) and reassign the random barcode sequence (read positions 1-5) and fixed barcode sequence (read positions 6-9) to the FASTQ read name (format: “[random]\_[fixed]”). A graphical representation of an example FASTQ read in a preprocessed .FASTQ file is below:



3. Trim any remaining adapter sequences from the right end of the fragment using cutadapt.
4. Filter all remaining reads to be at least 22 bp in length.

### Trimming adapters (FASTQ → FASTQ)

Using cutadapt (-m 22 -o 4 -e 0.2), trim the remaining ChIP-nexus adapters of the 3' end of the preprocessed fragment in the .FASTQ files. The -m 22 parameter removes reads that would be less than 22 bp after this trimming step.

### Aligning ChIP-nexus (FASTQ → BAM)

Align to appropriate reference genome using Bowtie/BWA/etc. and keep uniquely aligning reads. *We suggest a maximum of 2 mismatches.*

### **Deduplication of aligned reads (BAM → BAM/GRANGES/BIGWIG)**

To remove ChIP-nexus duplicates, remove reads with identical alignment coordinates (chromosome, start position and strand) and identical random barcode (which was assigned to the name of the FASTQ read in the preprocessing step). Tools for doing this can be found in the GitHub links above.

### **Conversion of aligned fragments to Nexus coverage (BAM/GRANGES/BIGWIG → BAM/GRANGES/BIGWIG)**

Split reads by strand orientation and calculate the genome-wide counts of the start positions (Lambda exonuclease's stop position) for each strand. This will result in a near single-bp resolution of ChIP-nexus mapping coverage on the forward and reverse strand, with read pileups found at TF binding sites.

### **Application of exo-specific peak callers (BAM/BIGWIG → BED/IGV/TSV)**

Due to the strand-specific nature of ChIP-nexus data, canonical peak callers often have difficulties accurately assigning peaks to ChIP-nexus coverage. However, there have been exo-specific peak callers published in recent years:

- PeakXus: Hartonen et al., Bioinformatics, 2016  
<https://www.ncbi.nlm.nih.gov/pubmed/27587683>
- Q-nexus: Hansen et al., BMC Genomics, 2016  
<https://www.ncbi.nlm.nih.gov/pubmed/27814676>

Some already-existing peak callers have published suggestions for application of their peak calling tools towards ChIP-exo/nexus data, such as GEM:

<http://groups.csail.mit.edu/cgs/gem/>

Depending on the needs of the investigator, the selection of a peak caller and associated parameter selection within the tools will vary.

## F. Reagents

### Lysis buffers

#### Lysis Buffer A1

15 mM HEPES, pH 7.5  
15 mM NaCl  
60 mM KCl  
4 mM MgCl<sub>2</sub>  
0.5% Triton X-100  
0.5 mM DTT (add fresh)

#### ChIP Buffer A2

15 mM HEPES, pH 7.5  
140 mM NaCl  
1 mM EDTA  
0.5 mM EGTA  
1% Triton X-100  
0.5% N-lauroylsarcosine  
0.1% sodium deoxycholate  
0.1% SDS

#### Standard ChIP Buffer

10 mM Tris, pH 8.0  
140 mM NaCl  
1% Triton X-100  
0.1% sodium deoxycholate  
0.5% N-lauroylsarcosine  
0.1% SDS

*Add protease inhibitors freshly to an aliquot of each buffer before starting the protocol.*

## Wash & elution buffers

### Wash Buffer A

10 mM Tris, pH 8.0  
1 mM EDTA  
0.1% Triton X-100

### Wash Buffer B

20 mM Tris, pH 8.0  
150 mM NaCl  
5 mM EDTA  
5.2% sucrose  
1% Triton X-100  
0.2% SDS

### Wash Buffer C

5 mM Tris, pH 8.0  
25 mM HEPES  
250 mM NaCl  
0.5 mM EDTA  
0.5% Triton X-100  
0.05% sodium deoxycholate

### Wash Buffer D

10 mM Tris, pH 8.0  
250 mM LiCl  
10 mM EDTA  
0.5% IGEPAL CA-630  
0.5% sodium deoxycholate

### Tris buffer

10 mM Tris, pH 8.0

### RIPA Buffer

50 mM HEPES, pH 7.5  
500 mM LiCl  
1 mM EDTA  
0.7% sodium deoxycholate  
1% IGEPAL CA-630

### Elution buffer

25 mM Tris, pH 8.0  
5 mM EDTA  
0.5% SDS



**Reagents to order**

<b>Vendor</b>	<b>Reagent</b>	<b>Catalog #</b>
New England Biolabs	NEBNext End Repair Module	E6050S
	NEBNext dA-Tailing Module	E6053S
	Quick Ligation Kit	M2200S
	phi29 DNA polymerase	M0269S
	Lambda exonuclease	M0262S
	Q5 High-Fidelity 2x Master Mix	M0492S
	10 mM dNTPs	N0447S
	Monarch DNA Gel Extraction Kit	T1020
Invitrogen	Proteinase K (20 mg/ml)	25530049
Roche	Glycogen (20 mg/ml)	10901393001
Lucigen	CirLigase ssDNA Ligase	CL4111K

## Nexus oligos to order

Name	Identity	Modification	Barcode	Sequence
Nex_adapter_U	Adaptor: universal	5' phosphate	/	/5Phos/GATCGGAAGAGCACACGTCTGGATCC ACGACGCTCTCC
Nex_adapter_1	Adaptor: barcoded	5' phosphate	<u>AGTCNNNNN</u>	/5Phos/ <u>AGTCNNNNN</u> NAGATCGGAAGAGCGTC GTGGATCCAGACGTGTGCTCTTCCGATCT
Nex_adapter_2	Adaptor: barcoded	5' phosphate	<u>CAGTNNNNN</u>	/5Phos/ <u>CAGTNNNNN</u> NAGATCGGAAGAGCGTC GTGGATCCAGACGTGTGCTCTTCCGATCT
Nex_adapter_3	Adaptor: barcoded	5' phosphate	<u>GTCANNNNN</u>	/5Phos/ <u>GTCANNNNN</u> NAGATCGGAAGAGCGTC GTGGATCCAGACGTGTGCTCTTCCGATCT
Nex_adapter_4	Adaptor: barcoded	5' phosphate	<u>TCAGNNNNN</u>	/5Phos/ <u>TCAGNNNNN</u> NAGATCGGAAGAGCGTC GTGGATCCAGACGTGTGCTCTTCCGATCT
Nex_primer_U	Primer: universal	3' phospho- thioate bond	/	AATGATACGGCGACCACCGAGATCTACACTCTT TCCCTACACGACGCTCTTCCGATC*T
Nex_primer_01	Primer: indexed	3' phospho- thioate bond	<u>ATCAGC</u>	CAAGCAGAAGACGGCATAACGAGAT <u>CCTGATGT</u> GACTGGAGTTCAGACGTGTGCTCTTCCGATC*T
Nex_primer_02	Primer: indexed	3' phospho- thioate bond	<u>CGATGT</u>	CAAGCAGAAGACGGCATAACGAGAT <u>ACATCGGT</u> GACTGGAGTTCAGACGTGTGCTCTTCCGATC*T
Nex_primer_03	Primer: indexed	3' phospho- thioate bond	<u>TTAGGC</u>	CAAGCAGAAGACGGCATAACGAGAT <u>GCCTAAGT</u> GACTGGAGTTCAGACGTGTGCTCTTCCGATC*T
Nex_primer_04	Primer: indexed	3' phospho- thioate bond	<u>TGACCA</u>	CAAGCAGAAGACGGCATAACGAGAT <u>TGGTCAGT</u> GACTGGAGTTCAGACGTGTGCTCTTCCGATC*T
Nex_primer_05	Primer: indexed	3' phospho- thioate bond	<u>ACAGTG</u>	CAAGCAGAAGACGGCATAACGAGAT <u>CACTGTGT</u> GACTGGAGTTCAGACGTGTGCTCTTCCGATC*T
Nex_primer_06	Primer: indexed	3' phospho- thioate bond	<u>GCCAAT</u>	CAAGCAGAAGACGGCATAACGAGAT <u>ATTGGCGT</u> GACTGGAGTTCAGACGTGTGCTCTTCCGATC*T
Nex_primer_07	Primer: indexed	3' phospho- thioate bond	<u>CAGATC</u>	CAAGCAGAAGACGGCATAACGAGAT <u>GATCTGGT</u> GACTGGAGTTCAGACGTGTGCTCTTCCGATC*T
Nex_primer_08	Primer: indexed	3' phospho- thioate bond	<u>ACTTGA</u>	CAAGCAGAAGACGGCATAACGAGAT <u>TCAAGTGT</u> GACTGGAGTTCAGACGTGTGCTCTTCCGATC*T

## Preparation of CHIP-nexus oligos

Nexus adapter: Oligos for the universal adapter (Nex\_adapter\_U) and the barcoded adapters (Nex\_adapter\_1 to \_4) are annealed to become the Nexus adapter. Each barcoded adapter contains a random barcode (NNNNN) followed by one of four fixed barcodes (AGTC, CAGT, GTCA, or TCAG). The random barcode is used to detect overamplification in the PCR and to determine the complexity of the final library.

To prepare 50  $\mu$ M Nexus adapter mix, anneal:

Nex_adapter_U (200 $\mu$ M)	50 $\mu$ l
Nex_adapter_1 (AGTC, 200 $\mu$ M)	12.5 $\mu$ l
Nex_adapter_2 (CAGT, 200 $\mu$ M)	12.5 $\mu$ l
Nex_adapter_3 (GTCA, 200 $\mu$ M)	12.5 $\mu$ l
Nex_adapter_4 (TCAG, 200 $\mu$ M)	12.5 $\mu$ l
10x TE	20 $\mu$ l
5 M NaCl	2 $\mu$ l
dH <sub>2</sub> O	78 $\mu$ l
total	200 $\mu$ l

Create the following adapter annealing program on a thermocycler:

95 °C            5 min  
cool to 25 °C   1 °C per 1 min  
25 °C            30 min  
hold at 4 °C

Dilute this stock 1:50 to obtain a working stock of 1  $\mu$ M Nexus adapter mix (aliquot to avoid repeated freeze-thaw-cycles).

PCR library amplification primers: Universal primer (Nex\_primer\_U) and index primers (Nex\_primer\_01 to \_08) are used during PCR amplification of the libraries. Prepare working stocks of 10  $\mu$ M of all primers.

## Other reagents and equipment

Beads (e.g. Dynabeads Protein A or Protein G from Invitrogen)

Protease inhibitor (e.g. Roche)

DMSO

Phenol:chloroform:IAA

Formaldehyde

100% Ethanol

70% Ethanol

10% Triton X-100

3 M NaOAC

5 M NaCl

10x TE buffer

TAE buffer

Agarose (ultrapure)

Agarose gel dye (e.g. GelRed)

6x DNA gel loading dye

DNA ladder for agarose gel

Microcentrifuge tubes (e.g. Eppendorf Safe-Lock)

15 ml tubes (e.g. Falcon)

50 ml tubes (e.g. Falcon)

PCR tubes

Disposable scalpels or razor blades

7 ml Dounce homogenizer (Wheaton)

Miracloth (filtration material for homogenates, e.g. Sigma-Aldrich)

Magnetic racks (DynaMag-2 Magnet, Invitrogen, #12321D) *\*expensive, but works well*

Bioruptor/ sonicator (e.g. Diagenode)

Benchtop centrifuges (both room temperature and 4 °C; e.g. Eppendorf)

Thermomixer (e.g. Eppendorf)

Rotator for 1.5 ml microcentrifuge tubes

Thermocycler (e.g. Eppendorf)

Equipment for agarose gel electrophoresis

*Agilent 2100 Bioanalyzer (optional)*