ChIP-exo Protocol

https://www.ncbi.nlm.nih.gov/pubmed/?term=22153082

ChIP-exo is the methodology that combines chromatin immunoprecipitation (ChIP) with lambda exonuclease digestion followed by high-throughput sequencing. ChIP-exo allows identification of a nearly complete set of the binding locations of DNA-binding proteins at near-single-nucleotide resolution with almost no background. The process is initiated by cross-linking DNA and associated proteins. Chromatin is then isolated from nuclei and subjected to sonication. Subsequently, an antibody against the desired protein is used to immunoprecipitate specific DNA-protein complexes. ChIP DNA is purified, sequencing adaptors are ligated, and the adaptor ligated DNA is then digested by lambda exonuclease, followed by high-throughput sequencing. The sequences of the fragments are mapped back to the reference genome to determine the binding locations. The 5 ends of DNA fragments on the forward and reverse strands indicate the left and right boundaries of the DNA-protein binding regions, respectively.

An updated protocol can be found at http://sites.utm.utoronto.ca/rhee/.

Outline

I. Cell Lysis and Sonication

Add complete protease inhibitors (CPI) to lysis buffers before use. Dissolve one Complete Protease Inhibitor Cocktail Tablet in a mL H_2O to make a 100x solution. Store in aliquots at -20°C.

1. Resuspend cell pellets (~**10 to 30 million cells**) in 4 mL of **Lysis Buffer 1**. Rock at 4°C for 10 minutes. Spin at 1,350 x g for 5 minutes at 4°C. Discard the supernatant.
2. Resuspend each pellet in 4 mL of **Lysis Buffer 2**. Rock gently 4°C for 10 minutes. Spin at 1,350 x g for 5 minutes at 4°C. Discard the supernatant.
3. Resuspend pellet in 2.9 mL **Sonication buffer**. Spin down.
4. Sonicate the suspension with the power amplitude at 70 (30W). Sonicate 6 cycles of 30 seconds ON and 30 seconds OFF.
5. Split into two 1.5 mL microfuge tubes. Spin at 20,000x g for 10 minutes at 4°C to pellet debris.

Lysis Buffer 1		Final Conc.
1 M HEPES-KOH (pH 7.5)	2.5 mL	50 mM
5 M NaCl	1.4 mL	140 mM
0.5 M EDTA (pH 8.0)	0.1 mL	1 mM
50% Glycerol	10.0 mL	10%
10% IGEPAL	2.5 mL	0.5%
10% Triton X-100	1.25 mL	0.25%
ddH_2O	up to 50 mL	Store at 4°C.

Lysis Buffer 2		Final Conc.
0.5 M Tris-HCl (pH 8.0)	1 mL	10 mM
5 M NaCl	2 mL	200 mM
0.5 M EDTA (pH 8.0)	0.1 mL	1 mM
ddH_2O	up to 50 mL	Store at 4°C.

Sonication Buffer		Final Conc.
1 M HEPES-KOH (pH 7.5)	2.5 mL	50 mM
5 M NaCl	1.4 mL	140 mM
0.5 M EDTA (pH 8.0)	0.1 mL	1 mM
10% Trition X-100	5.0 mL	1.0%
10% Na-deoxycholate	0.5 mL	0.1%
5% SDS	1 mL	0.1%
ddH_2O	up to 50 mL	Store at 4°C.

II. Chromatin Immunoprecipitation

a. ChIP

1. Obtain **Dynabeads Protein G** (Invitrogen) from 4°C storage. Mix the tube to make sure beads is fully re-suspended.
2. Transfer 20 uL of Dynabead slurry to a fresh 1.5 mL Lo-Bind tube for each immunoprecipitate.

3. Wash with 1 mL **Block Solution** (0.5% BSA). Place tube on a magnetic device for ~1 minute. Remove supernatant.
4. Wash the beads twice with 1.5 mL **Block Solution**. Remove supernatant.
5. Add 500 uL of **Block Solution** + **CPI**. Add 1 to ~5 uL antibody. Incubate samples on rotor torque, 4°C, overnight while slowly rotating.
6. Next day, wash the beads with **1 mL of Blocking Solution** three times. Remove supernatant.
7. Resuspend the beads in 50 uL of **Blocking solution**.
8. Add 50 uL of the above solution including the beads to a fresh 1.5 mL Lo-Bind tube.
9. Thaw sonicated material from -80°C storage. Make sure samples go on ice as soon as thawing is complete.
10. Pour the sonicated cell equivalent (~0.5 to 1.0 mL) into the prepared 1.5 mL tubes.
11. Incubate samples on rotor torque, 4C, overnight while slowly rotating.

Block Solution		Final conc.
BSA	250 mg	0.5% BSA
PBS	up to 100 mL	Store at 4°C.

b. ChIP Washes

1. Spin samples briefly in the tabletop centrifuge at ~400 rpm for about 15 seconds.
2. Place tube in the magnetic rack for 1 minute. Remove flow thru (pour off carefully). Add 0.5 mL Mixed Micelle Buffer + CPI (4C) to each sample and transfer to fresh 1.5ml Lo-Bind tube.
3. **Wash 1** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add pre-chill 1 ML **Sonication Buffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.
4. **Wash 2** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add pre-chill 1 mL **High Salt Wash Buffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.
5. **Wash 3** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add pre-chill 1 mL **LiCl Wash Buffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.
6. **Wash 4** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add 1 mL **Tris-EDTA Bufffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.
7. Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Gently re-suspend each sample by adding 47 uL of **10mM Tris-HCl, pH 8.0** (4°C).

High Salt Wash Buffer		Final Conc.
1 M HEPES-KOH, pH 7.5	2.5 mL	50 mM
5 M NaCl	5.0 mL	500 mM

0.5 M EDTA, pH 8.0	0.1 mL	1 mM
10% Trition X-100	5.0 mL	1%
5% Na-deoxycholate	1.0 mL	0.1%
5% SDS	1.0 mL	0.1%
ddH_2O	up to 50 mL	Store at 4°C.

LiCl Wash Buffer

0.5 M Tris-HCl, pH 8.0	2.0 ml	20 mM
0.5 M EDTA, pH 8.0	0.1 ml	1 mM
1 M LiCl	12.5 ml	250 mM
10% IGEPAL	2.5 mL	0.5%
5% Na-deoxycholate	5.0 mL	0.5%
ddH_2O	up to 50 mL	Store at 4°C.

Tris-EDTA Buffer

0.5 M Tris-HCl, pH 8.0	1.0 ml	10 mM
0.5 M EDTA, pH 8.0	0.1 ml	1 mM
ddH_2O	up to 50 mL	Store at 4°C.

10mM Tris-HCl pH 7.5

1M Tris-Cl pH 7.5	0.5 mL	10 mM
ddH_2O	up to 50 mL	Store at 4°C.

III. Enzymatic Reactions on Resin

a. Polishing & Kinase (End-repair) Reaction on Resin

1. Prepare Master Mix in 1.5 mL tube.

Master Mix	1x mix (uL)	[Final]
ChIP'd DNA	47	-
10x NEBuffer 2.1	3	1x
10x T4 PNK Reaction Buffer	3	-
10x BSA (1mg/ml)	3	50 ug/ml
3mM dNTPs	3	150 uM
T4 DNA polymerase (3 U/ul)	1	3 U
T4 Polynucleotide Kinase (10 U/ul)	0.5	5 U
SUM	60 uL	

2. In the Thermomixer, incubate samples for 20 minutes at 20 °C, 1100 rpm.
3. **Wash 1** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add pre-chill 1 mL **High Salt Wash Buffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.
4. **Wash 2** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add pre-chill 1 mL **LiCl Wash Buffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.

5. **Wash 3** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add 1 mL **Tris-EDTA Bufffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.
6. Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Gently re-suspend each sample by adding 46 uL of **10mM Tris-HCl, pH 7.5** (4°C).

b. 1st Adapter and Index Ligation on Resin

1. Prepare Master Mix in 1.5 mL tube.

Master Mix	1x mix (uL)	[Final]
Polished DNA	46	-
10x T4 DNA ligase buffer	6	1x
100 mM DTT	0.5	1 mM
T4 DNA Ligase (500 U/ul)	2	1 kU
SUM	55 uL	

2. Add **5ul of 1st adaptor*** (15 uM) to each sample and pipette mix gently. Incubate samples in Thermomixer for 2 hours at 25°C, 1400rpm.
3. **Wash 1** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add pre-chill 1 mL **High Salt Wash Buffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.
4. **Wash 2** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add pre-chill 1 mL **LiCl Wash Buffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.
5. **Wash 3** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add 1 mL **Tris-EDTA Bufffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.
6. Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Gently re-suspend each sample by adding 43 uL of **10mM Tris-HCl, pH 7.5** (4°C).

< Scheme >
* Sequencing with Illumina HiSeq 2000
(Note that the adaptor DNA sequences in the protocol are dependent on the deep sequencing platform)

1st adaptor with a multiplexing barcode (Barcode adaptor)

```
5' Phos-CAGAAGACGGCATACGAGATgcactaGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-ChIP DNA 3'
                              3'CACTGACCTCAAGTCTGCACACGAGAAGGCTAGA ChIP DNA 5'
```

c. Fill-in Reaction on Resin

1. Prepare Master Mix in 1.5 mL tube.

Master Mix	1x mix (uL)	[Final]
Ligated DNA	43	-
10x BSA (1 mg/ml)	6	200 ug/ml
10x phi29 rxn buffer	6	1x
3mM dNTPs	3	165 uM
phi29 DNA polyerase (10 U/ul)	2	20 U
SUM	60 uL	

2. Incubate samples in Thermomixer for 30 minutes at 30°C, 1400rpm.
3. **Wash 1** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add pre-chill 1 mL **High Salt Wash Buffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.
4. **Wash 2** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add pre-chill 1 mL **LiCl Wash Buffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.
5. **Wash 3** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add 1 mL **Tris-EDTA Bufffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.
6. Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Gently re-suspend each sample by adding 52 uL of **10mM Tris-HCl, pH 9.5** (4°C).

< Scheme >

1st adaptor ligation

```
5' Phos-CAGAAGACGGCATACGAGATgcactaGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-ChIP DNA 3'
                                  3'CACTGACCTCAAGTCTGCACACGAGAAGGCTAGA ChIP DNA 5'
```

Fill in reaction

```
5' Phos-CAGAAGACGGCATACGAGATgcactaGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-ChIP DNA 3'
     3' GTCTTCTGCCGTATGCTCTAcgtgatCACTGACCTCAAGTCTGCACACGAGAAGGCTAGA-ChIP DNA 5'
```

d. Lambda Exonulease Digestion on Resin

1. Prepare Master Mix in 1.5 mL tube.

Master Mix	1x mix (uL)	[Final]
Kinased DNA	52	-
10x lambda exo rxn buffer	6	1x
Lambda exonuclease (5 U/ul)	2	10 U
SUM	60	

2. Incubate samples in Thermomixer for 30 minutes at 37°C, 1400rpm.
3. **Wash 1** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add pre-chill 1 mL **High Salt Wash Buffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.
4. **Wash 2** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add pre-chill 1 mL **LiCl Wash Buffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.
5. **Wash 3** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add 1 mL **Tris-EDTA Bufffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.
6. Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Gently re-suspend each sample by adding 53 uL of **10mM Tris-HCl, pH 8.0** (4°C).

< Scheme >

1st adaptor ligation

```
5′ Phos-CAGAAGACGGCATACGAGATgcactaGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-ChIP DNA 3′
                                  3′CACTGACCTCAAGTCTGCACACGAGAAGGCTAGA-ChIP DNA 5′
```

Fill in reaction

```
5′ Phos-CAGAAGACGGCATACGAGATgcactaGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-ChIP DNA 3′
     3′ GTCTTCTGCCGTATGCTCTAcgtgatCACTGACCTCAAGTCTGCACACGAGAAGGCTAGA-ChIP DNA 5′
```

Lambda exonuclease digestion

```
                                                                  5′ Phos-ChIP DNA 3′
     3′ GTCTTCTGCCGTATGCTCTAcgtgatCACTGACCTCAAGTCTGCACACGAGAAGGCTAGA-ChIP DNA 5′
```

e. RecJf Exonuclease Digestion on Resin

(This step is optional, but recommended to further reduce contaminated DNA)

1. Prepare Master Mix in 1.5 mL tube.

Master Mix	1x mix (uL)	[Final]
Exo Digested DNA	53	-
10x NEBuffer 2	6	1x
RecJf exonuclease (30U/ul)	1	30 U
	60	

2. Incubate samples in Thermomixer for 30 minutes at 37°C, 1400rpm.
3. **Wash 1** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add pre-chill 1 mL **High Salt Wash Buffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.
4. **Wash 2** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add pre-chill 1 mL **LiCl Wash Buffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.

5. **Wash 3** – Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Add 1 mL **Tris-EDTA Bufffer** + CPI (4°C) to each tube. Mix gently on rotor torque at room temperature for 5 minutes.

IV. Elution and DNA extraction

1. Place tube in the magnetic rack for 1 minute and then aspirate off supernatant. Re-suspend beads in 450 uL **ChIP Elution buffer**.
2. Incubate 65˚C for 15 minutes on Thermomixer, 1400 rpm.
3. Spin down briefly. Place sample on magnetic rack for 1 minute.
4. Transfer eluate to a new 1.5ml LoBind tube.
5. Add 2 ul 20 mg/ml **Proteinase K** to eluate, vortex.
6. Incubate samples, 65˚C, on the heat block for 1 hour.
7. PCIA or EtOH Extraction.
8. RNAse 1uL (10 mg/mL) at 37˚C for 1 hour (optional).

ChIP Elution Buffer

0.5M Tris-HCl, pH 8.0	5 mL	50 mM
0.5M EDTA, pH 8.0	1 mL	10 mM
10% SDS	5 mL	1%
ddH_2O	up to 50 mL	Store at 4°C.

V. Library Preparation

a. Denature and Primer Extension

1. Prepare a Mater Mix in 1.5 mL tube.

Master Mix	1x mix	[Final]
Precipitated DNA	9	-
ddH_2O	3	-
10x phi29 rxn buffer	2	1x
1x BSA (1mg/ml)	4	200 ug/ml
3 mM dNTPs	0.5	100 uM
Library primer (20 uM)*	0.5	0.5 uM
	19	

2. Run samples in the thermocycler using the following program.

Temp (˚C)	Time
95	5 min
62	5 min
30	10 min
30	Forever*

3. Add 1 uL of phi29 DNA polymerase (10 U/ul) at 30˚C ("Forever" step*)

Temp (˚C)	Time
30	20 min
65	10 min
4	Forever

< Scheme >

1st adaptor ligation

```
5′ Phos-CAGAAGACGGCATACGAGATgcactaGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-ChIP DNA 3′
                              3′CACTGACCTCAAGTCTGCACACGAGAAGGCTAGA-ChIP DNA 5′
```

Fill in reaction

```
5′ Phos-CAGAAGACGGCATACGAGATgcactaGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-ChIP DNA 3′
     3′ GTCTTCTGCCGTATGCTCTAcgtgatCACTGACCTCAAGTCTGCACACGAGAAGGCTAGA-ChIP DNA 5′
```

Lambda exonuclease digestion

```
                                                                  5′ Phos-ChIP DNA 3′
     3′ GTCTTCTGCCGTATGCTCTAcgtgatCACTGACCTCAAGTCTGCACACGAGAAGGCTAGA-ChIP DNA 5′
```

Primer extension

```
     3′ GTCTTCTGCCGTATGCTCTAcgtgatCACTGACCTCAAGTCTGCACACGAGAAGGCTAGA-ChIP DNA 5′
 5′ CAAGCAGAAGACGGCATACGAG 3′ (Primer-2)
```

b. Second Adaptor Ligation

1. Prepare the Master Mix in 1.5 mL tube.

2nd Ligation Mix	1X (uL)
A tailed DNA	20
ddH_2O	13
10x T4 DNA ligase buff	4
100 mM DTT	0.5
2nd adaptor (15 uM)*	0.5
T4 DNA Ligase (400 U/ul)	2
SUM	40 uL

2. Add 10 uL of **2nd Adaptor Ligation Mix** to each sample and pipette mix gently.
3. Incubate ligation reactions in thermocycler for 2 hours at 25˚C.
4. Obtain AMPure beads from 4C and re-suspend on the rotor torque at RT for 15 minutes before proceeding to AMPure purification.

< Scheme >

2nd adaptor (Sequencing adaptor)

```
5′ AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 3′
                        3′ GATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA 5′
```

c. AMPure Purification

*You can use QIAGEN Purification Kit to remove un-ligated adaptor DNA instead of AMPure Kit.

1. Transfer above sample (40 uL) into 1.5 ml Lo-Bind tube.
2. Add 1.8 volumes of AMPure beads (72 uL) to the sample (Make sure AMPure beads are fully re-suspended before use). Pipette-mix it for 20 seconds.
3. Place the tube in the magnetic rack for 1 minutes. Remove the supernatant.
4. Add 400 uL of 70% EtOH (room temperature), Pipette-mix for 20 seconds.
5. Place the tube in the magnetic rack for 1 min. Remove the supernatant.
6. Repeat steps 4-5 three more times.
7. Place the tube in the magnetic rack. Remove the supernatant.
8. Dry the beads at room temp for 10 minutes.
9. Elute the DNA by adding 40 uL ddH_2O, pipette-mix for 20 seconds.
10. Place tubes in the magnetic rack. Save the eluted sample in a 1.5 mL Lo-bind tube. (keep at 4˚C).

d. LM-PCR to Check 1st and 2nd Adaptor Ligations

1. Prepare the PCR Mix in 1.5 mL tube.

PCR Mix	1x mix (uL)	[Final]
Purified DNA	18	-
ddH_2O	15	-
10x PCR Buffer	4	1x (1.5 mM $MgCl_2$)
25 mM dNTPs each	0.4	0.25 mM each
1st primer (20 uM)	1	0.3 uM
2nd primer (20 uM)	1	0.3 uM
Taq polymerase (2 U/uL)	0.5	2.5 U
SUM	40 uL	

2. Add 7ul of PCR Mix to each sample and pipette mix gently.
3. Keep on ice until ready to put samples in thermocycler. Run the following PCR program.

Temp (˚C)	Time	Cycles
72	10 min	1
98	20 sec	1
98	20 sec	24
52	1min	
72	1min	
72	5 min	1
4	Forever	Hold

< Scheme >

1st adapter (barcode adapter)

```
      3' GTCTTCTGCCGTATGCTCTAcgtgatCACTGACCTCAAGTCTGCACACGAGAAGGCTAGA-ChIP DNA 5'
 5' CAAGCAGAAGACGGCATACGAG 3' (Primer-2)
```

2nd adaptor (Sequencing adapter)

```
5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'
                        3' GATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA 5'

5' AATGATACGGCGACCACCGAG 3' (Primer-1-21)
```

Appendix

ChIP-exo Adapter and Primer sequences
(modified from Illumina TruSeq Adapters)

Adapter for 1st ligation
(with a multiplexing **barcode** in Enzymatic Reaction, Step b).

Index 1, A2-a1-p-**ATCACG**
```
5'[Phos]CAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'     A2-a-p
                              3'CACTGACCTCAAGTCTGCACACGAGAAGGCTAGA 5'    A2-b-34
```

Index 2, A2-a2-p-**CGATGT**
```
5'[Phos]CAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'
```

Index 3, A2-a3-p-**TTAGGC**
```
5'[Phos]CAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'
```

Index 4, A2-a4-p-**TGACCA**
```
5'[Phos]CAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'
```

Index 5, A2-a5-p-**ACAGTG**
```
5'[Phos]CAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'
```

Index 6, A2-a6-p-**GCCAAT**
```
5'[Phos]CAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'
```

Index 7, A2-a7-p-**CAGATC**
```
5'[Phos]CAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'
```

Index 8, A2-a8-p-**ACTTGA**
```
5'[Phos]CAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'
```

Index 9, A2-a9-p-**GATCAG**
```
5'[Phos]CAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'
```

Index 10, A2-a10-p-**TAGCTT**
```
5'[Phos]CAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'
```

Index 11, A2-a11p-**GGCTAC**
```
5'[Phos]CAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'
```

Index 12, A2-a12-p-**CTTGTA**
```
5'[Phos]CAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'
```

Adapter for 2nd ligation
(a sequencing adaptor) in Library Preparation, Step b.
```
5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'      A1-a-58
                        3' GATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA 5'      A1-b-35
```

Library primer (for primer extension) in Library Preparation, Step a.
& PCR primer (for LM-PCR) in Library Preparation, Step d.
```
3' GAGCATACGGCAGAAGACGAAC 5'                                          Primer-2
```

PCR primer (for LM-PCR) in Library Preparation, Step d.
```
5' AATGATACGGCGACCACCGAG 3'                                           Primer-1-21
```