LIMprep: Ligation-based Illumina Multiplex library PREParation method for low amount DNA using TruSeq adaptor and KAPA library preparation kit

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Quartz-Seq: a highly reproducible and sensitive single-cell RNA-Seq reveals non-genetic gene expression heterogeneity
Yohei Sasagawa and Itoshi Nikaido, et al.

Step1: End repair

☐	10x End repair buffer	5ul
☐	End repair enzyme mix	2.5ul
☐	Nuclease free water (X + Y = 42.5ul)	X ul
☐	Sheared DNA 10ng	Y ul
☐	Total volume	50ul

☐ 20℃ 30min (lid temp 60℃)→4℃

☐ End repaired 50ul + AMPure XP 90ul RT 15min
☐ Separate Ampure beads using magnetic stand for 10min.
☐ 80% EtOH 100ul wash x2times RT1min
☐ Elute with 10ul EB[1/10]

Alternative purification for removal of unexpected large size DNA (over 400-500bp)
Sometimes, ChIP DNA contains unexpected large size DNA.
You can remove large size DNA by alternative purification.
☐ End repaired 50ul + AMPure XP 30-35ul RT 15min
☐ Separate Ampure beads using magnetic stand for 10min.
☐ Supernatant 80-85ul + AMPure XP 65ul RT 15min
☐ Separate Ampure beads using magnetic stand for 10min.
☐ 80% EtOH 100ul wash x2times RT1min
☐ Elute with 10ul EB[1/10]
Note: There is a lot of DNA loss in size selection by using gel-purification (step4-2).
The alternative purification with step4-1 greatly improves sensitivity.

*1: EB[1/10]: 1mM TrisHCl pH8.0

<Safe Stopping Point (-80℃)>

Step2: dA-tailing

☐ A-tailing by KAPA AT mix

☐	10x A-tailing buffer	2.5ul
☐	A-tailing enzyme	1.5ul
☐	Nuclease free water	11ul
☐	End repaired DNA	10ul
☐	Total volume	25ul

☐ 30℃ 30min (lid temp 60℃)→4℃

☐ dA-tailing 25ul + AMPure XP 45ul RT 15min
☐ Separate Ampure beads using magnetic stand for 10min.
☐ 80% EtOH 100ul wash x2times RT1min
☐ Elute with 10ul EB[1/10]

<Safe Stopping Point (-80℃)>

Step3: Adaptor ligation

☐ Adaptor ligation by using KAPA AL mix and TruSeq Adaptor

☐ 5x ligation buffer	5ul
☐ DNA ligase	2.5ul
☐ Nuclease free water	6.5ul
☐ TruSeq Adaptor 10uM[*3]	1ul
☐ A-tailed DNA	10ul
☐ Total volume	25ul

*2: please dilute TruSeq adaptor for low amount DNA (<1ng sheared DNA), aim 1-2uM

You must thaw TruSeq Adaptor solution under 10℃.

You must handle this solution under 10℃.

You should mix TruSeq Adaptor and A-tailed DNA at 0℃ before addition of premix.

☐ 20℃ 15min (lid temp 40℃)→4℃

☐ immediately proceed purification step

Step4: Adaptor dimer removal

☐ You should choose alternative removal-step for your experiment.

Step4-1: Adaptor dimer removal by AmpureXP system

Purification 1st

☐ Adaptor ligation 25ul + Binding Support Buffer[*3] 25ul + AMPure XP 50ul RT15min

☐ Separate beads using magnetic stand for over 10min

☐ 80% EtOH 100ul wash x2times RT1min

☐ Elute with 25ul EB

Purification 2nd

☐ Eluted DNA 25ul + Binding Support Buffer[*3] 25ul + AMPure XP 50ul RT15min

☐ Separate beads using magnetic stand for over 10min

☐ 80% EtOH 100ul wash x2times RT1min

☐ Elute with 20ul ∅ EB[1/10]

*3: Binding Support Buffer (1M NaCl, 20mM $MgCl_2$, 20mM TrisHCl pH7.8)

<Safe Stopping Point (-80℃)>

Step4-2: Adaptor dimer removal by Gel-Size-selection (Alternative)

Purification

☐ Adaptor ligation 25ul + AMPure XP 45ul RT15min
☐ Separate beads using magnetic stand for 10min
☐ 80% EtOH 90ul wash x2times RT1min
☐ Elute with 10ul EB$^{1/10}$

<Safe Stopping Point (-80℃)>

☐ dsDNA conversion by KAPA HiFi

☐ 2xKAPA HiFi HS RM	25ul
☐ 10uM TPC	1.75ul
☐ Nuclease free water	13.25ul
☐ Adaptor ligated DNA	10ul
☐ Total volume	50ul

<KAPA HiFi> (lid temp 105℃)

☐ 98℃ 45sec→

→(98℃ 15sec→60℃ 30sec→72℃ 30sec)x5cycle→72℃ 1min→4℃

Purification

☐ Adaptor ligation 50ul + AMPure XP 90ul RT10-15min
☐ 80% EtOH 90ul wash x2times RT1min
☐ Elute with 10-15ul EB

<Safe Stopping Point (-80℃)>

Size selection by using Caliper LabChip XT or E-gel size selection

☐ Size selected by Caliper LabChip XT or E-gel size selection
☐ You can get eluted DNA. (up to 20ul with EB$^{1/10}$)

<Safe Stopping Point (-80℃)>

Step5: Determination of PCR cycle number

You use 3ul Eluted sample (Adaptor ligated sample) from 20ul Eluted sample for determination of optimal cycle number by using KAPA HiFi Real-time PCR Library amplification kit.

☐ 2xKAPA real-time PCR library	5ul
☐ 10uM TPC mix	0.35ul
☐ Nuclease free water	1.65ul
☐ Adaptor ligated DNA	3ul
☐ Total volume	10ul

qPCR detection by using ABI Prism7900 system

☐ 98℃ 45sec→

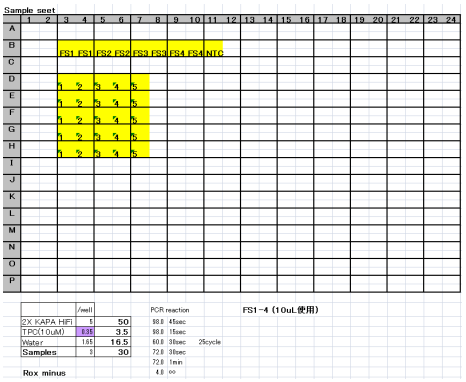
→(98℃ 15sec→60℃ 30sec→72℃ 30sec [data capture])x20cycle→72℃ 1min→4℃

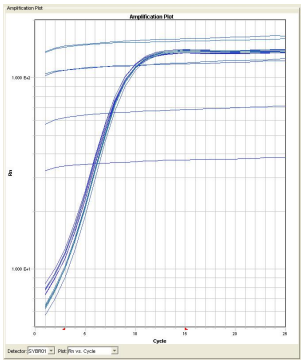
(Rox minus condition)

You use 10ul Fluorescence Standard control (1, 2, 3, 4) per well. (n=2)

Determination of cycle number

An aim of PCR cycle number:
FS2-FS3: yield of library DNA will be about 100ng< ⇒ QC BA1000 or BA High-sensitivity
FS1-FS2: yield of library DNA will be 50ng-100 ⇒ QC BA High-sensitivity
Under FS1 : yield of library DNA will be under 50ng
I recommend under FS1 condition or FS1-FS2 condition.





You should store qPCR-plate for adaptor dimer contamination check [option].

Step6: Adaptor dimer contamination check (option)

☐ 100bp ladder marker and qPCR sample are separated by electrophoresis.
For example, electrophoresis by using E-gel EX2.0% for 15min.
☐ Check the adaptor dimer band at 100-200bp(Theoretical 121bp)
☐ If adaptor dimer remain, you should purify again using 17ul adaptor ligated solution.

Step7: Enrichment of library DNA by PCR

☐ 2xKAPA HiFi HS RM	25ul
☐ 10uM TPC mix	1.75ul
☐ Nuclease free water	6.25ul
☐ Adaptor ligated DNA	17ul
☐ Total volume	50ul

<Temperature condition for KAPA HiFi>

☐ 98℃ 45sec→

→(98℃ 15sec→60℃ 30sec→72℃ 30sec)x_____→72℃ 1min→4℃

☐ PCR reaction 50ul + AMPure XP 90ul

☐ Elute with ____ul $TE^{1/2}$ *4 (Aim: 15ul)

*4: $TE^{1/2}$: Dilute TE with Nuclease-free water. Double dilution

<Safe Stopping Point (-80℃)>

Step8: Sample QC

Determination of Average size and DNA concentration

☐ You should check DNA pattern by BioAnalyzer DNA1000 or High-sensitivity kit. [n=1<]

1. Average size [bp]
2. Conc. [pg/ul] an Aim
3. Molarity [pmol/l]

☐ You check concentration of library DNA by Pico-green system. [n=2 / sample, Option]

☐ You should check by KAPA library quantification kit.
You should prepared two or three independent dilution sample per one library DNA.
(n=2 or 3 x2, dilution x qPCR-duplicate / sample)

☐ Mix library sample according to KAPA quantification data

☐ Re-check concentration of library DNA mixture by using KAPA library quantification kit [Option]

Materials

☐TruSeq Adaptor:

TruSeq adaptors and TruSeq primers were prepared by reference to Illumina company, Ethan-omics protocol [TruSeq ChIP-seq protocol http://ethanomics.wordpress.com/chip-seq-library-construction-using-the-illumina-truseq-adapters/] and Kozarewa et al. paper [Nat Methods. 2009 Apr;6(4):291-5].

Adaptor making

☐ Each primers are dissolved with Adaptor Buffer (10mM Tris-HCl pH7.8, 0.1mM EDTA pH8.0, 50mM NaCl) to 100uM concentration.

☐ TRSI-2 to -12 100uM	5ul
☐ TRSU 100uM	5ul
☐ Total volume	10ul

☐ 95℃ 2min→(95℃ 30sec, increment -0.5℃)x170cycle→4℃→Lid open. Cool tube lid.

☐ Add 40ul adaptor buffer (pre-chilled 0℃) at 0℃. You can obtain 50ul 10uM adaptor solution.
☐ Dispense to 1ul per tube under 10℃ and store at -80℃.

Sequence

```
TRSU : AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T
 TRSI-2 : (5'-phosphate)GATCGGAAGAGCACACGTCTGAACTCCAGTCACCGATGTATCTCGTATGCCGTCTTCTGCTT*G
 TRSI-4 : (5'-phosphate)GATCGGAAGAGCACACGTCTGAACTCCAGTCACTGACCAATCTCGTATGCCGTCTTCTGCTT*G
 TRSI-5 : (5'-phosphate)GATCGGAAGAGCACACGTCTGAACTCCAGTCACACAGTGATCTCGTATGCCGTCTTCTGCTT*G
 TRSI-6 : (5'-phosphate)GATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCAATATCTCGTATGCCGTCTTCTGCTT*G
 TRSI-7 : (5'-phosphate)GATCGGAAGAGCACACGTCTGAACTCCAGTCACCAGATCATCTCGTATGCCGTCTTCTGCTT*G
TRSI-12 : (5'-phosphate)GATCGGAAGAGCACACGTCTGAACTCCAGTCACCTTGTAATCTCGTATGCCGTCTTCTGCTT*G
 TRSI-1 : (5'-phosphate)GATCGGAAGAGCACACGTCTGAACTCCAGTCACATCACGATCTCGTATGCCGTCTTCTGCTT*G
 TRSI-3 : (5'-phosphate)GATCGGAAGAGCACACGTCTGAACTCCAGTCACTTAGGCATCTCGTATGCCGTCTTCTGCTT*G
 TRSI-8 : (5'-phosphate)GATCGGAAGAGCACACGTCTGAACTCCAGTCACACTTGAATCTCGTATGCCGTCTTCTGCTT*G
 TRSI-9 : (5'-phosphate)GATCGGAAGAGCACACGTCTGAACTCCAGTCACGATCAGATCTCGTATGCCGTCTTCTGCTT*G
TRSI-10 : (5'-phosphate)GATCGGAAGAGCACACGTCTGAACTCCAGTCACTAGCTTATCTCGTATGCCGTCTTCTGCTT*G
TRSI-11 : (5'-phosphate)GATCGGAAGAGCACACGTCTGAACTCCAGTCACGGCTACATCTCGTATGCCGTCTTCTGCTT*G

HPLC purification grade
* : phosphorothioate bond
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[For japanese user.....]
☐以下の情報にて、北海道システムサイエンスに HPLC グレードにて発注。

シーケンス名：TRSU
配列　　：AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
合成スケール：1μmol
精製方法　：HPLC 精製(1μmol)
塩基数　　：58 mer
修飾 1　　：部分 S 化 (1 ヶ所)
備考　　：57 塩基目の C と 58 塩基目の T の間 S 化

枝番：2
シーケンス名：TRSI-2
配列　　：GATCGGAAGAGCACACGTCTGAACTCCAGTCACCGATGTATCTCGTATGCCGTCTTCTGCTTG
合成スケール：1μmol
精製方法　：HPLC 精製(1μmol)
塩基数　　：63 mer
修飾１　　：5'リン酸化
修飾２　　：部分 S 化 (1 ヶ所)
備考　　：62 塩基目の T と 63 塩基目の G の間 S 化

枝番：3
シーケンス名：TRSI-4
配列　　：GATCGGAAGAGCACACGTCTGAACTCCAGTCACTGACCAATCTCGTATGCCGTCTTCTGCTTG
合成スケール：1μmol
精製方法　：HPLC 精製(1μmol)
塩基数　　：63 mer
修飾１　　：5'リン酸化
修飾２　　：部分 S 化 (1 ヶ所)
備考　　：62 塩基目の T と 63 塩基目の G の間 S 化

枝番：4
シーケンス名：TRSI-5
配列　　：GATCGGAAGAGCACACGTCTGAACTCCAGTCACACAGTGATCTCGTATGCCGTCTTCTGCTTG
合成スケール：1μmol
精製方法　：HPLC 精製(1μmol)
塩基数　　：63 mer
修飾１　　：5'リン酸化
修飾２　　：部分 S 化 (1 ヶ所)
備考　　：62 塩基目の T と 63 塩基目の G の間 S 化

枝番：5
シーケンス名：TRSI-6
配列　　：GATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCAATATCTCGTATGCCGTCTTCTGCTTG
合成スケール：1μmol
精製方法　：HPLC 精製(1μmol)
塩基数　　：63 mer
修飾１　　：5'リン酸化
修飾２　　：部分 S 化 (1 ヶ所)
備考　　：62 塩基目の T と 63 塩基目の G の間 S 化

枝番：6
シーケンス名：TRSI-7
配列　　：GATCGGAAGAGCACACGTCTGAACTCCAGTCACCAGATCATCTCGTATGCCGTCTTCTGCTTG
合成スケール：1μmol
精製方法　：HPLC 精製(1μmol)
塩基数　　：63 mer
修飾１　　：5'リン酸化
修飾２　　：部分 S 化 (1 ヶ所)
備考　　：62 塩基目の T と 63 塩基目の G の間 S 化

枝番：7
シーケンス名：TRSI-12
配列　　：GATCGGAAGAGCACACGTCTGAACTCCAGTCACCTTGTAATCTCGTATGCCGTCTTCTGCTTG
合成スケール：1μmol
精製方法　：HPLC 精製(1μmol)
塩基数　　：63 mer
修飾１　　：5'リン酸化
修飾２　　：部分 S 化 (1 ヶ所)
備考　　：62 塩基目の T と 63 塩基目の G の間 S 化

☐**PCR primer**:

☐ Dissolve in H2O or Buffer (10mM Tris-HCl pH7.8, 0.1mM EDTA pH8.0, 50mM NaCl) to 100uM concentration.
☐ 10uM TPC mix: 10ul 100uM TPC1 + 10ul 100uM TPC2 + 80ul H2O or Buffer
☐ Store at -80℃

Sequence
TPC1 : AATGATACGGCGACCACCGA*G
TPC2 : CAAGCAGAAGACGGCATACGA*G
HPLC purification grade
* : phosphorothioate bond

[For Japanese user]
☐以下の情報にて、北海道システムサイエンスに HPLC グレードにて発注。

枝番：1
シーケンス名：TPC1
配列　　：AATGATACGGCGACCACCGAG
合成スケール：1μmol
精製方法　：HPLC 精製(1μmol)
塩基数　　：21 mer
修飾 1　　：部分 S 化 (1 ヶ所)
備考　　：(*←S 化)AATGATACGGCGACCACCGA*G

枝番：2
シーケンス名：TPC2
配列　　：CAAGCAGAAGACGGCATACGAG
合成スケール：1μmol
精製方法　：HPLC 精製(1μmol)
塩基数　　：22 mer
修飾 1　　：部分 S 化 (1 ヶ所)
備考　　：(*←S 化)CAAGCAGAAGACGGCATACGA*G

☐ Commercial items:

☐ KAPA library preparation kit
KK8200 KAPA NGS library prep kit 10 or
KK8201 KAPA NGS library prep kit 50
☐ KAPA library amplification kit with real-time qPCR
KK2701 KAPA real-time
☐ Agencourt Ampure XP
Agencourt AMPure XP 60 mL Kit A63881
☐ EB: Elution Buffer (Qiagen)
250 ml Elution Buffer 19086 ¥3,500
☐ KAPA library quantification kit
KK4835 KAPA Library Quantification Kits for Illumina GA / ABI Prism®
☐ Bioanalyzer DNA1000 and High-sensitivity kit
☐ Nuclease-free water
Rnase-free water, non DEPC, 500ml BioDynamics DR127

☐ 5M NaCl Sigma S5150-1L

☐ 0.5M EDTA (pH8.0) nippon gene 311-90075

☐ 1M TrisHCl pH7.8 Sigma T2569-100ML

☐ LabChip XT
☐ E-gel Ex2.0%
☐ KAPA HiFi DNA polymerase KK2601 KAPA HiFi HS ReadyMix
(same content: =KAPA library amplification kit: KK2611)
☐ KAPA standard DNA KK4808 DNA standard for Illumina GA/HiSeq
☐ Pico-green (Invitrogen)
☐ DynaMag 96 Side Invitrogene 123.31D