Primer ID MiSeq Library Prep Version 3

Shuntai Zhou

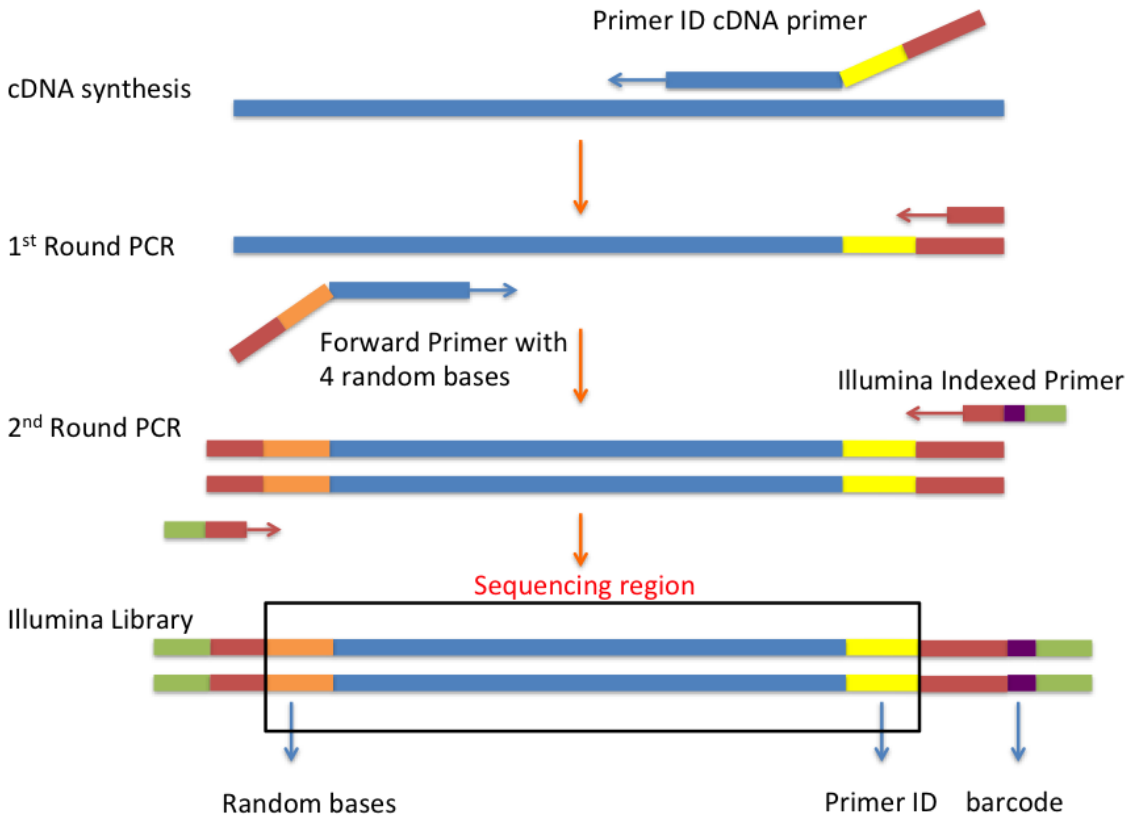
Abstract

This is the protocol to prepare Primer ID MiSeq sequencing library. Viral RNA was first extracted using QIAamp viral RNA extraction kit. The block of random nucleotides (Ns) in the cDNA primers served as the Primer ID. The Superscript III kit was used for the cDNA synthesis. We used two rounds of PCR to amplify the cDNA and incorporate Illumina indexed adapters with KAPA2G Robust and KAPA HiFi PCR kits, respectively.

Citation: Shuntai Zhou Primer ID MiSeq Library Prep. **protocols.io**
dx.doi.org/10.17504/protocols.io.sjdeci6
Published: 12 Aug 2018

Guidelines

Viral RNA extraction using QIAamp vRNA mini kit.



Primers (NOTE: HIV-1 ENV REGION AS AN EXAMPLE, SUBSTITUTE THE BLUE Sequences in the forward primer and cDNA primer)

V1F(forward) 5'-3'	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNTTATGGGATC AAAGCCTAAAGCCATGTGTA
BV3R Uni (cDNA Primer) 5'-3'	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNCAGTCCATTT TGCTCTACTAATGTTACAATGTGC
Universal Adapter	AATGATACGGCGACCACCGAGATCTACACGCCTCCCTCGCGCCATCAGAGATGT G
Indexed Adapter	CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCAGACGTGT GCTC
ADPT_2a	GTGACTGGAGTTCAGACGTGTGCTC

Note: Primer ID primer and forward primer use random bases. Indexed primers have 24 fixed barcodes.

Table of Indexed Primers

Indexed Primer	Index	Index Sequence	Sequence
PCR Primer, Index 1	1	ATCACGA	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 2	2	CGATGTA	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 3	3	TTAGGCA	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 4	4	TGACCAA	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 5	5	ACAGTGA	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 6	6	GCCAATA	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 7	7	CAGATCA	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 8	8	ACTTGAA	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 9	9	GATCAGA	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 10	10	TAGCTTA	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 11	11	GGCTACA	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 12	12	CTTGTAA	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 13	13	TCCATAA	CAAGCAGAAGACGGCATACGAGATTATGGAGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 14	14	GTACTAA	CAAGCAGAAGACGGCATACGAGATTAGTACGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 15	15	ACAGTAA	CAAGCAGAAGACGGCATACGAGATTACTGTGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 16	16	CTCATGA	CAAGCAGAAGACGGCATACGAGATCATGAGGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 17	17	ACGATAA	CAAGCAGAAGACGGCATACGAGATTATCGTGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 18	18	TGCAGAA	CAAGCAGAAGACGGCATACGAGATTCTGCAGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 19	19	TTCATAA	CAAGCAGAAGACGGCATACGAGATTATGAAGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 20	20	TGCTGTA	CAAGCAGAAGACGGCATACGAGATACAGCAGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 21	21	TATCACA	CAAGCAGAAGACGGCATACGAGATGTGATAGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 22	22	TGGATAA	CAAGCAGAAGACGGCATACGAGATTATCCAGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 23	23	CGCATTA	CAAGCAGAAGACGGCATACGAGATAATGCGGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 24	24	GCCTTAA	CAAGCAGAAGACGGCATACGAGATTAAGGCGTGACTGGAGTTCAGACGTGTGCTC

Materials

- QIAamp vRNA mini kit 51304 by Qiagen
- Agencourt RNAClean XP A63987 by Beckman Coulter
- 70% ethanol by Contributed by users
- DNase-free water by Contributed by users
- AmpureXP PCR cleanup kits A63880 by Beckman Coulter

QIAquick gel extraction kit 28704 by Qiagen

Qubit dsDNA BR Assay kit Q32850 by Invitrogen - Thermo Fisher

Protocol

Prepare Primer Mix (Optional, only for multiplexed Primer ID library prep)

Step 1.

For multiplexing sequencing, first, prepare Primer Mix.

Example (For HIV drug resistance pipeline).

Regions	**DR cDNA** primer	**DR F** primer
PR	R2614_PID	F2163AD
RT	R3284_PID11	F2620_AD
INT	R4752_PID11	F4383_AD
V3	V3R_Buni_11	V1F_AD

Make 10 µM primer mix: mix 10 µL of each primer in one set and 60 µL of dH_2O.

Primer Tables

primer	sequence (5'-3')
R2614_PID	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNCAGTTTAACTTTTGGGCCATCCATTCC
R3284_PID11	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNCAGTCACTATAGGCTGTACTGTCCATTTATC
R4752_PID11	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNATCGAATACTGCCATTTGTACTGC
R7209_PID11	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNCAGTCCATTTTGCTYTAYTRABVTTACAATRTGC
F2163AD	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNTCAGAGCAGACCAGAGCCAACAGCCCCA
F2620_AD	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNGGCCATTGACAGAAGAAAAAATAAAAGC
F4383_AD	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNAAAAGGAGAAGCCATGCATG
V1F_AD	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNTTATGGGATCAAAGCCTAAAGCCATGTGTA

“N” in this Primer table is a random nucleotide.

P1	AATGATACGGCGACCACCGAGATCTACACGCCTCCCTCGCGCCATCAGAGATGTG
Indexed Adapter	CAAGCAGAAGACGGCATACGAGAT**NNNNNN**GTGACTGGAGTTCAGACGTGTGCTC
ADPT_2a	GTGACTGGAGTTCAGACGTGTGCTC

The 6 “N”s are not random nucleotides. They are a set of 24 pre-designed indexed sequences.

Step 2.

Mix well.

cDNA synthesis

Step 3.

Pipette the following components into a 0.5 ml RNase-free tube:

µl/tube		[stock]	[final]	[mastermix]
3.0	dNTP Mix	10 mM each	0.5	
1.5	cDNA primer	10 µM	0.25 µM	
34.5	RNA template			
39.00	Total volume			

AMOUNT
3 µl : dNTPMix
AMOUNT
1.5 µl : cDNA primer
AMOUNT
34.5 µl : RNA template

Step 4.

Place tube in 65°C heat block for 3-5’.

DURATION
00:05:00 : 65°C heat block

Step 5.

Place the tube on ice for 1'.

DURATION
00:01:00 : on ice

Step 6.

Add the following components:

µl/tube		[stock]	[final]	[mastermix]
12.0	5x buffer	5x	1x	
3.0	DTT	100 mM	5	
3.0	RNaseOUT	40 u/µl	2	
3.0	SSIII RT	200 u/µl	10	
21.0	Per tube			

AMOUNT
12 µl : 5x buffer
AMOUNT

3 µl : DTT
AMOUNT
3 µl : RNaseOUT
AMOUNT
3 µl : SSII RT

Step 7.

Mix and incubate at 50°C for 1 hr.

DURATION
01:00:00 : Incubation at 50°C

Step 8.

Increase to 55°C and incubate for 1 hr.

DURATION
01:00:00 : Incubation at 55°C

Step 9.

Inactivate SSIII RT by heating at 70°C for 15’.

DURATION
00:15:00 : Heating at 70°C

Step 10.

To each tube, add 1 µl RNase H, incubate at 37°C for 20’.

AMOUNT
1 µl : RNase H
DURATION
00:20:00 : Incubation at 37°C

Purification

Step 11.

Purify cDNA using Agencourt RNAClean XP.

Resuspend the beads and take an aliquot out. Keep at room temperature for at least **30 minutes** before use. (Should be in 1ml aliquots)

DURATION
00:30:00 : Room temperature

Step 12.

Transfer the cDNA reactions into 1.7 mL RNase-free tubes.

Step 13.

Resuspend the beads (Vortex). Add **42 µl of beads to 60 µl cDNA (Ratio: 0.6 – 0.8)** Agencourt RNAClean XP beads to each cDNA reaction.

AMOUNT
42 µl : beads
AMOUNT
60 µl : cDNA

Step 14.

Mix the Agencourt RNAClean XP and sample thoroughly by pipette mixing 15 times. No **vortexing**. Let the tube incubate at room temperature for **20 minutes** before proceeding to the next step.

DURATION
00:20:00 : Room temperature

Step 15.

Place the tube onto the magnetic tube rack for **5 minutes** to separate the beads from solution.

DURATION
00:05:00 : Magnetic tube rack

Step 16.

Slowly aspirate the cleared solution from the tube and discard. This step should be performed while the tube is situated on the rack. Do not disturb the magnetic beads, which have formed a spot on the side of the tube.

Step 17.

Dispense 500 µL of 70% ethanol into the tube and incubate for **30 seconds** at room temperature. Aspirate out the ethanol and discard. (1/3)

It is important to perform this step with the tube situated **on the rack**. **Do not disturb the separated magnetic beads**. Be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants.

DURATION
00:00:30 : Incubation at room temperature

Step 18.

Dispense 500 µL of 70% ethanol into the tube and incubate for **30 seconds** at room temperature. Aspirate out the ethanol and discard. (2/3)

It is important to perform this step with the tube situated **on the rack**. **Do not disturb the separated magnetic beads**. Be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants.

DURATION
00:00:30 : Incubation at room temperature

Step 19.

Let the reaction tube air-dry **10 minutes** on the rack with the cap open. The tube(s) should air-dry until the last visible traces of ethanol evaporate. Over drying the sample may result in a lower recovery.

DURATION
00:10:00 : Air-drying

Step 20.

(Optional for clinical samples) Elute in 60 µl water. Repeat step 12 to 19.

GOTO
Optional for clinical samples -> go to step #13

Step 21.

Remove the tube from the rack and resuspend beads in 24 µl DNase-free water by pipetting up and down. Place tube back on the rack and leave for **3 minutes**.

AMOUNT
24 µl : DNase-free water
DURATION
00:03:00 : Magnetic rack

Step 22.

Pipette the eluant from the tube while it is situated **on the magnetic tube rack.**

PCR 1

Step 23.

Complete thaw and vortex KAPA reagents (except for enzyme) before use.

Step 24.

µl/tube		[stock]	[final]	[mastermix]
10.0	5x **Buffer A**	5x	1x	
10.0	Enhancer	5x	1x	
1.0	dNTPs	10 mM	0.2 mM	
2.5	Forward primer	10 µM	0.5 µM	
2.5	ADPT_2a	10 µM	0.5 µM	
0.5	KAPA Robust polymerase	5 U/µl	2.5 U	
23.5	Template cDNA			

Prepare mastermix in cold box and use repeater pipette to add to each tube in the cold box. Add template cDNA to each tube and pipette up and down to mix.

AMOUNT
10 µl : 5x Buffer A
AMOUNT
10 µl : Enhancer

AMOUNT
1 µl : dNTPs
AMOUNT
2.5 µl : Forward primer
AMOUNT
2.5 µl : ADPT_2a
AMOUNT
0.5 µl : KAPA Robust polymerase
AMOUNT
23.5 µl : Template cDNA

Step 25.

Cycle

95˚C	1 min
95˚C	15 s
58˚C	1 min
72˚C	30 s
15-25 cycles	
72˚C	3 min
4˚C	On hold

Purification PCR products

Step 26.

Purify PCR products using AmpureXP PCR cleanup kits.

Vortex the 1 ml aliquot and remove the needed volume. Keep at room temperature for at least **30 minutes** before use.

DURATION
00:30:00 : Room temperature

Step 27.

Transfer the PCR1 reactions into 1.7 mL RNase-free tubes.

Step 28.

Resuspend the beads. Add **40 µl (Ratio: 0.6 – 0.8: 1, 36µl – 48µl)** Ampure XP beads to each cDNA reaction.

AMOUNT
40 µl : Ampure XP beads

Step 29.

Mix the Ampure XP and sample thoroughly by **vortexing**. Let the tube incubate at room temperature for **5 minutes** before proceeding to the next step (incube off the rack).

DURATION
00:05:00 : Incubation at room temperature

Step 30.

Place the tube onto the magnetic tube rack for **5 minutes** to separate the beads from solution.

DURATION
00:05:00 : Incubation magnetic rack

Step 31.

Slowly aspirate the cleared solution from the tube and discard. This step should be performed while the tube is situated on the rack. Do not disturb the magnetic beads, which have formed a spot on the side of the tube.

Step 32.

Dispense 500 µL of 70% ethanol into the tube and incubate for **30 seconds** at room temperature. Aspirate out the ethanol and discard. (1/2)

AMOUNT
500 µl : 70% ethanol
DURATION
00:00:30 : Incubation at room temperature

NOTES

It is important to perform this step with the tube situated **on the rack**. **Do not disturb the separated magnetic beads**. Be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants.

Step 33.

Dispense 500 µL of 70% ethanol into the tube and incubate for **30 seconds** at room temperature. Aspirate out the ethanol and discard. (2/2)

AMOUNT
500 µl : 70% ethanol
DURATION
00:00:30 : Incubation at room temperature

NOTES

It is important to perform this step with the tube situated **on the rack**. **Do not disturb the separated magnetic beads**. Be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants.

Step 34.

Let the reaction tube air-dry **10 minutes** on the rack with the cap open. The tube(s) should air-dry until the last visible traces of ethanol evaporate. Over drying the sample may result in a lower recovery.

DURATION
00:10:00 : Air-drying

Step 35.

Remove the tube from the rack and resuspend beads in 50 µl DNase-free water by pipetting up and down. Place tube back on the rack and leave for **3 minutes.**

AMOUNT
50 µl : DNase-free water
DURATION
00:03:00 : Magnetic rack

Step 36.

Pipette the 45 µl eluant from the tube while it is situated **on the magnetic tube rack**.

PCR 2

Step 37.

Complete thaw and vortex KAPA reagents (except for enzyme) before use.

Step 38.

µl/tube		[stock]	[final]	[mastermix]
5.0	5x KAPA HiFi Fidelity Buffer	5x	1x	
1.0	dNTP Mix	10 mM	0.4 mM	
1.0	Uni Adapter (ADPT_P1)	10 µM	0.4 µM	
0.5	KAPA HiFI polymerase	1 U/µl	0.5 U	
1.0	Indexed Adapter	10 µM	0.4 µM	
2.0	Purified template DNA			
14.5	Water			

Prepare mastermix in cold box and use repeater pipette to add to each tube in the cold box. Add Indexed Adapter to each tube.

AMOUNT
5 µl : 5x KAPA HiFi Fidelity Buffer
AMOUNT
1 µl : dNTP Mix

AMOUNT
1 µl : Uni Adapter (ADPT_P1)
AMOUNT
0.5 µl : KAPA HiFI polymerase
AMOUNT
1 µl : Indexed Adapter
AMOUNT
2 µl : Purified template DNA
AMOUNT
14.5 µl : Water

Step 39.

Cycle (PCR machine #5 SZ -> ILM2):

95˚C	2 min
98˚C	20 s
63˚C	15 s
72˚C	30 s
25 - 35 cycles	
72˚C	3 min
4˚C	On hold

Gel Purification and quantification

Step 40.

Before gel purification, run 2 µl products on 1% agarose gel to check the bands.

Step 41.

Gel purification. (Qiagen QIAquick gel extraction kit)

Run 2nd round PCR products on 1.2% agarose gel. E = 4 V/cm, T = 60 min.

Step 42.
Excise DNA fragment.

Step 43.
Weight the gel; add 3 volume of Buffer QG to 1 volume of gel.

Step 44.

Incubate at 50 ˚C for **10 minutes** to completing dissolve. Vortex every **2-3 minutes** to help dissolve.

DURATION
00:10:00 : Incubation at 50 ˚C
DURATION
00:02:30 : Vortex

Step 45.
Check the color of gel solution (should be yellow, otherwise add 10 µl 3M sodium acetate).

Step 46.

Place **MinElute** column, apply the sample to the column and centrifuge for **1 minute**.

DURATION
00:01:00 : Centrifugation

Step 47.

Add 500 µl buffer QG and centrifuge for **1 minute.**

AMOUNT
500 µl : Buffer QG
DURATION
00:01:00 : Centrifugation

Step 48.

Add 0.75 ml buffer PE, **incubate for 5 minutes** at room temperature, centrifuge for **1 minute**.

DURATION
00:05:00 : Incubation at room temperature
DURATION
00:01:00 : Centrifugation

Step 49.

Discard the fluid, centrifuge for additional **3 minutes**.

DURATION
00:03:00 : Centrifugation

Step 50.

Put the column in a new 1.7 ml tube, add **10** µl buffer EB. Stand for **4 minutes**, centrifuge for **2 minutes.**

DURATION
00:04:00 : Stand

Step 51.

Quantification using Invitrogen Qubit dsDNA BR Assay kit. See Qubit dsDNA BR assay protocol. **Don’t use Nanodrop to quantify!**

Step 52.
After quantification, pool libraries in equal amount. Use AMPure XP beads to purify pooled libraries (2:3, two washes), elute in 20 to 30 µL Elution Buffer.

Step 53.
Quantify the pooled library using Qubit dsDNA BR assay kit. Check the quality of the library by Agilent Bioanalyzer or Bio-rad Experion. Repeat purification if primer dimmer is present (200bp).