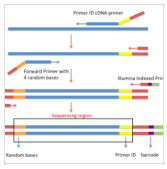

Version 4

Oct 17, 2018

Working

Primer ID MiSeq Library Prep Version 4

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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.

TAGS

Library prep

NGS

Show tags

amplicon protocol_v1.5.docx

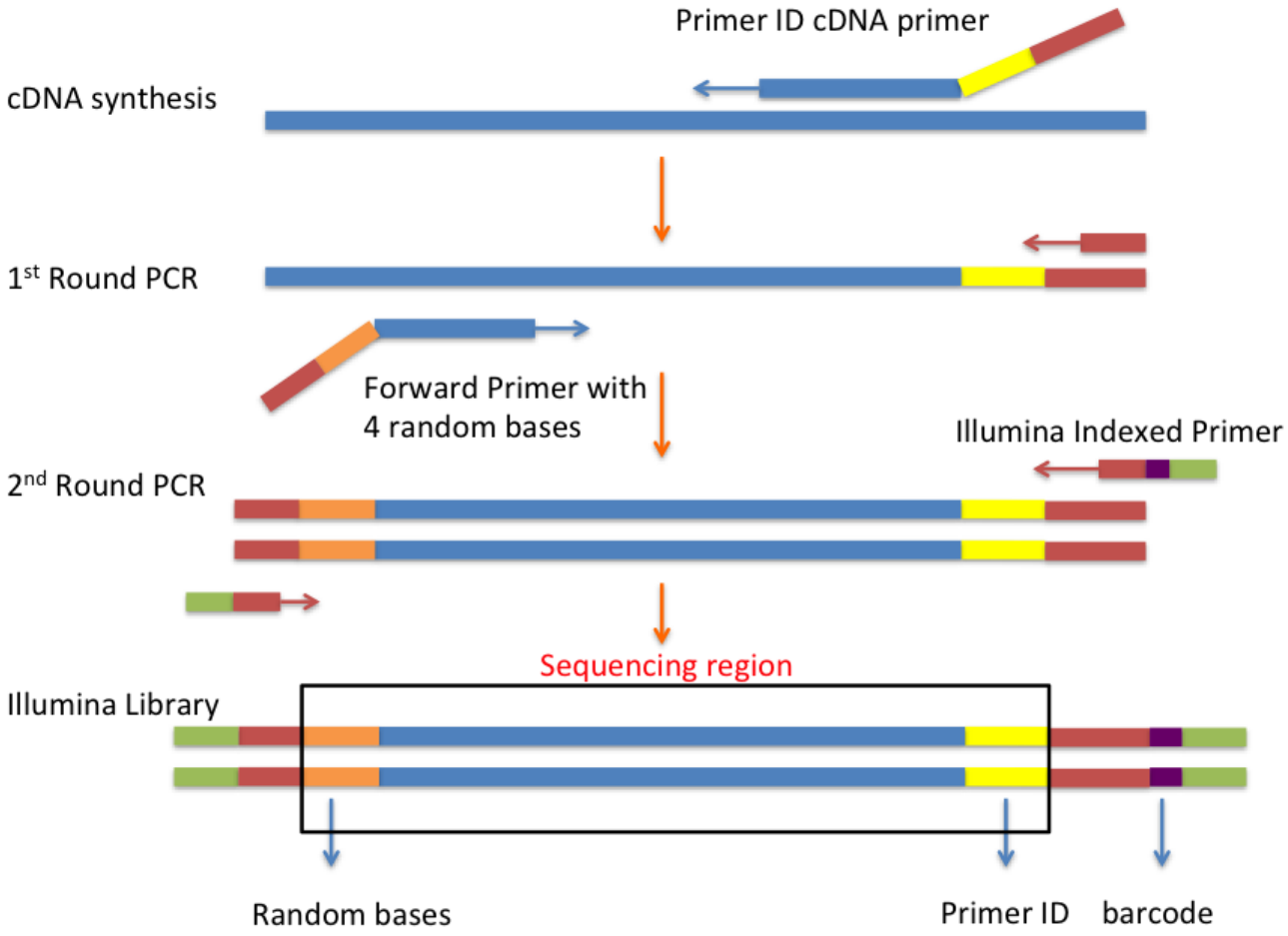
PROTOCOL STATUS

Working

We use this protocol in our group and it is working

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

NAME	CATALOG #	VENDOR
QIAamp vRNA mini kit	51304	Qiagen
Agencourt RNAClean XP	A63987	Beckman Coulter
70% ethanol		Contributed by users
DNase-free water		Contributed by users
AmpureXP PCR cleanup kits	A63880	Beckman Coulter
QIAquick gel extraction kit	28704	Qiagen
Qubit dsDNA BR Assay kit	Q32850	Invitrogen - Thermo Fisher

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

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
IN	R4752_PID11	F4383_AD
V3	R7209_PID11	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	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNN NNNNCAGTTTAACTTTTGGGCCATCCATTCC
R3284_PID11	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNN NNNNNNCAGTCACTATAGGCTGTACTGTCCATTTATC
R4752_PID11	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNN NNNNNNATCGAATACTGCCATTTGTACTGC
R7209_PID11	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNN NNNNNNCAGTCCATTTTGCTYTAYTRABVTTACAATRTGC
F2163AD	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNN NNTCAGAGCAGACCAGAGCCAACAGCCCCA
F2620_AD	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNN NNGGCCATTGACAGAAGAAAAAATAAAAGC
F4383_AD	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNN NNAAAAGGAGAAGCCATGCATG
V1F_AD	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNN NNTTATGGGATCAAAGCCTAAAGCCATGTGTA

"N" in this Primer table is a random nucleotide.

P1	AATGATACGGCGACCACCGAGATCTACACGCCTCCCTCGC GCCATCAGAGATGTG
Indexed Adapter	CAAGCAGAAGACGGCATACGAGAT**NNNNNN**GTGACTGG AGTTCAGACGTGTGCTC
ADPT_2a	GTGACTGGAGTTCAGACGTGTGCTC

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

2 Mix well.

cDNA synthesis

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			

3 µl dNTPMix

1.5 µl cDNA primer

34.5 µl RNA template

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

00:05:00 65°C heat block

5 Place the tube on ice for 1'.

00:01:00 on ice

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			

12 µl 5x buffer

3 µl DTT

3 µl RNaseOUT

3 µl SSII RT

7 Mix and incubate at 50°C for 1 hr.

01:00:00 Incubation at 50°C

8 Increase to 55°C and incubate for 1 hr.

01:00:00 Incubation at 55°C

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

00:15:00 Heating at 70°C

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

1 µl RNase H

00:20:00 Incubation at 37°C

Purification

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)

00:30:00 Room temperature

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

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.

42 µl beads

60 µl cDNA

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.

00:20:00 Room temperature

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

00:05:00 **Magnetic tube rack**

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.

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.

00:00:30 **Incubation at room temperature**

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.

00:00:30 **Incubation at room temperature**

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.

00:10:00 **Air-drying**

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

goto can't be rendered without protocol or steps

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**.

24 µl **DNase-free water**

00:03:00 **Magnetic rack**

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

PCR 1

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

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.

10 µl **5x Buffer A**

10 µl **Enhancer**

1 µl **dNTPs**

2.5 µl **Forward primer**

2.5 µl **ADPT_2a**

0.5 µl **KAPA Robust polymerase**

23.5 µl **Template cDNA**

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

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.

00:30:00 **Room temperature**

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

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

40 µl **Ampure XP beads**

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).

00:05:00 **Incubation at room temperature**

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

00:05:00 **Incubation magnetic rack**

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.

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)

NOTE

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.

500 µl 70% ethanol

00:00:30 Incubation at room temperature

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)

NOTE

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.

500 µl 70% ethanol

00:00:30 Incubation at room temperature

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.

00:10:00 Air-drying

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.**

50 µl DNase-free water

00:03:00 Magnetic rack

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

PCR 2

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

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.

5 µl 5x KAPA HiFi Fidelity Buffer

1 µl dNTP Mix

1 µl **Uni Adapter (ADPT_P1)**

0.5 µl **KAPA HiFI polymerase**

1 µl **Indexed Adapter**

2 µl **Purified template DNA**

14.5 µl **Water**

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

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

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.

42 Excise DNA fragment.

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

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

00:10:00 **Incubation at 50 ˚C**

00:02:30 **Vortex**

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

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

00:01:00 **Centrifugation**

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

500 µl **Buffer QG**

00:01:00 **Centrifugation**

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

00:05:00 **Incubation at room temperature**

00:01:00 **Centrifugation**

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

00:03:00 **Centrifugation**

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

00:04:00 **Stand**

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

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.

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).