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Protocol status: Working
We use this protocol and it's working

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Primer ID MiSeq Library Prep V.3

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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.

ATTACHMENTS

amplicon
[protocol_v1.5.docx](#)

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'	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNCCAGTCCATT TGCTCTACTAATGTTACAATGTGC
Universal Adapter	AATGATACGCGCACCACCGAGATCTACACGCCTCCCTCGCGCCATCAGAGATGT 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	CAAGCAGAAGACGGCATACGAGATGGGTCAAGTGACTGGAGTTCAGACGTGTGCTC
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	CAAGCAGAAGACGGCATACGAGATCAAGTGTGACTGGAGTTCAGACGTGTGCTC
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	CTTGTA	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 13	13	TCCATAA	CAAGCAGAAGACGGCATACGAGATTATGGAAGTGACTGGAGTTCAGACGTGTGCTC
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	CAAGCAGAAGACGGCATACGAGATTCTGCAAGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 19	19	TTCATAA	CAAGCAGAAGACGGCATACGAGATTATGAAGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 20	20	TGCTGTA	CAAGCAGAAGACGGCATACGAGATACAGCAAGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 21	21	TATCACA	CAAGCAGAAGACGGCATACGAGATGTGATAGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 22	22	TGGATAA	CAAGCAGAAGACGGCATACGAGATTATCCAAGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 23	23	CGCATTA	CAAGCAGAAGACGGCATACGAGATAATGCGGTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 24	24	GCCTTAA	CAAGCAGAAGACGGCATACGAGATTAAGCGGTGACTGGAGTTCAGACGTGTGCTC

MATERIALS

MATERIALS

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

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

- For multiplexing sequencing, first, prepare Primer Mix.
Example (For HIV drug resistance pipeline).

Regions	DRCDNA primer	DRF primer
P R	R 2614 P I D	F 2163 A D
R T	R 3284 P I D 11	F 2620 A D
I N T	R 4752 P I D 11	F 4383 A D
V 3	V 3 R B u n i 1 1	V 1 F A D

Make 10 μ M primer mix: mix 10 μ L of each primer in one set and 60 μ L of dH₂O.

Primer Tables

pr i m e r	s e q u e n c e (5' - 3')
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GTGACTGGAGTTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNCAGTTTAACTTTTGGGCCATCCATTCC

R2614
PI
D

GTGACTGGAGTTTCAGACCTGTTGCTCTTCCGATCTNNNNNNNNNNNCAGTCACTATTAGGCTGTACTGTCCATTATTC

GTGACTGGAGTTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNATCGAATACTGCCATTGTACTGC

R4752
PI D11

R7209
P1D11

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I n d e x e d A d a p t e r	C A G C A G A A A G A C G G C C A T A C G A G A T T N N N N G T G A C T G G A G T T C A G A C G T G T G C T C
	G T G A C T G G A G T T C A G A C G T G T G C T C

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:

μ / t u b e		[s t o c k]	[f i n a l]	[m a s t e r m i x]
3 .0	dNTP Mix	10 mM each	0.5	
1 .5	cDNA primer	10 μ M	0.25 μ M	
34 .5	RNA template			
39 .00	Total volume			

🧪 3 μ L dNTP Mix

🧪 1.5 μ L cDNA primer

🧪 34.5 μ L 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:

μ / t u b e		[s t o c k]	[f i n a l]	[m a s t e r m i x]
12 .0	5x buffer	5x	1x	
3 .0	DTT	100 mM	5	
3 .0	RNaseOUT	40 u/ μ L	2	
3 .0	SSIRT	200 u/ μ L	10	

		P			
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	1	r			
	0	t			
		u			
		b			
		e			

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.

go to step #13 undefined

- 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

µ/ t u b e	[s t o c k]	[f i n a l]	[m a s t e r m i x]
5 x 1 0 . 0 B u f f e r A	5 x 1 x		
1 0 . 0 E n h a n c e r	5 x 1 x		
1 . 0 d N T P s	1 0 m M	0. 2 m M	
2 5 F o r w a r d p r i m e r	1 0 µ M	0. 5 µ M	

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

9 5 C	1 m in
9 5 C	1 5 s
5 8 C	1 m in
7 2 C	3 0 s
1 5 - 2 5 c y c l e s	
7 2 C	3 m in
4 C	O n h o l d

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 (incubate 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.

μl/ tube		5 x KAPPA HIF iFidel ity Buffer	[s to c k]	[fi nal]	[ma ster mix]
5 .0			5 x	1 x	
1 .0	d NTP Mix		1 0 mM	0. 4 mM	
1 .0	Uni Adapter (ADPT p1)		1 0 μM	0. 4 μM	
0 .5	KAPPA HIF polymer ase		1 U/ μl	0. 5 U	
1 .0	Index ed Ad apter		1 0 μM	0. 4 μM	
2 .0	Pur ified tem plate DNA				
1 4 .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

- Before gel purification, run 2 µl products on 1% agarose gel to check the bands.
- Gel purification.** (Qiagen QIAquick gel extraction kit)
Run 2nd round PCR products on 1.2% agarose gel. E = 4 V/cm, T = 60 min.
- Excise DNA fragment.
- Weight the gel; add 3 volume of Buffer QG to 1 volume of gel.
- 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 dimer is present (~200bp).