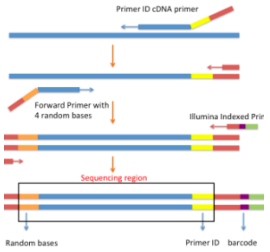
Oct 18, 2018 Version 4

Primer ID MiSeq Library Prep V.4



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

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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

DOCX

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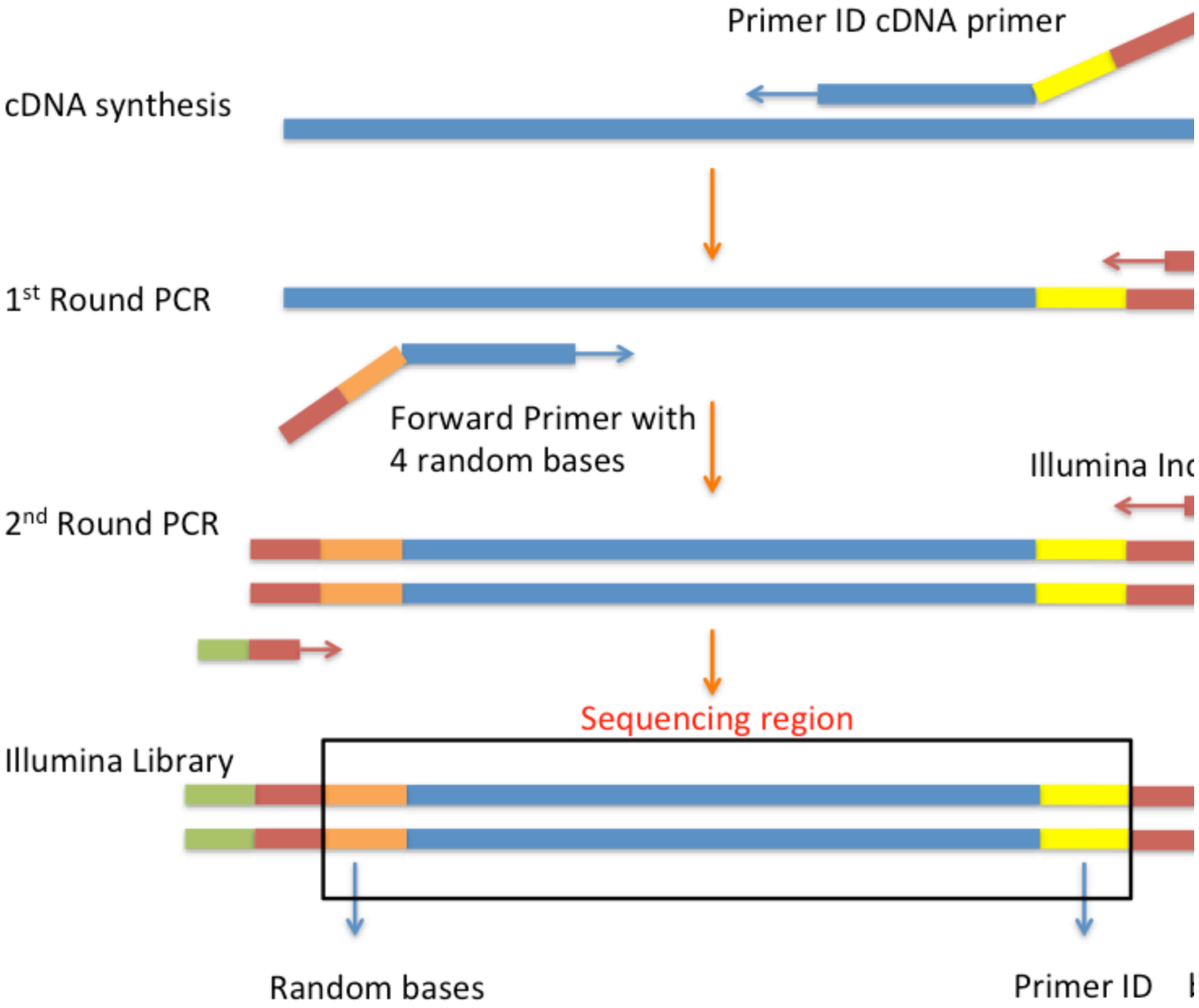
222KB

Guidelines

Viral RNA extraction using QIAamp vRNA mini kit.



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

V1F(forward) 5'-3'	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAG AAAGCCTAAAGCCATGTGTA
BV3R Uni (cDNA Primer) 5'-3'	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNN TGCTCTACTAATGTTACAATGTGC
Universal Adapter	AATGATACGGCGACCACCGAGATCTACACGCCTCCCTC G
Indexed Adapter	CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACT 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	CAAGCAGAAGACGGCATACGAGATCGTGATGT
PCR Primer, Index 2	2	CGATGTA	CAAGCAGAAGACGGCATACGAGATACATCGGT
PCR Primer, Index 3	3	TTAGGCA	CAAGCAGAAGACGGCATACGAGATGCCTAAGT
PCR Primer, Index 4	4	TGACCAA	CAAGCAGAAGACGGCATACGAGATTGGTCAGT
PCR Primer, Index 5	5	ACAGTGA	CAAGCAGAAGACGGCATACGAGATCACTGTGT
PCR Primer, Index 6	6	GCCAATA	CAAGCAGAAGACGGCATACGAGATATTGGCGT
PCR Primer, Index 7	7	CAGATCA	CAAGCAGAAGACGGCATACGAGATGATCTGGT
PCR Primer, Index 8	8	ACTTGAA	CAAGCAGAAGACGGCATACGAGATTCAAGTGT
PCR Primer, Index 9	9	GATCAGA	CAAGCAGAAGACGGCATACGAGATCTGATCGT
PCR Primer, Index 10	10	TAGCTTA	CAAGCAGAAGACGGCATACGAGATAAGCTAGT
PCR Primer, Index 11	11	GGCTACA	CAAGCAGAAGACGGCATACGAGATGTAGCCGT
PCR Primer, Index 12	12	CTTGTAA	CAAGCAGAAGACGGCATACGAGATTACAAGGT
PCR Primer, Index 13	13	TCCATAA	CAAGCAGAAGACGGCATACGAGATTATGGAGT
PCR Primer, Index 14	14	GTACTAA	CAAGCAGAAGACGGCATACGAGATTAGTACGT
PCR Primer, Index 15	15	ACAGTAA	CAAGCAGAAGACGGCATACGAGATTACTGTGT
PCR Primer, Index 16	16	CTCATGA	CAAGCAGAAGACGGCATACGAGATCATGAGGT
PCR Primer, Index 17	17	ACGATAA	CAAGCAGAAGACGGCATACGAGATTATCGTGT
PCR Primer, Index 18	18	TGCAGAA	CAAGCAGAAGACGGCATACGAGATTCTGCAGT
PCR Primer, Index 19	19	TTCATAA	CAAGCAGAAGACGGCATACGAGATTATGAAGT
PCR Primer, Index 20	20	TGCTGTA	CAAGCAGAAGACGGCATACGAGATACAGCAGT
PCR Primer, Index 21	21	TATCACA	CAAGCAGAAGACGGCATACGAGATGTGATAGT
PCR Primer, Index 22	22	TGGATAA	CAAGCAGAAGACGGCATACGAGATTATCCAGT
PCR Primer, Index 23	23	CGCATTA	CAAGCAGAAGACGGCATACGAGATAATGCGGT
PCR Primer, Index 24	24	GCCTTAA	CAAGCAGAAGACGGCATACGAGATTAAGGCGT

Materials

MATERIALS

- QIAamp vRNA mini kit **Qiagen Catalog #**51304
- Agencourt RNAClean XP **Beckman Coulter Catalog #**A63987
- 70% ethanol
- DNase-free water
- 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

Troubleshooting

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	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNCAGTTTAACTTTG

		GGCCATCCATTCC
	R3284_PID11	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNCAGTCACTAGCTGTACTGTCCATTTATC
	R4752_PID11	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNATCGAATACTGCCATTTGTACTGC
	R7209_PID11	GTGACTGGAGTTCAGACG

		TGT GCT CTT CCG ATC TNN NNN NNN NNN CAG TCC ATT TTG CTY TAY TRA BVT TAC AAT RTG C
	F216 3AD	GCC TCC CTC GCG CCA TCA GAG ATG TGT ATA AGA GAC AGN NNN TCA GAG CAG ACC AGA GCC AAC AGC CCC A
	F26 20_A D	GCC TCC CTC GCG CCA TCA GAG ATG TGT ATA AGA GAC AGN NNN GGC CAT TGA

		CAG AAG AAA AAA TAA AAG C
	F4383_AD	GCC TCC CTC GCG CCA TCA GAG ATG TGT ATA AGA GAC AGN NNN AAA AGG AGA AGC CAT GCA TG
	V1F_AD	GCC TCC CTC GCG CCA TCA GAG ATG TGT ATA AGA GAC AGN NNN TTA TGG GAT CAA AGC CTA AAG CCA TGT GTA

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

	P1	AAT GAT ACG

		GCG ACC ACC GAG ATC TAC ACG CCT CCC TCG CGC CAT CAG AGA TGT G
	Indexed Adapter	CAA GCA GAA GAC GGC ATA CGA GAT **NNN NNN** GTG ACT GGA GTT CAG ACG TGT GCT C
	ADPT_2a	GTG ACT GGA GTT CAG ACG TGT GCT 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:

	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.

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

	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]	[masterm ix]

	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).