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RVI-seq - Multi-respiratory virus sequencing protocol V.1

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Andrew Wong[1], Ben Hyatt[1], Diana Rajan[1], Sarah Frances Field[1], Emma Dawson[1], Tobi Ajenifuja[1], Scott Thurston[1], Katherine L Bellis[1], Josef Wagner[1], Matthew Forbes[1], Andrea Frick-Kretschmer[1], Frank Schwach[1], Florent Lassalle[1], David K Jackson[1], Sara Stott[1], Ian Johnston[1], Ewan M Harrison[1]

[1]Wellcome Sanger Institute

Andrew Wong
Wellcome Sanger Institute

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We use this protocol and it's working

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Abstract

RVI-seq is a protocol for sequencing a range of clinically important respiratory viruses in a single workflow. This SOP describes the procedure for generating Illumina compatible libraries from total nucleic acid extracted from upper respiratory tract swabs (nose and throat). Up to 200ng of each library is combined in 8-plex hyb pools prior to enrichment with the Twist Respiratory Viral Research Panel. Captured sequences are cleaned up and further amplified before sequencing on the Illumina NovaSeq 6000.

Guidelines

Note: Throughout the protocol we have indicated the overage required for liquid handling automation in use at Sanger, often based on customised kits. Lower excess volumes may be possible.

For some protocol steps, in-house reagent stocks that are used across multiple NGS pipelines at Sanger are referenced (e.g., adapters, UDI indexing primers, blockers). In many instances equivalent reagents are either provided in the kits listed or off-the-shelf substitutes are available, however we have not validated these alternatives.

Materials

Adapters/UDI indexing primers and blockers will have to be purchased separately as they are not included in the protocol materials listed.

Protocol materials

- 2x Kapa HiFi Hotstart Readymix **Kapa Biosystems** **Catalog #**KK2602
- Elution Buffer (EB) **Qiagen** **Catalog #**19086
- Dynabeads MyOne Streptavidin T1 **Thermo Fisher Scientific** **Catalog #**65602
- Twist Hybridisation and Wash Kit **Twist Bioscience** **Catalog #**101025
- Twist Universal Blockers **Twist Bioscience** **Catalog #**100578
- Twist Respiratory Virus Research Panel **Twist Bioscience** **Catalog #**102957
- NEBNext Ultra II Directional RNA Library Prep Kit for Illumina - 96 rxns **New England Biolabs** **Catalog #**E7760L
- SPRIselect **Beckman Coulter** **Catalog #**B23318
- NEBNext Ultra II FS DNA Library Prep Kit for Illumina - 96 rxns **Catalog #** E7805L

Troubleshooting

cDNA Generation

2h 49m 30s

1

Important! This step must be performed in an RNase free, pre-PCR environment in which post-PCR amplicons are not present, in order to minimise risk of sample contamination.

Decontaminate bench surfaces, pipettes and gloves with RNaseZAP or similar before starting work. Keep reagents and samples chilled throughout the process.

2 Defrost PCR plate containing 8 µL extracted total nucleic acid on ice.

3 NEBNext Ultra II Directional RNA Library Prep Kit for Illumina - 96 rxns **New England Biolabs Catalog #**E7760L

Defrost NEB first strand and second strand reagents on ice.

Do NOT vortex enzymes - mix by inverting/flicking before use.

4 Prepare first strand mastermix on ice:

	A	B	C
	Reagent	**1X rxn**	**96X rxn + 30% overage**
	Strand Specificity Reagent	8 µL	1,000 µL
	First Strand Synthesis Reaction Buffer	4 µL	500 µL
	First Strand Synthesis Enzyme Mix	2 µL	250 µL
	Total	**14 µL**	**1,750 µL**

Note

We use high overage volumes due to the use of automated liquid handlers. Lower excess volumes may be possible.

Mix thoroughly by pipette mixing. Keep on ice.

5 Pulse vortex and spin down random primers.

Dispense 1 µL into each sample. Mix thoroughly by pipetting.

6 Heat seal plate and briefly centrifuge at 800 x g for 30 seconds. 30s

7 Place plate on a thermocycler and run the following programme: 10m

65 °C for 00:05:00 , then cool to 4 °C

When the thermocycler reaches 4 °C , remove plate and immediately place on ice for 5 minutes.

Briefly centrifuge plate after 5 minute crash-cool at 800 x g for 1 minute. **Keep plate on ice**.

8 Pipette 14 µL of first strand mastermix into each sample **on ice**, mixing thoroughly by pipette mixing.

Heat seal plate and briefly centrifuge at 800 x g for 30 seconds.

9 Place plate on a thermocycler and run the following programme: 1h 15m

Lid Temp Constant: 80 °C
Volume: 23 µL

	A	B
	Temperature	**Time**
	25 °C	10 minutes
	42 °C	50 minutes
	70 °C	15 minutes
	4 °C	∞

10 While the plate is on the thermocycler, prepare the second strand mastermix on ice:

	A	B	C
	Reagent	**1X rxn**	**96X rxn + 20% overage**
	Nuclease Free Water	48 µL	5,529.6 µL
	Second Strand Synthesis Reaction Buffer	8 µL	921.6 µL
	Second Strand Synthesis Enzyme Mix	4 µL	460.8 µL
	Total	**60 µL**	**6,912 µL**

Pipette mix 10 times with a 5 mL pipette.

11 When the first strand thermocycler step is finished, retrieve the plate and centrifuge at 800 x g for 1 minute.

12 Pipette 60 µL of second strand mastermix into each sample **on ice**, mixing thoroughly by pipette mixing.

Heat seal plate and briefly centrifuge at 800 x g for 30 seconds.

13 Place plate on a thermocycler and run the following programme: 1h

16 °C for 01:00:00 , then cool to 4 °C

When the thermocycler is cooled to 4 °C, retrieve the plate and centrifuge at 800 x g for 1 minute.

14 Perform a SPRI Clean-up as follows.
Note: if working in a PCR plate, it may be necessary to move to a deepwell to accommodate the volumes below.

15 SPRIselect **Beckman Coulter Catalog #**B23318 10m

Vortex SPRIselect beads well, ensuring they are homogenous prior to use.

Add 144 µL SPRI Select to each sample, mixing well by pipetting.

Incubate for 00:10:00 at room temperature.

16 Place plate on a magnet and wait 4 minutes to allow beads to pellet. 5m

Leaving the plate on the magnet, carefully remove and discard supernatant, taking care not to disturb the beads.

On the magnet, add 180 µL of freshly prepared 80% ethanol to the beads. Wait 1 minute then carefully remove ethanol and discard.

17 Repeat ethanol wash. 5m

Carefully remove as much residual ethanol as possible without disturbing the beads.

Dry beads for 5 minutes at room temperature.

18 Remove plate from the magnet and resuspend with 28 µL 1X TE buffer, mixing well by pipetting. 2m

Incubate at room temperature for 2 minutes .

19 Place plate on magnet and wait for 2 minutes to allow solution to clear. 2m

Carefully transfer 26 µL of supernatant into a new PCR plate.

Final volume of sample after cleanup = 26 µL

When finished, seal plate and centrifuge at 800 x g for 30 seconds.

20 **PAUSE POINT** cDNA can be stored at 4 °C (same day) or -20 °C (up to a week).

Library prep 2h 46m

21 NEBNext Ultra II FS DNA Library Prep Kit for Illumina - 96 rxns **Catalog #** E7805L

Defrost Ultra II FS Reaction Buffer and Ultra II FS Enzyme Mix on ice. Briefly vortex **both** the enzyme and buffer, spin down.

Note: Ultra II FS Reaction Buffer may contain white precipitate. Break this up as much as possible by pipette mixing.

22 Prepare Fragmentation mastermix on ice:

	A	B	C
	Reagent	**1X rxn**	**96X rxn + 30% overage**
	Ultra II FS Reaction Buffer	7 µL	873.6 µL
	Ultra II FS Enzyme Mix	2 µL	249.6 µL
	Total	**9 µL**	**1,123.2 µL**

Vortex briefly to mix, spin down.

23 Add 9 µL fragmentation mastermix into each cDNA sample on ice. Mix thoroughly by pipette mixing.

24 Heat seal plate and centrifuge at 800 x g for 30 seconds.

25 Place plate on a thermocycler and run the following program: 45m

Lid Temp Constant: 85 °C

Volume: 35 µL

	A	B
	Temp	**Time**
	37 °C	12 minutes
	65 °C	30 minutes
	4 °C	∞

26 While the thermocycler is running, defrost the following reagents on ice:
Ultra II Ligation Master Mix
Ligation Enhancer
TruSeq adapter

Vortex and spin the reagents.

Note

We use an alternative TruSeq compatible adapter which does not require the USER enzyme incubation step in the NEB protocol. If using the NEBNext adapter instead (purchased separately in a NEBNext Multiplex Oligo kit), follow the steps in the NEBNext Ultra II FS DNA Library Prep Kit manual to add USER enzyme to the ligation reaction.

The alternative TruSeq adapter sequence has been published here:
https://doi.org/10.1099/mgen.0.001228
(Supplementary Table 1)

27 Prepare ligation mastermix on ice:

Note: Use low retention tips and pipette slowly as the Ligation Mix is extremely viscous.

	A	B	C
	Reagent	**1X rxn**	**96X rxn + 20% overage**
	Ultra II Ligation Master Mix	30 µL	3,456 µL
	Nuclease Free Water	2.25 µL	259.2 µL
	Ligation Enhancer	1 µL	115.2 µL
	TruSeq Adapter (100 µM)	0.25 µL	28.8 µL
	Total	**33.5 µL**	**3,859.2 µL**

Mix by pipetting the entire volume at least 10 times. Insufficient mixing can affect ligation efficiency. Check the solution is completely clear and homogenous.

28 Once the thermocycler has reached 4 °C , remove the plate and centrifuge at 800 x g for 1 minute.

Add 33.5 µL Ligation mastermix into each sample. Mix thoroughly by pipette mixing.

29 Incubate at 20 °C for 00:20:00 20m

30 Once the incubation is complete, perform a 0.8X SPRI clean up as follows:

Vortex SPRIselect beads well, ensuring they are homogenous prior to use.

31 Add 55 µL SPRIselect to each sample, mixing well by pipetting. 5m

Incubate for 5 minutes at room temperature.

32 Place plate on a magnet and wait 5 minutes to allow beads to pellet.

5m

Carefully remove and discard supernatant, taking care not to disturb to beads.

33 On the magnet, add 180 µL of 75% ethanol to the beads.

Wait 1 minute then carefully remove ethanol and discard.

Repeat ethanol wash.

Carefully remove as much residual ethanol as possible without disturbing the beads.

34 Keeping the plate on a magnet, dry beads for 5 minutes at room temperature. 7m

Remove plate from the magnet and resuspend with 26.5 µL of NFW, mixing well by pipetting.

Incubate for 2 minutes at room temperature.

35 Place plate back on a magnet, and wait for solution to clear, ~ 2 minutes. 2m

Note

Keep the plate on the magnet while proceeding with steps 36-38 below.

36 2x Kapa HiFi Hotstart Readymix **Kapa Biosystems Catalog #**KK2602

Defrost KAPA HiFi Master Mix and a lyophilised UDI tag plate on ice.

Note

We use lyophilised UDI primers purchased from IDT allowing for 25 µL of sample eluate to be added directly to the primer plate. Additionally, we use KAPA HiFi Hotstart Readymix instead of the Ultra II Q5 Master Mix provided in the NEB kit.

If using different UDI primers (e.g. from a NEBNext Multiplex Oligo kit), follow the PCR set-up volumes from the NEBNext Ultra II FS DNA Library Prep Kit manual.

The UDI primer sequences we use have been published here:
https://doi.org/10.1099/mgen.0.001228
(Supplementary Table 1)

37 Centrifuge the UDI tag plate at 800 x g for 2 minutes. Vortex and spin the KAPA HiFi Master Mix. 2m

38 Add 25 µL KAPA HiFi Master Mix into each well of the UDI tag plate on ice.

39 Carefully transfer 25 µL of supernatant from each sample from step 35 into the UDI tag plate on ice. Mix thoroughly by pipette mixing.

The final concentration of each UDI in the 50 µL PCR reaction is [M] 2 micromolar (µM) .

40 Heat seal plate and centrifuge at 800 x g for 30 seconds.

41 Transfer the plate to a Post-PCR lab. Place plate on a thermocycler and run the following program: 1h

Lid Temp Constant: 100 °C

Volume: 50 µL

	Step	Temperature	Time
	1	95.0°C	5 minutes
	2	98.0°C	30 seconds
	3	65.0°C	30 seconds
	4	72.0°C	2 minutes
	5	Repeat from step 2, 11 more times	-
	6	72.0°C	10 minutes

7	4°C	∞

42 When the thermocycler is finished, retrieve plate and centrifuge at 800 x g for 1 minute.

43 Perform a 1X SPRI clean up as follows:

43.1 Vortex SPRIselect beads well, ensuring they are homogenous prior to use. 5m

Add 50 µL SPRIselect to sample, mix well by pipetting.

Incubate for 5 minutes at room temperature.

43.2 Place plate on a magnet and wait 5 minutes to allow beads to pellet. 5m

Leaving plate on the magnet, carefully remove and discard supernatant, taking care not to disturb the beads.

On the magnet, add 180 µL of 75% ethanol to the beads. Wait 30 seconds then carefully remove ethanol and discard.

43.3 Repeat ethanol wash. 5m

Carefully remove as much residual ethanol as possible without disturbing the beads.

Dry beads for 5 minutes at room temperature.

43.4 Remove plate from the magnet and resuspend with 32 µL elution buffer, mixing well by pipetting. 3m

Elution Buffer (EB) **Qiagen Catalog #**19086

Incubate at room temperature for 3 minutes.

43.5 Place the plate on magnet and wait for 2 minutes to allow solution to clear. 2m

Carefully transfer 30 µL of supernatant into a new plate.

Final volume of sample after cleanup = 30 µL

When finished, seal plate and centrifuge at 800 x g for 30 seconds.

44 **PAUSE POINT** Libraries can be stored at 4 °C (same day) or at -20 °C.

Library QC and Pooling

45 Quantify purified DNA libraries with a fluorescence based assay. We have an automated assay setup using the AccuClear Ultra High Sensitivity dsDNA Quantitation kit with 7 DNA standards (Biotium) and BMG FLUOstar Omega plate reader. However, any equivalent assay may be used, e.g. Qubit dsDNA Broad Range Quantitation Assay (Thermo Fisher Scientific)

46 Dilute and re-quantify libraries as necessary for accurate pooling.

47 Take 200 ng or up to 12 µL per indexed library and combine them into 8-plex hybridisation pools. The total mass per pool will be <= 1.6µg.

Note

We take a maximum of 12µl per library to allow for a repeat if needed.

48 **PAUSE POINT** Pooled libraries can be stored at 4 °C (same day) or -20 °C (up to a week).

Hybridisation

19h 42m

49 **Important!** It is essential to ensure that all required reagents are available prior to beginning this step due to the lack of stopping points.

The following steps follow the Twist Target Enrichment Standard Hybridization v1 protocol.

Twist Hybridisation and Wash Kit **Twist Bioscience Catalog #**101025

Twist Universal Blockers **Twist Bioscience Catalog #**100578

Twist Respiratory Virus Research Panel **Twist Bioscience Catalog #**102957

50 If frozen, defrost sample plate on ice and centrifuge at 800 x g for 1 minute.

Defrost custom blockers on ice.

Note

We add custom blockers (200 µM stock) to each pool prior to the dry-down step. Twist Universal Blockers can be used instead (7 µL of Twist Universal Blockers should be used as per the Twist Target Enrichment Standard Hybridization v1 Protocol instead of 25 µL of custom blockers). If using Twist Universal Blockers, these should be added **after** the dry down at step 56, in place of nuclease free water.

The custom blocker sequences have been published here:
https://doi.org/10.1101/2024.10.30.24316422
(Supplementary Note 1)

51 Add 25 µL of custom blockers (200 µM) to each pool of samples.

Make sure to mix during addition, and to change tips after each individual addition to avoid contaminating sample pools.

52 Seal plate and centrifuge briefly at 200 x g for 1 minute.

Inspect wells to ensure there are no bubbles and that the samples are sitting at the bottom of each well.

53 Carefully remove seal and place plate into SpeedVac with an appropriate counterbalance plate. 3h 30m

Set the SpeedVac to 45 °C for 03:30:00 and start.

Sample pools must be completely dried down.

Note

Typically this process should take 3-5 hours depending on the sample pool volumes. After 3.5 hours it is recommended to periodically check the progress every 15 minutes to gain an appreciation as the when the plate may be ready to remove. Set the SpeedVac to 15 minute increments beyond this point.

54 **PAUSE POINT** dried pools can be sealed with a PCR adhesive seal and stored at -20 °C for **up to 24 hours.**

55 If proceeding to hyb setup, defrost the following reagents on ice towards the end of dry-down:

- Twist bait panel (probes)
- Twist blocker solution
- Hybridisation mix

When defrosted, transfer the Hybridisation mix to a heat block set to 65 °C

56 Near the end of the dry-down, prepare the **Twist blocker solution mix**. This will be used to re-suspend the dried down custom blocker solution and sample pools.

Note

Replace nuclease free water with 7µl Twist Universal blockers if custom blockers have not been added prior to dry down.

	A	B	C
	Reagent	**1X rxn**	**12X rxn + 15% overage**
	Blocker Solution	5 µL	69 µL
	Nuclease Free Water	7 µL	96.6 µL
	Total	**12 µL**	**165.6 µL**

Pipette mix solution 10 times.

57 When the sample plate has been dried down, remove it from the SpeedVac. There may be a small, dry pellet or smear at the bottom of the wells.

58 Re-suspend sample pools by adding 12 µL of the **Twist blocker solution mix** to each well requiring resuspension.

Avoid touching the well sides/bottom during addition. Make sure to change tips after each individual addition to avoid contaminating the sample pools.

Do not pipette mix following blocker solution addition.

59 Seal the re-suspended sample plate with an adhesive PCR seal and vortex at medium speed for 1 minute on a plate shaker.

Centrifuge plate at 800 x g for 1 minute and visually inspect the plate to ensure samples are at the bottom of the wells.

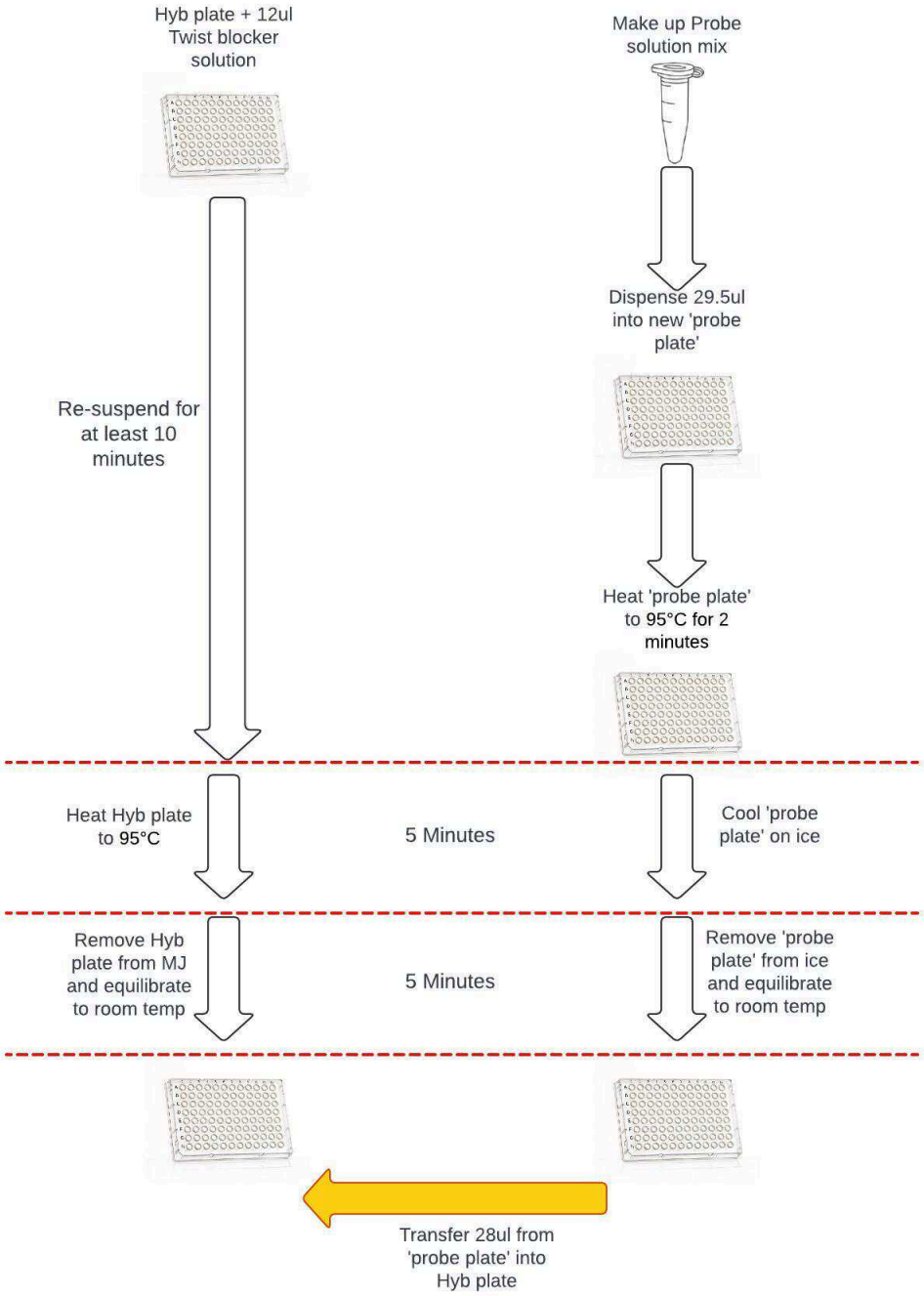
60 Allow the plate to resuspend for **at least 10 minutes.**

If you are doing this step in the afternoon, proceed with the next steps of hyb preparation. If not, wait until at least 2 pm before starting hyb preparation.

61

Note

The following steps should be performed in parallel.



62 Ensure the Hybridisation mix has been heated to 65°C for at least 15 minutes - this is to ensure all salts are fully incorporated into the liquid and no residue remains. Pulse vortex and spin down.

The solution should look clear.

63 Remove the hyb mix tube from the heat block and equilibrate to room temperature for 2 minutes. Immediately proceed to preparing the mastermix at room temperature.

Hybridisation mix is very viscous - aspirate the desired volume slowly to ensure accurate pipetting.

Do not vortex probes - mix well by flicking and inversion.

If hyb mix becomes cloudy before pipetting, heat again to 65°C to dissolve precipitate.

	A	B	C
	Reagent	**Volume 1 rxn**	**Volume 12 rxn + 15% overage**
	Twist bait panel (probes)	4 µL	55.2 µL
	Water	4 µL	55.2 µL
	Hybridisation Mix	20 µL	276 µL
	Total	**28 µL**	**386.4 µL**

Pipette 10x carefully and keep at room temperature.

64 Take the Probe solution mix and aliquot 29.5 µL per well into a new plate, mirroring the layout of the sample plate.

Note

Small white particles may be present in the Twist bait panel (probes); this will not impact the final capture product.

65 Seal plate and centrifuge at 800 x g for 30 seconds.

66 Set a thermocycler to heat to 95 °C forever, lid constant 105 °C forever.

67 Once the thermocycler has reached 95 °C for block and 105 °C for the lid, place the plate containing the Probe solution mix onto the heated block position and tighten the lid. 2m

Set a timer for **2 minutes** as soon as the thermocycler lid has been closed.

68 After the 2 minutes has expired **immediately remove plate from the thermocycler and cool on ice for 5 minutes.** 5m

Do not cancel the thermocycler programme as it is about to be used again. While the Probe solution is cooling proceed with the next step.

69 Take the re-suspended Hyb plate and place it onto the heated block position and tighten the lid.

Set a timer for **5 minutes.**

> Note
>
> It is more prudent to set the timer for 5 minutes once the re-suspended hyb plate is on, rather than setting a 5 minute timer for the crash cool, then another timer for denaturation of the resuspended plate, to avoid confusion.

70 After the 5 minutes, remove the re-suspended Hyb plate from the thermocycler and equilibrate **BOTH** the Probe solution mix and the re-suspended Hyb plate on the benchtop for **5 minutes at room temperature.** 5m

The 95 °C thermocycler block can now be stopped.

71 At this point, take the Twist hybridisation enhancer out of the freezer and keep on ice. **Keep covered as this reagent is light sensitive.**

72 Once both plates have been resting for 5 minutes at room temperature, briefly centrifuge both plates and transfer 28 µL of the Probe solution mix to the corresponding wells in the re-suspended Hyb plate, mixing the Probe solution thoroughly before and after addition.

73 Seal plate with an adhesive seal and pulse-centrifuge to 200 x g to ensure the solution is at the bottom of the wells.

Check for the absence of bubbles, and repeat centrifuge if necessary.

74 Carefully de-seal plate and **add** 30 µL **of Hybridisation enhancer to the top of each active well**, making sure to change tips after each individual addition to prevent contamination.

DO NOT MIX - the hybridisation enhancer will settle on top of the reaction, helping to prevent evaporation.

75 Heat seal the plate and inspect to ensure there are no bubbles, with samples sitting at the bottom of the wells.

DO NOT SPIN this plate down - the hybridisation enhancer is an oil-based solution that may have a tendency to spill into neighboring wells, compromising seal integrity and resulting in cross contamination / evaporation .

76 Set a thermocycler to run at 70 °C forever, lid constant 85 °C forever, reaction volume 70 µL . 16h

Once the block has reached temperature, place the plate on the block and close the lid - ensure the lid is firmly shut.

The hybridisation reactions will now incubate for 16 hours at 70C.

Hybridisation Capture and Enrichment 1h 1m

77 **Important!** Inspect the thermocycler and check that the hybridisation reaction has been allowed at least **16 hours** to incubate before proceeding. **DO NOT stop the thermocycler program - keep the hybridisation reactions at 70 °C until they are transferred directly to the washed streptavidin beads in step 81 below.**

78 Dynabeads MyOne Streptavidin T1 **Thermo Fisher Scientific Catalog** #65602

Allow Dynabeads MyOne Streptavidin T1 beads to acclimate to room temperature prior to use (~30 minutes).

Preheat the following tubes at 48 °C until any precipitate is dissolved:

- Binding Buffer

- Wash Buffer 1
- Wash Buffer 2

For each hybridisation reaction (well):

- Equilibrate 800 µL Binding Buffer to room temperature
- Equilibrate 200 µL Wash Buffer 1 to room temperature
- Leave 700 µL Wash Buffer 2 at 48 °C

79 Thoroughly vortex the room temperature Dynabeads until homogeneous.

Prepare the Dynabeads as follows:

79.1

	A	B	C
	Reagent	**1X rxn**	**12X rxn + 25% overage**
	Dynabeads	100 µL	1,500 µL
	Binding Buffer (per wash)	200 µL	3,000 µL

Add Binding Buffer to Dynabeads in a tube according to the table above. Mix thoroughly by vortexing.

79.2 Place the tube containing the resuspended beads in a magnet rack for 2 minutes. 2m

79.3 After 2 minutes, discard the supernatant. Remove the tube from the magnet.

79.4 Repeat the Binding Buffer wash two more times for a total of three washes. 5m

79.5 After removing the supernatant from the third Binding Buffer wash, add a final Binding Buffer re-suspension. Do not place the tube on the magnet, mix the solution by pipetting and gently vortexing until homogenous.

80 Pipette 200 µL of the prepared Dynabeads solution into the required number of columns in a deep well plate, mirroring the layout of the hybridisation plate.

81 Perform a final check of the thermocycler to ensure it is at least **16 hours** from the start of hybridization.

Keeping the hybridisation reaction on the thermocycler at 70 °C, transfer the whole volume (70 µL) of each of the hybridisation reaction into the prepared Dynabeads plate. Pipette mix thoroughly.

Note

It is critical to keep the hybridisation reaction at 70°C and do this transfer quickly, as a drop in temperature can increase off-target binding.

82 Mix the deep well plate now containing the hybridisation reaction and Dynabeads for 30 minutes at room temperature on a plate shaker at a speed sufficient to keep the solution mixed. 30m

Do not vortex - aggresive mixing is not required.

83 After the 30 minute mix period, pulse-spin the plate to ensure all the solution is at the bottom of the wells.

84 Place the plate on a plate magnet for 1 minute. 1m

85 Keeping the plate on the magnet, remove and discard the clear supernatant including the hybridisation enhancer.

Do not disturb the bead pellet.

Note

Some Hybridisation Enhancer may be visible after supernatant removal and throughout each wash step - it will not affect the final capture product.

86 Remove the plate from the magnetic stand and add 200 µL Wash Buffer 1. Mix by pipetting.

87 Pulse-spin plate to ensure all solution is at the bottom of the wells.

88 Transfer the entire volume, ~ 200 µL into a new plate and place on a plate magnet for 1 minute. 1m

89 Keeping the plate on the magnet, remove and discard the clear supernatant.

Make sure to not disturb the bead pellet.

90 Remove the plate from the magnet and add 200 µL of 48 °C Wash Buffer 2.

Mix by pipetting, then pulse-spin to ensure all solution is at the bottom of the wells.

91 Incubate the plate for 5 minutes at 48 °C 5m

92 Place the plate on a plate magnet for 1 minute. 1m

93 Keeping the plate on the magnet, remove and discard the clear supernatant. 1m

Make sure to not disturb the bead pellet.

94 Repeat the Wash Buffer 2 wash (steps 90 to 93) two more times, for a total of three washes. 15m

95 After the final wash, use a 10 µl pipette to remove all traces of supernatant. Proceed immediately to the next step and do not allow the beads to dry.

96 Remove the plate from the magnetic stand and add 25 µL water. Mix by pipetting until homogenised, then keep on ice.

This solution is referred to as the Streptavidin Binding Bead Slurry.

Post-capture PCR amplification and cleanup 22m

97 Defrost KAPA 2x HiFi HotStart ReadyMix and p5/p7 amplification primers on ice.

Note

We use p5/p7 primers purchased from IDT as opposed to the amplification primers from the Twist kit. The primers are used at a final concentration of 2.5 µM each in the final 50 µL PCR reaction.

The primers are standard p5/p7 sequences but can also be found here: https://doi.org/10.1101/2024.10.30.24316422 (Supplementary Note 1)

98 Vortex and spin the reagents, and prepare the following PCR mix on ice:

	A	B	C
	Reagent	**Volume Per Reaction**	**12X rxn + 1.5X rxn overage**
	Amplification Primers (p5/p7) (50 µM)	2.5 µL	33.75 µL
	KAPA 2X HiFi HotStart Readymix	25 µL	337.5 µL
	Total	**27.5 µL**	**371.25 µL**

Mix thoroughly by pipette mixing.

99 Into a new 96 well PCR plate, pipette 27.5 µL of PCR mix, mirroring the layout of the Streptavidin Binding Bead Slurry plate.

100 Inspect the Streptavidin Slurry. If the beads have separated at the bottom, resuspend them using a pipette. It should be opaquely brown once thoroughly mixed.

101 Once homogenised, transfer 22.5 µL from each well of Streptavidin Slurry into the PCR mix plate. Mix thoroughly by pipette mixing.

Heat seal the plate. Check if there are beads on the sides of the wells.

Note

If there are beads on the sides of the wells, pulse spin very gently (up to 5 seconds). If a harder spin is required, re-homogenise the beads by placing on a plate shaker until fully resuspended.

102 Place plate on a thermocycler and run the following program:

Lid Temp Constant: 100 °C

Volume: 50 µL

	A	B	C
	Step	**Temperature**	**Time**
	1	98.0 °C	45 seconds
	2	98.0 °C	15 seconds
	3	60.0 °C	30 seconds
	4	72.0 °C	30 seconds
	5	Repeat from step 2, 13 more times	-
	6	72.0 °C	1 minute
	7	4 °C	∞

103 Vortex the plate on a plate shaker at ~2,000rpm for 30 seconds to re-suspend the beads. Pulse centrifuge the plate very gently ~up to 5 seconds.

104 Perform a 0.8X SPRI clean up as follows:

104.1 Vortex SPRIselect beads well, ensuring they are homogenous prior to use. 5m

Add 40 µL SPRI Select to sample, mix well by pipetting.
Incubate for 5 minutes at room temperature.

104.2 Place plate on a magnet and wait 5 minutes to allow beads to pellet. 5m

Leaving plate on the magnet, carefully remove and discard supernatant, taking care not to disturb the beads.

On the magnet, add 180 µL of 75% ethanol to the beads. Wait 30 seconds then carefully remove ethanol and discard.

104.3 Repeat ethanol wash. 5m

Carefully remove as much residual ethanol as possible without disturbing the beads.

Dry beads for 5 minutes at room temperature.

104.4 Remove plate from the magnet and resuspend with 52 µL elution buffer, mixing well by pipetting. 5m
Elution Buffer (EB) **Qiagen Catalog #**19086

Incubate at room temperature for 5 minutes.

104.5 Place the plate on magnet and wait for 2 minutes to allow solution to clear. 2m

Carefully transfer 50 µL of supernatant into a new plate.

Final volume of sample after cleanup = 50 µL

When finished, seal plate and centrifuge at 800 x g for 30 seconds.

105 **PAUSE POINT** Enriched libraries can be stored at 4 °C (same day) or -20 °C (up to a week).

Post Capture QC, Pooling and Normalisation

106 Quantify enriched DNA libraries with a fluorescence based assay. We have an automated assay setup using the AccuClear Ultra High Sensitivity dsDNA Quantitation kit with 7 DNA standards (Biotium) and BMG FLUOstar Omega plate reader. However, any equivalent assay may be used, e.g. Qubit dsDNA Quantitation Assay (Thermo Fisher Scientific)

107 Run the enriched libraries on an Agilent Bioanalyzer with either the D1000 or High Sensitivity assay kit, depending on library concentration.

The average peak size should be roughly 350 - 400 bp. Check there is no primer-dimer present.

108 Pool the enriched libraries into one 1.5 mL tube in an equimolar fashion.

109 Dilute the equimolar pool to [M] 2.8 nanomolar (nM) .

Run the diluted pool on the Agilent Bioanalyzer with the High Sensitivity DNA assay kit in triplicate to confirm the concentration.

Note

We currently sequence samples on an Illumina NovaSeq 6000, SP flow cell (150PE), using the XP workflow. We have plexed up to 96 samples per lane, this could be increased further depending on coverage requirements.

Pools are diluted to 0.8nM for sequencing, a minimum of 18 µL is required for one sequencing attempt.

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