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We use this protocol and it's working

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Ovation RRBS Methyl-seq library prep

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ABSTRACT

This protocol describes a library prep procedure for 1 to 96 samples with the Ovation RRBS Methyl-seq System. The original Tecan Ovation protocol describes a low throughput procedure, therefore this protocol is especially targeted for manual high-throughput preps from 24 to 96 samples, although it can be used for small sample numbers too. Importantly, the Ovation RRBS kit is currently sold as oxBS only (identifying both 5-methyl-C and 5-hydroxymethyl-C), whilst this protocol is modified to exclude the oxidation step and describes a basic RRBS, where both 5mC and 5hmC appear as the same positive signal and are indistinguishable. This protocol has a few further modifications, specifically in the bisulfite conversion step and bead purifications, which have performed better in our hands.

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GUIDELINES

Ovation RRBS Methyl-seq library prep (up to 96 samples)

This protocol is based on Tecan's Ovation oxBS RRBS protocol, but without the oxidation step, making it suitable for analysing methylation and not hydroxy-methylation. Tecan do not provide a protocol for their 96-sample kit, they have a protocol for 1-16 samples. This protocol is therefore adjusted for a higher-throughput for 24 up to 96 sample preparation.

Tecan recommend processing not more than 3 strips (24 samples) at a time manually, which means splitting a 96-sample kit in 4 batches. This consideration is mainly because of the washing steps, where timing is crucial and there is a risk of over-drying the beads and reducing yield. This protocol describes a procedure for the washes, which works for manual handling, but does require good pipetting and laboratory experience. The protocol is not designed by Tecan for automated steps (with liquid handling systems), although automation is possible in principle, however, some volumes are too low for most robots. In our personal experience from performing automated wash steps, however, yields can be lower due to loss of beads - a careful manual prep can deliver a 10 to 100-fold higher library yields and much higher quantity consistency between the samples.

Unmethylated DNA control must be purchased separately – this is optional but advisable to include.

MATERIALS

Reagents

1. Qubit DNA Quantification Assay kit (High Sensitivity) or Quant-iT PicoGreen dsDNA Assay Kit with Lambda DNA standard (Invitrogen/Thermo Fisher Scientific)
2. Ovation RRBS Methyl-Seq System (for 32 or 96 samples)
3. Unmethylated DNA control (any supplier)
4. 100 % ethanol
5. Agencourt Magnetic Beads
6. 20X EvaGreen (any supplier) - if doing qPCR

Materials

1. A set of standard single-channel pipettes with low retention nuclease-free tips
2. ESSENTIAL Multichannel pipettes: manual 0.5-10 µl, 2-20 µl and 20-200 µl 8-channel pipettes and an electronic dispensing 100-1200 µl 8-channel pipette with a buffer reservoir
3. ESSENTIAL Electronic multi-dispenser pipette, such as Eppendorf Multipette E3 with associated combitips of different sizes
4. Pipet-aid/pipettor with stripettes (for the ethanol wash prep)
5. Clear 0.2 ml PCR strip tubes or clear 96-well PCR plates
6. Adhesive seals for PCR plates and seal applicators
7. Plastic tubes of different sizes – 0.5 ml to 5 ml tubes, and 15- or 50-ml conical centrifuge tubes (for ethanol)
8. Ice bucket with ice or cooling racks and cooling plate stands

Laboratory equipment

1. Qubit fluorometer or a fluorescent microplate reader
2. Magnetic separation rack or plate
3. PCR cycler
4. Benchtop microcentrifuges: for 0.2 ml tubes and plates
5. Bioanalyzer or TapeStation or other fragment analyzer, together with associated reagents and consumables (always use the ‘High Sensitivity’ DNA assays)
5. Vortex
6. Lab fume hood

SAFETY WARNINGS

Wear gloves and a laboratory coat at all times.

This high-throughput manual protocol is demanding on the hands and thumbs for tip ejection. Make sure to choose multichannel pipettes, which do not require extreme force for tip ejection by the thumb (ideally tip ejection should *NOT* be performed with the thumb - see CAPP multichannel pipettes design). Excessive thumb workload by multichannel pipetting is a major cause of repetitive strain injuries.

BEFORE START INSTRUCTIONS

Timing suggestions (allow more time until well familiar):
The calculations can be prepared the day before (Day1).
Sample preparation, MspI digestion, Adaptor ligation - 6 hours with 48 samples; full day with 96 samples (Day 2).
End repair, Purification and Bisulfite conversion - 6 hours (48 samples); full day (96 samples); BS reaction left O/N (Day 3)
Bisulfite-converted DNA desulfonation and purification, library amplification (left O/N) - 6 hours (48 samples); full day (96 samples) (add extra time for optional qPCR) (Day 4)
Amplified library purification and quality control – half a day (48-96 samples); (Day 5)

Calculations

1 Prepare a list of samples and their concentrations, calculate the required volumes for 100 ng DNA and nuclease-free water for a final reaction volume of 8.5 µL.

Note

This step should not be underestimated and can take a while if preparing half or a full plate. 48 to 96 samples can be done at once, but it is advisable to have a second person around to shadow and swap with when needed, especially when doing 96 samples.
DNA concentration must be measured by Qubit or Picogreen assay before the library prep (Nanodrop is too inaccurate for this) an must be above 12.5 ng/µl.

2 Calculate the volume of a methylation conversion control to spike in each sample. This is usually

unmethylated Lambda virus DNA, added in 1:1000 – 1:5000 ratio to each DNA sample – i.e. up to 0.1 ng unmethylated DNA control per sample. The starting unmethylated DNA stock will be highly concentrated and a dilution will be required.

Note

The easiest and least error-prone approach to do this step, is to calculate in MS Excel the exact amounts of DNA, unmethylated control and water, ordered by ascending DNA quantities. In this way, there will be slight to no changes of pipette volume after each sample, which makes the pipetting of 96 samples much faster, and reduces chances of error – even if one forgets to change the pipette volume it will be very close to the required volume anyway and easy to fix.

Put a mark for every 8 samples on the list and plot in this order, 8 samples per column, onto a plate format, to know the exact location of each sample on the plate. Also, prepare plate schematics with sample loading quantities for DNA and water separately for reference.

Sample Preparation

3 Prepare the unmethylated DNA control – add the necessary total volume to the total volume of water (from the calculations, sum up the unmethylated control DNA and water volumes for all samples), and pre-mix well.

Note

The amounts of unmethylated control to be added per sample are so low that it is practically best to pre-mix with the water, even if that means slightly different final ratios per sample.

4 Add water (+ unmethylated control) into 0.2 mL PCR strip tubes (if doing 24 samples) or a plate - usually starting with the highest amount of water going down the sample list, column after column. Cross-check for each with the order on the plate schematic, re sample ID, required volume and correct well position.

5 Add DNA samples in the same sample order, column by column, starting from low to high volume. Cross-check for each with the order on the plate schematic, re sample ID, required DNA volume and correct well position. It is a good practice to cover the completed plate columns as going forward. Keep DNA samples On ice.

MspI Digestion

1h

6 Thaw MspI Buffer Mix at Room temperature. Mix MspI Buffer Mix by vortexing, spin and place On ice.

7 Spin down the MspI Enzyme Mix and place On ice.

8 Prepare a master mix by combining MspI Buffer Mix and MspI Enzyme Mix in a 0.2ml PCR tube within an 8-tube PCR strip, according to the volumes shown in Table 1. Mix by pipetting, spin down briefly and immediately place On ice.

Table 1. MspI Master Mix (advisable extra volume included for high sample number preps).

	A	B	C	D	E
	REAGENT	**1X RXN VOLUME**	**48X RXN VOLUME (55)**	**96X RXN VOLUME (110)**	**STORAGE**
	MspI BUFFER MIX (BLUE)	1.0 µL	54 µL	110 µL	–20°C
	MspI ENZYME MIX (BLUE)	0.5 µL	27 µL	55 µL	–20°C

9 Distribute equal amounts of master mix to the 8 tubes in the PCR strip for multichannel pipetting.

10 Add 1.5 µL of MspI Master Mix to each sample tube for a total of 10 µL (with a reliable 0.5-10 µL multichannel and low retention tips). Mix by pipetting (increase pipette volume for the mixing), spin down and place On ice.

11 Place the tubes in a thermal cycler programmed to run Program 1 (MspI Digestion): 1h

- 37 °C – 01:00:00,
- hold at 4 °C.

12 In the meantime take out to thaw at RT the water and buffers for adapter ligation – see **Adaptor Ligation**.

13 Remove the tubes from the thermal cycler, spin to collect condensation and place On ice.

Note

It is OK to freeze and store the samples after this step if necessary.

Adaptor Ligation

40m

14 Thaw Ligation Buffer Mix L1 at Room temperature. Mix well by vortexing, spin and place On ice.

15 Thaw the Ligation Adaptor Plate On ice, spin down, and return to ice.

Note

Important: Do not warm Ligation Adaptor Mixes above Room temperature. Heating will severely degrade performance.

16 Spin down the Ligation Enzyme Mix L3 and place On ice or in a cooling rack.

17 Pierce the Ligation Adaptor Plate seal with the tips and aspirate 3 µL (the entire amount) of the Ligation Adaptor Mix L2 (with a 0.5-10ul multichannel and low retention tips). Add one adaptor barcode per sample, noting the adaptor plate positions per each sample. Mix thoroughly by pipetting and keep On ice.

18 Just prior to use, prepare a master mix by combining D1, L1 and L3, according to the volumes shown in Table 2. Mix by pipetting slowly, without introducing bubbles, spin and place On ice. Use the master mix immediately.

Note

L1 is extremely viscous. Pipet this reagent slowly and mix thoroughly. Ensure it is well mixed after thawing, and that the Ligation Master Mix and ligation reactions are well-mixed.

Table 2. Ligation Master Mix (advisable extra volume included for high sample number preps)

	A	B	C	D	E
	REAGENT	**1X RXN VOLUME**	**48X RXN VOLUME (55)**	**96X RXN VOLUME (105)**	**STORAGE**
	NUCLEASE-FREE WATER (GREEN: D1)	2.0 µL	110 µL	210 µL	-
	LIGATION BUFFER MIX (YELLOW: L1)	4.0 µL	220 µL	420 µL	–20°C
	LIGATION ENZYME MIX (YELLOW: L3)	1.0 µL	55 µL	105 µL	–20°C

19 Add 7 µL Ligation Master Mix to each reaction tube for a total of 20 µL (with a multi-dispenser or a reliable 0.5-10µl/2-20µl multichannel and low retention tips). Mix thoroughly by pipetting slowly and gently, spin down and place on ice. Proceed immediately with the incubation.

20 40m

Place the tubes in a thermal cycler programmed to run Program 2 (Ligation):

- 25 °C – 00:30:00,
- 70 °C – 00:10:00,
- hold at 4 °C.

21 Remove the tubes from the thermal cycler, spin to collect condensation and place On ice.

Note

It is OK to store the samples at 4 °C O/N after this step.

Final Repair

20m

22 Remove the Magnetic Bead Solution and Binding Buffer 1 from 4 °C and place at Room temperature for use in the next step.

23 Thaw Final Repair Buffer Mix (FR1) at Room temperature. Mix by vortexing, spin down and place On ice.

24 Spin down Final Repair Buffer Enzyme (FR2) and place On ice.

25 Prepare a master mix by combining FR1, FR2 and Nuclease-free Water (D1) according to the volumes shown in Table 3.

Table. 3 Final Repair Master Mix (advisable extra volume included for high sample number preps)

	A	B	C	D	E
	REAGENT	**1X RXN VOLUME**	**48X RXN VOLUME (54)**	**96X RXN VOLUME (100)**	**STORAGE**
	FINAL REPAIR BUFFER MIX (PURPLE: FR1 ver 4)	6 µL	324 µL	600 µL	–20 °C
	FINAL REPAIR ENZYME MIX (PURPLE: FR2)	0.5 µL	27 µL	50 µL	–20 °C
	NUCLEASE-FREE WATER (GREEN: D1)	13.5 µL	729 µL	1350 µL	–

26 Add 20 µL of the Final Repair Master Mix to each sample for a total of 40 µL (use a reliable multichannel pipette and low retention tips). Mix by pipetting each time, spin down and place On ice.

27 Place the tubes in a thermal cycler pre-heated to 60 °C (if possible) and programmed to run 20m Program 3 (Final Repair):

- 60 °C – 00:10:00,
- 70 °C – 00:10:00,
- hold at 4 °C.

28 Remove the tubes from the thermal cycler, spin to collect condensation and place On ice.

Note

Samples can be stored O/N at -20 °C before continuing with purification.

DNA Purification

40m

29 Remove Magnetic Bead Solution and Binding Buffer 1 from storage and place on bench top. Ensure they have reached Room temperature before use.

Note

If continuing with **Bisulfite Conversion** immediately after purification, prepare the reagents (steps 51-55) at the start of the purification, since dissolving the bisulfite reagent can take 1-2 hours.

30 Once warmed to room temperature, mix Binding Buffer 1 by inversion until homogenized.

31 Vortex Magnetic Bead Solution until homogenized.

32 Prepare a master mix of Magnetic Bead Binding Solution 1 (MBBS1) as directed in Table 4.
Binding
Buffer 1 is supplied in 5.4 mL bottles suitable for a 48 sample mix, use two bottles for a full plate.

Note

MBBS1 should be prepared fresh on the day of use. Do not store for longer than 1 week.

Table. 4 Magnetic Bead Binding Solution 1 Master Mix.

	A	B	C	D	E

	A	B	C	D	E
	REAGENT	**1X RXN VOLUME**	**48X RXN VOLUME (54)**	**96X RXN VOLUME (108)**	**STORAGE**
	BINDING BUFFER 1	100 µL	5.4 mL	2 x 5.4 mL	4 °C
	MAGNETIC BEAD SOLUTION	2.0 µL	108 µL	2 x 108 µL	4 °C

33 Vortex MBBS1 master mix thoroughly to ensure the beads are homogenized in solution.

34 At Room temperature, add 10 µL of Ultra Pure water to each sample for a total of 50 µL (with a reliable 0.5-10 µl/2-20 µl multichannel and low retention tips or an Eppendorf dispenser, without touching the wells, 1.0 ml tip).

35 Add 100 µL of MBBS1 master mix to each 0.2 mL tube/well containing 50 µL sample for a total of 150 µL (with a dispenser, 2-10 ml tip). Mix by pipetting with a multichannel pipette set at 130 µL and centrifuge briefly.

36 Incubate at Room temperature for 00:20:00 (can be longer if a break is needed). 20m

37 Prepare a fresh stock of 80% ethanol, using the Ultra Pure water provided with the kit. Mix by inversion and place at Room temperature.

Note

Suggested: 40 mL Et-OH + 10 mL H_2O for a full plate.

38 Transfer tubes/plate to a magnetic separation plate and incubate at Room temperature for 00:10:00 or until the solution of beads is completely clear. 10m

39 Keeping the tubes on the magnet, carefully remove the supernatant and discard it – with a manual multichannel pipette set at 140 µL.

40 With the tubes/plate still on the magnet, carefully add 200 µL of 80% ethanol wash to the wells without disturbing the bead pellet (with a dispensing multi-channel set at 1200 µL, 6x strips/half a plate at a time).

41 Remove and discard the 200 µL 80% ethanol wash, avoiding aspiration of the bead pellet (with a manual 20-200 µL multichannel, regular tips). Complete this step as quickly as possible, ideally within 2 minutes.

42 Repeat Steps 40 and 41 to perform 2 x 200 µL 80% ethanol washes in total. Remove as much of the final wash as possible – first with a 20-200 µL tip, and a second time with a 10/20 µL
tip if necessary.

43 Air dry the bead pellets for 5-10 minutes at Room temperature.

Note

Ensure the tubes are dry without visible ethanol droplets before continuing the protocol. Aspirating any remaining ethanol on the sides and bottom of the wells with a 0.5 – 10ul multichannel (without touching the pellet) can help remove residual droplets and help not over-dry the pellet. Ideally the pellet should be matte (not shiny) and not cracking, i.e. over-dried.

44 Remove the tubes from the magnet.

45 Add 11 µL of Elution Buffer directly onto the bead pellet.

Note

Use a multi-dispenser and aim at the pellet without touching the well, also helps avoid wells with visible ethanol whilst not letting the other wells over-dry.

46 Mix thoroughly with a 0.5-10µl multichannel to ensure all beads are resuspended. Don't centrifuge if there are drops on the walls!

Note

Beads might be stuck to the walls and quite high above the buffer. Make sure all beads are dissolved in the buffer before ejecting the tips.

47 Incubate at Room temperature for at least 00:05:00 to elute the TrueMethyl converted DNA from the beads. 5m

48 Seal the plate and centrifuge briefly to collect all drops at the bottom of the tubes.

49 Transfer tubes/plate to a magnetic separation plate and incubate at Room temperature for 5-10 minutes or until the solution of beads is completely clear.

50 Removing the seal one column/strip at a time, carefully aspirate 10 µL of the eluate, ensuring as few beads as possible are carried over, and transfer to a new microcentrifuge plate/tubes (use a 0.5-10uL multichannel with low retention tips). Keep at Room temperature if continuing with section **Bisulfite Conversion**. Can store in fridge or freezer if an Overnight break is needed. 5m

Note

Eluting can be slow and may require repetitions if beads are sucked in the tip. The elution volume is very low and keeping the seal on ensures that there is no evaporation in the remaining wells, which facilitates the transfer of equal amounts of eluate/yield. Sealing or covering the plate/strips with the ready eluate is also advisable to avoid mistakes.

Bisulfite Conversion

2h 25m

51 Set a heat block or heated orbital incubator to 70 °C.

52 Remove Bisulfite Diluent and Bisulfite Reagent aliquots from storage and place on bench top. Remove 1 aliquot of Bisulfite Reagent for every 25 reactions to be processed and spin quickly to remove any powder from cap (i.e. a full 96-well plate uses 4 Bisulfite Reagent vials).

53 Prepare Bisulfite Reagent Solution by adding 700 µL of Bisulfite Diluent to each aliquot of Bisulfite Reagent.

Note

Note: Each aliquot of Bisulfite Reagent Solution is sufficient for up to 25 samples (kit manual says 20 but this is not accurate). A fresh aliquot of BS solution should be prepared each time the kit is used and disposed of immediately after use.

54 Seal the lid of each aliquot with Bisulfite Reagent Solution tightly.

55 

Incubate the aliquots of Bisulfite Reagent Solution for minimum 00:30:00 at 70 °C and 3h 30m vortex regularly until the Bisulfite Reagent Solution is completely (or nearly completely) dissolved. This step can take up to 03:00:00 for the reagent to dissolve.

56 Spin down Bisulfite Reagent Solution briefly and place at Room temperature.

57 Ensure purified DNA samples from previous step are at Room temperature before proceeding.

58 Prepare Bisulfite Conversion Reaction mix by adding 30 µL of Bisulfite Reagent Solution to each 10 µL of DNA for a total of 40 µL (with multi-dispenser, 1 mL tips).

59 Mix by pipetting with a 20-200 µl multichannel, spin down and place at Room temperature.

60 Place the tubes in a pre-warmed thermal cycler programmed to run Program 5 (Bisulfite Conversion): 1h 55m

- 99 °C – 00:10:00,
- 65 °C – 01:45:00,
- hold at 20 °C.

Note

Optional stopping point: You may hold samples at Room temperature (20°C) for up to 16:00:00. Do not store below 20 °C.

61 Once the bisulfite conversion is complete, centrifuge samples briefly to collect the solution at bottom of the tubes.

62 Continue to **Bisulfite-Converted DNA Desulfonation and Purification**.

Bisulfite-Converted DNA Desulfonation and Purification

42m

63 Remove Desulfonation Buffer, Binding Buffer 2, Magnetic Bead Solution and Elution Buffer from storage and place at Room temperature for a minimum of 00:30:00 before use. 30m

64 Prepare a fresh stock of 80% Ethanol. Mix by vortexing or inversion.

65 Mix Binding Buffer 2 by inversion until homogenized.

66 Vortex Magnetic Bead Solution until homogenized.

67 Prepare a master mix of Magnetic Bead Binding Solution 2 (MBBS2) as directed in Table 5. Each kit BB2 bottle is filled with exactly 9 mL solution ready to use for a 48 sample prep; use two bottles for a full plate.

Table. 5 Magnetic Bead Binding Solution 2 Master Mix.

	A	B	C	D	E
	REAGENT	**1X RXN VOLUME**	**48X RXN VOLUME**	**96X RXN VOLUME**	**STORAGE**
	BINDING BUFFER 2	160 µL	9 mL	2 x 9 mL	4 °C
	MAGNETIC BEAD SOLUTION	1.92 µL	108 µL	2 x 108 µL	4 °C

Note

- MBBS2 should be prepared fresh on the day of use. Do not store for longer than 1 week.
- MBBS2 is a viscous solution. Pipet this reagent slowly and mix thoroughly. Ensure that MBBS2 and the MBBS2-sample mix are well-mixed.

68 Vortex MBBS2 thoroughly to ensure the solution is homogenous before aliquoting.

69 Carefully add 160 µL of MBBS2 to each tube containing 40 µL of bisulfite converted sample for a total of 200 µL (with multi-dispenser, 5 mL tip). Mix thoroughly by pipetting slowly and gently, spin down and place at Room temperature.

70 Incubate at Room temperature for 00:30:00 to 01:00:00, vortexing if necessary, 1h 30m or alternating plate position by rotation upwards-downwards after sealing VERY WELL.

71 Prepare desulfonation buffer by adding 7 mL 100% ethanol to 3 mL of Desulfonation Buffer for up to 48 samples, or 14 mL EtOH + 6 mL Desulfonation buffer for up to 96 samples, respectively.

72 Centrifuge briefly to collect the solution at the bottom of the tubes/wells. To bring down the beads from the liquid remaining above the magnet, centrifuge at 2200 rcf / 4700 rpm, Room temperature, 00:02:00. 2m

73 Place the tubes/plate onto the magnet and incubate at Room temperature for 10-15 minutes to completely clear the solution of beads.

74 Carefully remove the supernatant and discard it, without discarding any beads.

75 Add 200 µL of 80% Ethanol to each sample tube/well (with a dispensing multichannel up to 1200 µL), 6 columns at a time).

Note

Do NOT resuspend the beads in the Ethanol.

76 Carefully remove the 80% Ethanol wash and discard it. Remove as much of the wash as possible.

77 Add 200 µL of Desulfonation Buffer with added Ethanol onto the bead pellet. Incubate at Room temperature for 00:05:00. 5m

Note

Be sure that the Ethanol has been added to the Desulfonation Buffer, as described in step 72. Mixing the beads with Desulfonation solution (as instructed in the manufacturer's protocol) will result in dehydrating and precipitating the beads irreversibly and decreasing the yield.

78 Carefully remove 200 µL of the Desulfonation Buffer and discard it. Remove as much of the Desulfonation Buffer as possible without disturbing the bead pellet.

79 With the tubes/plate still on the magnet, add 200 µL of 80% Ethanol to each sample tube without disturbing the bead pellet and incubate for 00:00:30 (with a dispensing multichannel set at 1200 µL, 6x strips/half a plate at a time). 30s

Note

When doing 24 - 96 samples the given 30 sec incubation time is surpassed during the pipetting alone and must be proceeded to next step immediately.

80 Remove the 200 µL 80% Ethanol wash and discard it, avoiding aspiration of the bead pellet (with a manual 20-200 µL multichannel, regular tips). This step must be completed as quickly as possible (ideally within 00:02:00) 2m

81 Repeat steps 79–80 to perform 2 x 200 µL 80% Ethanol washes in total. Remove as much of the final wash as possible – first with a 20-200 µL tip, and a second time with a 10/20 µL tip, if necessary.

Note

Note: All Ethanol washes in this purification in the original Tecan protocol are performed with resuspension of beads in the ethanol. This, however, has been highly advised against from most bead manufacturers and inevitably leads to bead precipitation and sample loss.

82 Air-dry the the beads on the magnet for 5-10 minutes. Inspect each tube carefully to ensure that all of the Ethanol has evaporated – the pellet should be non-shiny (matt look) when it is ready. Using a pipette with 10 µl tips can help with aspirating Ethanol drops while still on the magnet.

83 Remove the tubes/plate from the magnet.

84 Quickly add Elution Buffer with a multi-dispensing pipette aiming directly onto the bead pellet:

84.1 For Library Amplification Optimzation with qPCR (recommended), resuspend the beads in 25 µL of Elution Buffer.

84.2 If qPCR optimization is not required, resuspend the beads in 21 µL of Elution Buffer.

85 Mix and resuspend completely the beads in the Elution Buffer with a multichannel pipette pipette – make sure all beads are removed from the well walls and brought in solution.

86 Incubate at Room temperature for 00:05:00 to elute the bisulfite converted DNA from the beads. 5m

87 Seal the plate and centrifuge briefly to collect the samples at the bottom of the tubes/wells.

88 Place the tubes/plate onto the magnet and incubate at Room temperature for 5-10 minutes to completely clear the solution of beads.

89 Opening the seal one column/strip at a time, carefully transfer eluate into a fresh 0.2 mL PCR plate:

89.1 For Library Amplification Optimzation with qPCR (recommended first time only), transfer 24 µL of eluate.

89.2 If qPCR optimization is not required, transfer 20 µL of eluate.

Note

Aspirate directly 20/24 µL (riskier), or aspirate 10 µL twice, with thin 10 µL tips - the latter can make easier the handling of the last few microliters nearer the beads during the second
aspiration.

Eluting can be slow and may require repetitions if beads are sucked in the tip. The seal ensures there is no evaporation from the remaining wells and facilitates the transfer of equal volumes of eluate/yield. Sealing or covering the plate/strips with the ready eluate is also advisable.

Library Amplification Optimization with qPCR (optional)

3m 45s

90

Note

Note: qPCR optimization should be performed when running the kit for the first time, when using a new sample type or input, and any time degraded or low input samples are used.

Prepare a master mix by combining P2, P3 and 20x EvaGreen in an appropriately sized capped tube according to the volumes shown in Table 6. Add P3 at the last moment and mix well by pipetting, taking care to avoid bubbles. Spin down and place On ice.

Table. 6 Library Amplification qPCR Master Mix (with extra volumes for high sample number).

	A	B	C	D	E
	REAGENT	**1X RXN VOLUME**	**4X RXN VOLUME**	**96X RXN VOLUME**	**STORAGE**
	AMPLIFICATION PRIMER MIX (RED: P2 ver 8)	1.0 µL	4.2 µL	100 µL	−20 °C
	AMPLIFICATION ENZYME MIX (RED: P3 ver 3)	4.5 µL	18.9 µL	450 µL	−20 °C
	20X EvaGreen	0.5 µL	2.1 µL	50 µL	−20 °C

91

Aliquot 6 µL of master mix per sample into an appropriate optically clear PCR plate. Spin down and place On ice.

92 On ice, add 4 µL of sample to each 6 µL of Library Amplification qPCR Master Mix for a total of 10 µL per reaction. Keep the remaining 20 µL of sample On ice.

93 Mix well by pipetting, spin down and place On ice.

94 Perform qPCR with the following cycling conditions: 3m 45s

- 95 °C – 00:02:00,
- 35 x (95 °C – 00:00:15, 60 °C – 00:01:00, 72 °C – 00:00:30).

95 Examine the log fluorescence vs. cycle number plot from the qPCR system to determine the appropriate number of library amplification cycles. Select a cycle number within the middle to late exponential phase of the amplification plot. Examples are provided in Figure 8 of the original Tecan kit manual.

Library Amplification

8m 45s

96 Remove Agencourt Beads from 4 °C and DR1 from -20 °C and place at Room temperature for use in the next step.

97 Spin down Amplification Enzyme Mix (P3) and place On ice.

98 Thaw Amplification Primer Mix (P2) at Room temperature. Mix by vortexing, spin down and place On ice.

99 Prepare a master mix by combining P2 and P3 according to the volumes shown in Table 7. Mix well by pipetting, taking care to avoid bubbles, spin down and place On ice.

Table. 7 Library Amplification Master Mix (with extra volumes for high sample number).

	A	B	C	D	E
	REAGENT	**1X RXN VOLUME**	**48X RXN VOLUME (50)**	**96X RXN VOLUME (100)**	**STORAGE**
	AMPLIFICATION PRIMER MIX (RED: P2 ver 8)	5.0 µL	275 µL	500 µL	−20 °C
	AMPLIFICATION ENZYME MIX (RED: P2 ver 3)	25.0 µL	1.375 µL	2.500 µL	−20 °C

100 On ice, add 30 µL of Amplification Master Mix to each sample for a total of 50 µL.

101 8m 45s

Place tubes in a pre-warmed thermal cycler programmed to run Program 6 (Library Amplification):

- 95 °C – 00:02:00,
- N(95 °C – 00:00:15,
- 60 °C – 00:01:00,
- 72 °C – 00:00:30),
- 72 °C – 00:05:00,
- hold at 10 °C.

102 Remove the tubes from the thermal cycler, spin to collect condensation and place On ice.

Note

Optional stopping point: Store samples at -20 °C.

Amplified Library Purification

20m

103 Ensure the Agencourt beads and DR1 Resuspension Buffer have reached Room temperature before proceeding.

104 Resuspend the beads by inverting and tapping the tube. Ensure the beads are fully resuspended before adding to samples. After resuspending, do not spin the beads.

105 Add 50 µL (1 volume) of the bead suspension to each reaction. Mix thoroughly by pipetting 10 times.

106 Incubate at Room temperature for 00:10:00. 10m

107 Prepare fresh 80% EtOH solution in a 50 mL falcon tube – 40 mL for a full plate, 20 mL for a half plate.

108 Transfer the tubes to the magnet and let stand 00:05:00 to completely clear the solution of beads. 5m

109 If doing your own QC, prepare a plate for Tapestation aliquots and take out Tapestation kit reagents to equilibrate to Room temperature (see Library Quality Control step).

110 Carefully remove 90 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

Note

The binding buffer can be kept in another plate until the QC results are available (it is possible to retrieve a sample from there if necessary to save an experiment).

The beads should not disperse but have to remain on the walls of the tubes. Significant loss of beads at this stage will impact the final yield, so ensure beads are not removed with the binding buffer or the wash.

111 With samples still on the magnet, add 200 µL of of 80% ethanol to each sample tube and incubate for 00:00:30 (with a dispensing multichannel set at 1200 µL, 6x strips/half a plate at a time). 30s

> **Note**
>
> When doing 24 - 96 samples the 30s incubation time is surpassed during the pipetting alone and must be proceeded to next step immediately.

112 Remove the 200 µL of 80% Ethanol wash and discard it (with a manual 20-200 µL multichannel, regular tips). This step must be completed as quickly as possible (ideally within 2 minutes).

113 Repeat steps 111 and 112 for a total of two washes. Remove all remaining traces of ethanol after the second wash.

> **Note**
>
> **Note**: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.

114 Air dry the beads on the magnet for 5-10 minutes. Inspect each tube carefully to ensure that all of the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing, but it is also critical to not let beads over-dry as this reduces yield (they have over-dried when they crack).

115 Remove the tubes from the magnet and add 20 µL DR1 Resuspension buffer to the dried beads (with dispenser aiming at the beads).

116 Mix thoroughly with a multichannel pipette, set at 10 µL, to ensure all beads are resuspended – if the beads have cracked this may require mixing for a few minutes. Take any beads remaining on the sides and make sure all beads are covered in solution.

117 Seal the plate/close the tubes and centrifuge briefly to collect sample at bottom of the tubes/wells.

118 Transfer the tubes to the magnet and let the samples stand for 00:05:00 or until the solution is completely clear of beads. 5m

119 Carefully remove the seal one column/strip at a time, and aspirate 19 µL of the eluate, ensuring as few beads as possible are carried over, and transfer to a fresh plate / set of PCR tubes. Keep the eluate on ice.

Note

Pipetting options: Aspirate directly 19 µL (riskier), or aspirate 9.5 µL twice, with thin 10 µL low retention tips - the latter can make easier handling of the last few microliters nearer the beads during the second aspiration.
Eluting can be slow and may require repetitions if beads are sucked in the tip. The seal ensures there is no evaporation in the remaining wells and facilitates the transfer of equal amounts of eluate/yield. Sealing or covering the plate/strips with the ready eluate is also advisable to avoid mistakes.

120 Once the elution is complete, proceed with preparing library aliquots for a Tapestation QC run in the next section **Library Quality Control (QC)**. If QC is not planned soon, seal the library plate well and store at -20 °C .

Prepare Library Aliquots For QC

121 Aliquot 1 µL of nuclease-free water in the wells of a 96-well plate, matching the number and positions of eluted libraries (this is for a 1 in 2 dilution, respectively, higher dilutions can also be used, depending on QC equipment sensitivity).

Note

Be quick with this step because of evaporation.

122 From the elution plate from Step 120, take 1 µL of purified library with a multichannel pipette, transfer to the QC plate with water and mix.

123 The plate with purified libraries can now be sealed and stored long term at -20 °C.

124 If performing a Tapestation run, continue with the High Sensitivity DNA 1000 Tapestation kit protocol (procedure not described here). If taking the DNA to be measured elsewhere, seal the QC plate and take to the facility. The QC plate can be stored at -20 °C, although evaporation can occur during long term storage.