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HyDrop v2 Bead Generation & Ligation Barcoding V.1

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We use this protocol and it's working

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Abstract

This protocol details hydrogel bead generation and barcoding for HyDrop v2. The barcodes are build by split-pool and ligation strategy (3×96). Here, we will create an emulsion of acrylamide monomers in a carrier oil containing TEMED. The monomer droplets will polymerise and form hydrogel beads. Ideally, you have a bead stock of around 3 mL of beads before you barcode, but 2 mL can work as well. It is best to produce the beads in one single run, from a single bead mixture to prevent disparities in sizes and/or primer concentration within the bead from occurring.

Attachments

HyDrop-buffer-listin...	plate-1-Fwd-96.xls	plate-1-Rev-96.xls	plate-2-Fwd-96.xls	plate-2-Rev-96.xls	plate-3-ATAC-96.xls	acrydite primer.xlsx
12KB	35KB	37KB	36KB	36KB	37KB	8KB

Materials

- 96-well plate
- Cassette HyDrop-v2-ATAC-plate
- Tris
- Tris-HCl
- NaCl
- NaOH
- $MgCl_2$
- Tween-20
- dH_2O
- Oligo-Mix BC1
- ATP
- T4 ligase
- Oligo-Mix B
- Brij-35
- TX-100

Before start

On the days before the barcoding prepare the primer cassette for the stage 1 and stage 2 barcoding.

Hydrogel bead generation

1 **Prepare monomer mix**. The volume used here will approximately equate the final volume of dense bead stock that you will produce. The beads will be slightly larger than the droplets generated, but you will also generate small losses during the wash steps.

	A	B	C	D	E
		Vol (uL)	**Stock**	**Final**	**Unit**
	TBSET	110	1	0.1	X
	Acrylamide	269.5	40	10	%w/v
	N,N'-Bis(acryloyl)cystamine	45.83	4.8	0.2	%w/v
	Ammonium persulfate	66	10	0.6	%
	Acrydite primer bead handle	44	100	4	uM
	Nuclease free water	564.67			
	TOTAL	1100			

The volumes used here equals to 10% acrylamide (%T) and 2% bisacryloylcystoylamine (%C). Note that N,N'-Bis(acryloyl)cystamine is dissolved w/v in methanol.

2 Add 1000 µL of filtered HFE-7500 Novac oil to the syringe (3 mL) and add the monomer mix and layer it on top of the HFE and prepare the syringe for injection.

3 Mix 2900 µL of the EA008 surfactant with 14.5 µL of TEMED (=0.5% TEMED). Mix the content well.

4 Prepare two 1.5 mL Eppendorf collection tubes and put 200 µL of mineral oil in the tubes. This will also serve as protection against evaporation during the overnight incubation afterwards.

5 Run the droplet generation for the preparation of the hydrogel beads.

Flow rates:

	A	B	C
		start	**stable**
	monomer mix	400	400
	oil	400	750

Collect the emulsion for 55 minutes in two 1.5 mL Eppendorf tubes.

6 Remove excess oil from the bottom of the collection tubes and double check if the layer of mineral oil is on top of the emulsions.

7 Put the emulsions on a 65°C heat block for 14 hours. Leave beads at 12°C until they are washed.

8 Remove top layer of mineral oil and bottom layer of EA008.

9 Wash the beads:
- 3 times with 20% PFO in HFE-7500.
- 3 times with 1% SPAN80-Hexane.
- 3 times with with TBSET buffer.
- 3 times with TET buffer and combine all aliquots in the last wash.

10 Filter the beads through a 70 um filter to exclude contamination of large beads.

11 Store the beads at 4°C.

Oligo preparation (done at least a day prior to barcoding)

12 Take 3 fresh 96 well plates. And label as:
- Cassette 1-HyDrop-v2-ATAC-plate (200 µM)
- Cassette 2-HyDrop-v2-ATAC-plate (200 µM)
- Plate 3-HyDrop-v2-ATAC-plate (500 µM)

13 For **Cassette 1-HyDrop-v2-ATAC-plate**:
Mix 20 µL of primers from plate-1-Fwd-96 of the [M] 400 micromolar (µM) stock primer plate to 20 µL of primers from plate-1-Rev-96 of the

[M] 400 micromolar (µM) stock primer.

14 For **Cassette 2-HyDrop-v2-ATAC-plate**:
Mix 20 µL of primers from plate-2-Fwd-96 of the [M] 400 micromolar (µM) stock primer plate to 20 µL of primers from plate-2-Rev-96 of the [M] 400 micromolar (µM) stock primer.

15 For **Plate 3-HyDrop-v2-ATAC-plate**:
Aliquot 18 µL of primers from plate-3-ATAC-96 of the [M] 500 micromolar (µM) stock primer plate to a new plate.

Barcoding Round 1

3h 38m

16
- Spin down the beads stored in TET buffer.
- Remove supernatant and wash the beads one more time with TET buffer.
- Centrifuge at 1000 x g, 00:03:00, brake 8, and discard the supernatant.

3m

17 Prepare the pre-Ligation buffer (PL buffer) below and filter with 0.2 µm filter (if not available).

	A	B	C	D	E	F
	Component	**Stock**	**Unit**	**Final**	**Unit**	**Volume**
	Tris 8.0	1000	mM	10	mM	5000
	NaCl	5000	mM	30	mM	3000
	$MgCl_2$	1000	mM	1	mM	500
	Tween-20	10	%	0.1	%	5000
	dH_2O					486500
	TOTAL					500000

18
- Wash the beads with PL buffer 2X times.
- Centrifuge @ 1000 x g, 00:01:00, brake 8, discard the supernatant.

1m

19 Using Hamilton, aliquot 22 µL of the compacted beads to each of the wells of 96 well plate.
Total compacted bead volume: 22×1.2×96 = 2534.4 µL .

20 Prepare the 2X Ligation-primer buffer (T4) below:

	A	B	C	D	E	F	G
	Component	**Stock**	**Unit**	**Final**	**Unit**	**Volume**	**96×1.2**
	Tris 7.5	1000	mM	100	mM	2	230.4
	$MgCl_2$	1000	mM	20	mM	0.4	46.08
	Cassette 1-HyDrop-v2-ATAC	**200**	**µM**	**40**	**µM**	**4**	**NO ADD**
	dH_2O					13.6	1566.7
						20	16 µl per reaction
							1843.2 µl

µl per reaction

21 Using Hamilton, aliquot 16 µL of the 2X ligation-primer buffer to the PCR plate.

22
- Add 4 µL (Hamilton) of **Cassette 1-HyDrop-v2-ATAC-plate** [M] 200 micromolar (µM) , spin and mix well by vortexing and place on PCR block pre-heated to 75 °C (with heated lid to 105 °C).

23 Heat the thermoblock to 75 °C and keep it ready.

24
- Heat the mix at 75 °C for 00:03:00 on the PCR and transfer and switch off the heat block to cool to Room temperature , to anneal the oligo to bead (~2.5 hours).
- Every 30 mins vortex the plate at max speed (2000x RPM) for 00:00:30 to resuspend the beads. Keep on looking after switching off for 02:30:00 (reaches 33 °C).

2h 33m 30s

- After 02:00:00 , leave the plate at Room temperature .

Note

Take it from block and keep it at Room temperature for 00:10:00 .

25 In the meantime, prepare the 1X ligase mix below (T4):

	A	B	C	D	E	F	G
	Component	**Stock**	**Unit**	**Final**	**Unit**	**Volume**	**96×1.05**
	Tris 7.5	1000	mM	50	mM	0.5	52.8
	$MgCl_2$	1000	mM	10	mM	0.1	10.6
	ATP	10	mM	1	mM	5.1	538.6
	T4 ligase	400	U/µl	20	U/µl	2.5	252
	dH_2O					1.8	181.4
						10	10 µl per reaction
							1035.4 µl

26
- Using Mantis, aliquot 10 µL of the ligase mix to the 96 well plate, after sealing the plate vortex the content in a vortex shaker, centrifuge at 1000 x g, 00:00:30 .
- On thermoblock shake 2000 rpm, 00:00:30 , to mix the beads.

30s

27 Perform ligation at 25 °C for 01:00:00 with a shaking of 1000 rpm (every minute shake at 1600 rpm, 00:00:30).

1h

28 Perform Overnight incubation at 16 °C on a thermoblock with shaking of 1000 rpm, 00:00:30 . <Pause point - overnight>

Barcoding Round 2

3h 59m 30s

29 Remove the plate from 16 °C incubation and leave at 4 °C till the next processing step (at least 30 min).

30 Prepare 2X ligation-primer buffer.

	A	B	C	D	E	F	G
	Component	**Stock**	**Unit**	**Final**	**Unit**	**Volume**	**96×1.2**
	Tris 7.5	1000	mM	100	mM	2	230.4
	$MgCl_2$	1000	mM	20	mM	0.4	46.1
	dH_2O					13.6	1566.7
						20	16 µl per reaction
							1843.2 µl

31 Aliquot 16 µL of the above 2X ligation-primer buffer mix to a fresh 96 well plate.

32
- Add 4 µL of **Cassette 2-HyDrop-v2-ATAC-plate** [M] 200 micromolar (µM) .
- Mix well (vortex) and place on a PCR block pre-heated to 75 °C (lid 105 °C).

33 2h 33m
- Heat the plates at 75 °C for 00:03:00 , transfer the plate to thermoblock at 75 °C and switch off the heat block to cool to Room temperature , to anneal the oligos.
- Keep on looking after switching off for 02:30:00 .
- After 2.5 hours, leave the plate at Room temperature .

Note

Take it from block and keep it at Room temperature for 00:10:00 .

34 20m
- In the meantime, perform the STOP-25 cleanup on the Hamilton.
- Transfer to a 50 ml falcon.
- Incubate for 00:20:00 after collection.

35 Spin the falcon at 3220 x g, 00:03:00 s and braking of 7. Transfer the beads to 15 ml falcon. 3m

36 Wash the beads with Stop-10 buffer. Centrifuge @ 1000 x g, 00:01:00 and discard the supernatant. 1m

37 Wash 3X times with TET buffer. Centrifuge @ 1000 x g, 00:01:00 and discard the supernatant. 1m

38 Wash the beads with PL buffer 2X times. Centrifuge @ 1000 x g, 00:01:00 and discard the supernatant. Set the level of beads to 2534.4 µL. 1m

39 Using Hamilton, aliquot 22 µL of the compacted beads to each of the wells of 96 well plate of a new deep well 96 well plate.

40 After the completion of the oligo annealing, spin the plate to collect condensate and using Hamilton transfer the contents of the annealed oligo cassette plate to the bead plate.

41 Prepare the ligase mix below:

	A	B	C	D	E	F	G
	Component	**Stock**	**Unit**	**Final**	**Unit**	**Volume**	**96×1.1**
	Tris 7.5	1000	mM	50	mM	0.5	52.8
	$MgCl_2$	1000	mM	10	mM	0.1	10.6
	ATP	10	mM	1	mM	5.1	538.6
	T4 ligase	400	U/µl	20	U/µl	2.5	252
	dH_2O					1.8	181.4
						10	10 µl
							1035.4

42 Using Mantis, aliquot 10 µL of the ligase mix to the 96 well plate, after sealing the plate vortex the content in a vortex shaker, centrifuge 1000 x g, 00:00:30. On thermoblock shake 1600 rpm, 00:00:30, to mix the beads. 30s

43 Perform ligation at 25 °C for 01:00:00 with 1000 rpm rotation shaking (every minute shake at 1000 rpm, 00:00:30). 1h

44 Perform Overnight incubation at 16 °C on a thermoblock with 1000 rpm rotation shaking. <Pause point - overnight>

Barcoding Round 3 1h 32m 30s

45 Remove the plate from 16 °C incubation and leave at 4 °C till ready for subsequent processing step.

46 Perform the STOP-25 cleanup on the Hamilton. Transfer to a 50 ml falcon. Incubate for 00:20:00 after collection. 20m

47 Spin the falcon at 3220 x g, 00:03:00 and braking of 7. Transfer the beads to 15 ml falcon. 3m

48 Wash the beads with Stop-10 buffer. Centrifuge @ 1000 x g, 00:03:00 and discard the supernatant. 3m

49 Wash 3X times with TET buffer. Centrifuge @ 1000 x g, 00:03:00 and discard the supernatant. 3m

50 Wash the beads with PL buffer 2X times. Centrifuge @ 1000 x g, 00:03:00 and discard the supernatant. Set the level of beads to 2534.4 µL . 3m

51 Using Hamilton, aliquot 23 µL of the compacted beads to a fresh 96 deep well plate.

52 Prepare the 2X Ligation buffer below:

	A	B	C	D	E	F	G
	Component	**Stock**	**Unit**	**Final**	**Unit**	**Volume**	**96×1.3**

	A	B	C	D	E	F	G
	Tris 7.5	1000	mM	100	mM	2	249.6
	$MgCl_2$	1000	mM	20	mM	0.4	49.92
	plate 3-HyDrop-v2-ATAC-plate	**500**	**µM**	**30**	**µM**	**3**	**NO ADD**
	dH_2O					14.6	1822.1
						20	17

53 Using Hamilton, aliquot 17 µL of the 2X ligation-primer buffer to the PCR plate.

54 Add 3 µL of **plate 3-HyDrop-v2-ATAC-plate** [M] 500 micromolar (µM) .

55 Prepare the ligase mix below:

	A	B	C	D	E	F	G
	Component	**Stock**	**Unit**	**Final**	**Unit**	**Volume**	**96×1.1**
	Tris 7.5	1000	mM	50	mM	0.5	52.8
	$MgCl_2$	1000	mM	10	mM	0.1	10.6
	ATP	10	mM	1	mM	5.1	538.6
	T4 ligase	400	U/µl	20	U/µl	2.5	252
	dH_2O					1.8	181.4
						10	10 µl
							1035.4

56 Using Mantis, aliquot 10 µL of the ligase mix to the 96 well plate, after sealing the plate vortex the content in a vortex shaker, centrifuge 1000 x g, 00:00:30 . On thermoblock shake 2000 rpm, 00:00:30 , to mix the beads. 30s

57 Perform ligation at 25 °C for 01:00:00 with rotation mixing of 1000 rpm . 1h

Note

Every minute shake at 1600 rpm, 00:00:30 .

58 Perform Overnight incubation at 16 °C on thermoblock with rotation mixing of 1000 rpm . <Pause point - overnight>

Bead Cleanup 29m

59 Remove the plate from 16 °C incubation and leave at 4 °C till ready for subsequent processing step.

60 Perform the STOP-25 cleanup on the Hamilton. Transfer to a 50 ml falcon. Incubate for 00:20:00 after collection. 20m

61 Spin the falcon at 3220 x g, 00:03:00 and braking of 7. Transfer the beads to 15 ml falcon. 3m

62 Wash the beads with Stop-10 buffer. Centrifuge @ 1000 x g, 00:03:00 and discard the supernatant. 3m

63 Wash 3X times with TET buffer. Centrifuge @ 1000 x g, 00:03:00 and discard the supernatant. 3m

Denaturation and Final Steps 24m

64 Prepare the following denaturation buffer and filter with 0.2 µm filter.

	A	B	C	D
	Component	**Stock**	**Final**	**Volume**
	Water	-	-	60.5 ml

	A	B	C	D
	NaOH	10 N		938 µl
	Brij-35			1050 µl
				62.5

65 Add 15 mL of denaturation solution to beads and incubate for 00:10:00 on a rotator at Room temperature . 10m

66 Wash the denaturation buffer 3X times with denaturation solution with 15 mL buffer.

67 Add 15 mL of Neutralization solution to beads and incubate for 00:10:00 on a rotator at Room temperature . 10m

68 Wash the beads with Neutralization buffer 1X more time.

69 Wash 3X times with TET buffer and proceed to QC.

70 Filter using 70 µm filter.

71 Prepare the following.

71.1 Wash 3X 15 mL falcon 3 times with distilled water to clear of the dust in the tube and keep (3X 15 ml Falcon) 2X Falcon is for TET buffer; 1X for Bead collection.

71.2 Wash 4X 50 mL falcon 3X times with distilled water to clear of the dust in the tube and keep (4X 50 ml Falcon) 2X Falcon is for bead filtering; 1X for Bead refiltering and 1X for filtered lysis buffer collection.

72 Sequential transfer of beads to 70 µm filter for 2 stage filtration.

73 Collect the final filtrate and transfer to the dust clean 15 mL falcon. Keep On ice .

74 Prepare the 1X lysis mix in fresh 50 ml falcon.

Prepare the 1X Lysis mix

	A	B	C	D	E	F
		Stock	**Unit**	**Final drop**	**Final**	**Volume**
	Tris-HCl (pH 7)	1000	mM	25	125	5000
	NaCl	5000	mM	30	150	1200
	$MgCl_2$	1000	mM	2.5	12.5	500
	TX-100	100	%	0.25	1.25	500
	BSA	30	%	0.08	0.4	533
	dH_2O	-				32800
						40000

75 Filter the 1X lysis mix to a 2nd pre-clean 50 ml falcon using 0.2 µm disc filter.

76 Spin down the falcon with beads in TET at 800 x g, 00:01:00 , remove as much as supernatant as possible. 1m

77 Add 10 mL of filter 1X lysis mix. Vortex well. Spin down the falcon with beads in TET at 1000 x g, 00:01:00 . Remove and discard the supernatant. 1m

78 Add 10 mL of filter 1X lysis mix. Vortex well. Incubate the beads at 4 °C Overnight for equilibration.

79 Next day wash the 8-strip Eppendorf tubes 2X times with the DI water. Remove as much liquid as possible with the p200 multichannel.

80 Spin down the bead at 1000 x g, 00:02:00 and aliquot 40 µL beads to 8-strip tube.
Put the strips to -80 °C storage.

2m