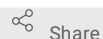# ⊕ HyDrop Bead Generation & PCR Barcoding v1.0

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1 Works for me   ⚛ Share   This protocol is published without a DOI.

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ABSTRACT

Protocol for producing dissolvable barcoded hydrogel beads used in HyDrop experiments.

Hydrogel bead generation

1   Hydrogel bead generation

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.

1.1   Prepare TEMED-oil mix by combining 🗋**6.6 µl** TEMED with 🗋**1650 µl** oil. 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.

Put the mix in syringe and filter with a 0.2 um disc filter

Prepare bead mix:

A	B	C	D	E
Bead mix	**Vol (uL)**	**Stock**	**Final**	
dH2O	920			
Acrylamide	300	40%	6%	
Bisacryloylcystoylamine	220	5%	0.55%	
TBSET	200	100%	10.00%	
Acrydite primer	240	100	12	uM
Ammonium persulfate	120	10%	0.60%	
	2000			

1.2 Transfer the TEMED-oil mix and bead mix mix to 3 mL syringes. Load mix in p1000, insert p1000 tip in the tip of the syringe, withdraw the plunger to suck the prepared mixes into the syringe.
Attach a 25 gauge needle and connect tubing (x cm) to the needles using tweezers

Prime the syringe by **hand**: keep the in a upright position to get rid of air and slowly push the plunger
Prime the syringe by **pump**: attach syringe to pump

1.3 Use the following flow rates as guidance:

Monomer mix: 600 uL/h
Oil mix: 600 uL/h

This will produce beads ~50 um in diameter.
Collect aliquots of 1 mL of beads in a 2 mL eppendorf tube.
Remove excess oil from the bottom and add 400 uL of pure mineral oil to the top to prevent evaporation.

1.4 Put aliquots on 65C heat block for 14 hours.
Put polymerised beads in 4C fridge until clean-up can begin.

Buffer mixing

2 Prepare all the buffers below. Most of these buffers are identical to Zilionis 2017, though small changes (such as the PCR buffer) may be present. These buffers may be stored at 4C for at least 3 months. We highly recommend you to filer these solutions using a 0.2 um cell medium strainer.

A	B	C	D	E
1. TBSET - Soln 1010 - 500 ml				
Component	**Volume**	**Stock**	**Final**	
Water	465.85	-	-	
Tris HCl (8.0)	5	1	0.01	M
NaCl	13.7	5	0.137	M
KCL	0.45	3	0.0027	M
EDTA	10	0.5	0.01	M
TX-100	5	10.00%	0.10%	%
	500			

	A	B	C	D	E
2. TET - Soln 1020 - 500 ml					
Component	**Volume**	**Stock**	**Final**	
Water	480	-	-	
Tris HCl (8.0)	5	1	0.01	M
EDTA	10	0.5	0.01	M
TW-20	5	10.00%	0.10%	%
	500			

	A	B	C	D	E
3. BWB - Soln 1030 - 500 ml					
Component	**Volume**	**Stock**	**Final**	
Water	490	-	-	
Tris HCl (8.0)	5	1	0.01	M
TW-20	5	10.00%	0.10%	M
	500			

	A	B	C	D	E
4. PCR Buffer - Soln 1040 - 500 ml					
Component	**Volume**	**Stock**	**Final**	
Water	481	-	-	
Tris HCl (8.0)	5	1	0.009999	M
KCl	8.3	3	0.04979502	M
MgCl2	0.75	1	0.00149985	M
TW-20	5	0.1	0.001	
	500.05			

	A	B	C	D	E
5. STOP-25 - Soln 1050 - 500 ml					
Component	**Volume**	**Stock**	**Final**	
Water	448.3	-	-	
Tris HCl (8.0)	5	1	0.01	M
EDTA	25	0.5	0.025	M
TW-20	5	10.00%	0.10%	%
KCl	16.7	3	0.1002	M
	500			

	A	B	C	D	E
6. STOP-10 - Soln 1060 - 500 ml					
Component	**Volume**	**Stock**	**Final**	
Water	463.3	-	-	
Tris HCl (8.0)	5	1	0.01	M
EDTA	10	0.5	0.01	M
TW-20	5	10.00%	0.10%	
KCl	16.7	3	0.1002	M
	500			

	A	B	C	D	E
8. Neutralization buffer - Soln 1080 - 500 ml					
Component	Volume	Stock	Final	
Water	425	-	-	
Tris HCl (8.0)	50	1	0.1	M
EDTA	10	0.5	0.01	M
TW-20	5	10.00%	0.10%	%
NaCl	10	5	0.1	M
	500			

	A	B	C	D	E
9. QC buffer					
Component	Volume	Stock	Final	
Water	32.33	-	-	
Tris HCl (8.0)	0.25	1	0.005	
EDTA	0.5	0.5	0.005	
TW-20	0.25	10.00%	0.05%	
KCl	16.67	3	1.0002	
	50			

Barcoded bead synthesis: clean-up

3   **Bead clean-up**
The beads are now polymerised, but they are suspended in carrier oil. We need to separate the beads from the oil phase by first breaking the emulsion and then washing in degreasing solutions.

3.1   Remove the excess mineral oil from the top and RAN oil from the bottom using a syringe with a tube attachment. This allows you to remove oil without pulling away too many beads.

3.2   Add 1 mL of 20% PFO in HFE, vortex at 2000 xg and remove the bottom oil phase. Perform this PFO wash for a total of 3 times. The HBs should appear as a solid, packed gel. This is a convenient step to pool the beads: the solid gels can easily be dropped into a common 15 mL tube.

3.3   Per 1 mL of beads, add 1 mL of filtered 1% SPAN-80 in Hexane under a chemical hood. Vortex well, centrifuge at 5000 xg, 30s and remove oil phase. HBs should appear as a packed gel at the bottom of the tube. Remove the top hexane layer. Perform this hexane wash for a total of 2 times.

3.4   Add 10 mL of TBSET and vortex well. Centrifuge 3000 xg, 3 min. and remove supernatant above bead pellet. Be sure to collect all hexane, which appears as a cloudy layer on top of the aqueous phase. Perform this TBSET wash for at least 3 times or further until all hexane is gone.
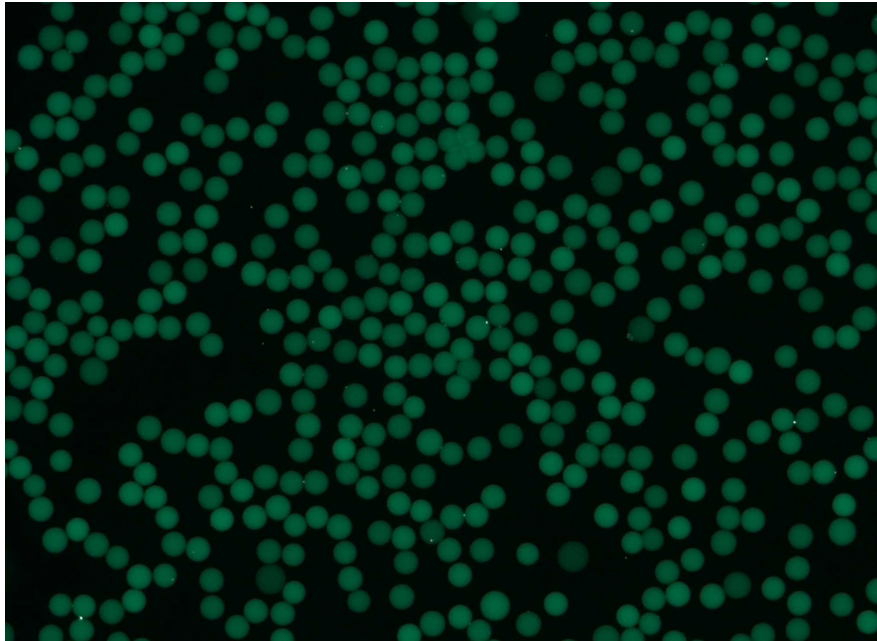
Barcoding

4   **QC**
Before proceeding to barcoding, perform acrydite QC according to Zilionis 2017 to check for loss of acrydite primers. You can take a 10x bead sample for intensity reference.

1. Take 10 uL of packed bead stock
2. Wash twice with QC buffer
3. Remove supernatant, leaving about 40 uL left
4. Add 2 uL of 200 uM specific FAM probe

5. Vortex and put on rotator for 30 min at room temperature
6. Wash beads three times with QC buffer
7. Visualise beads under Zeiss Axioplan
8. Settings: 5-FAM filter, 80% light source intensity, 300 ms exposure

You want uniformity in size and intensity. A good QC looks like so:

Proceed with the barcoding if QC is satisfactory.

5   **Pre-PCR washes**

The beads need to be washed several times throughout the barcoding process. A wash entails the following steps:
1. Centrifuge beads at 700 xg, 1 min.
2. Check supernatant for any suspended beads. If beads are visibly suspended, centrifuge again with lower centrifugation speed and braking rate.
3. Remove supernatant with a 10 mL pipette
4. Vortex pellet shortly
5. Add 1 mL of the buffer with which you want to wash
6. Vortex thoroughly
7. Add an additional 9 mL of the buffer
8. Vortex thoroughly
9. Inspect beads for possible clump formation and vortex further if necessary

5.1   Wash the beads twice in Bead Wash Buffer as described above

5.2   Wash the beads twice in PCR buffer as described above

6   **Bead split-pooling using Hamilton Liquid Handler**

These steps can also be performed manually with an 8- or 12-channel pipette. In that case, distribute all 22.5 uL of beads over a 96 well plate, add 25 uL of KAPA mix to each well, and finally add 2.5 L of 100 uM unique barcode oligo to each well. You can also pre-mix the KAPA and beads, and dispense 47.5 uL of this mixture instead. Then, perform the PCR program, manually pool the beads and proceed with step 8 of the protool.

We describe the use of the Hamilton robot to distribute beads, KAPA and primers to 96 wells. Please enquire with us if

you would like to copy our Hamilton protocol. It is highly likely that the protocol will have to be adapted to your labware and machine specifications.

All 96 well plates must be of the deep well variant and must be placed into a 96 well adapter, otherwise the hamilton coordinates will not match.

6.1 Open the latest version of protocol in Hamilton control software.
Prepare pipet tips and place in correct position according to protocol
Put full 96 tip rack in column 20, 3rd row counting from the front.
Put 8 tip rack in column 20, 4th row counting from the front.
Place empty working 96 well plate (VWR cat 82006-636) in the first column, 4th row from the front.

6.2 Estimate the volume of beads that you have. Without too many failures, the monomer volumes used above will produce around 2 mL of beads. If you use a volume of 22.5 uL of beads per well, you need 2160 uL total. To account for hamilton pipetting losses, add 20% extra. This means you need 2592 uL of beads. If you don't have enough beads, top off the ~2 mL of packed beads to 2592 uL using PCR wash buffer and dispense this slightly diluted bead stock isntead.

6.3 In the hamilton program, enter the primer, bead and KAPA mix volumes which you will use. For the above volumes:
Beads: 22.5 uL
KAPA: 25 uL
100 uM primer plate: 2.5 uL

KAPA must always be 50%. The final primer concentration here is 2.5 uM, but this may vary in the future.

6.4 Fill column 1 of the bead reservoir plate with the indicated volume of beads.
Place reservoir plate in the first column, 2nd row from the front
Proceed with the program and watch as the machine dispenses 20 uL of beads from the reservoir plate into the working plate.

6.5 Refill the reservoir plate with beads as indicated and continue the program.
Run the last step where the remainders of the beads left in the reservoir plate are distributed over the working plate.

6.6 Fill column 1 of the bead reservoir plate with the indicated volume of KAPA and watch as the hamilton dispenses KAPA in all working plate wells. Refill the KAPA in the bead reservoir plate when prompted.

6.7 Place the primer plate in the correct slot and watch how the hamilton dispenses 5 uL of primer to the working plate.

6.8 Seal the primer and bead plate with BioRad seals.
Spin down the bead plate at 300 xg for 1 minute.

7 **PCR**
Sub-barcodes are integrated into the hydrogel matrix using PCR.

Shake the plate 30s at 2000 rpm on a room temperature heat block.
Put plate in PCR block and start HB PCR program.

A	B	C
a. 95C	: 3 mins	
b. 98C	: 20 s	
c. 38C	: 4 mins	
d. 72C	: 2 mins	repeat b-d for 5 cycles
e. 98C	: 1 min	
f. 38C	: 10 min	
g. 72C	: 4 mins	
h. 4C	: Inf	

Meanwhile, set heat block to 38 C

During the 38 C annealing step, take the plate out of the PCR block and shake it for 30s at 2000 rpm on the 38 C heat block. Return the plate and continue. This is crucial, as lack of convection due to the beads will lead to uneven bead barcoding if you don't do this!

8 Post-PCR washes

Now, the hamilton is used again to pool all 96 wells. Then, STOP-25 is added to stop all enzymatic activity. After this, we wash the beads several times to remove free primers. Then, the beads will be incubated and washed in a denaturation solution which removes oligos hybridised to the bead barcodes.

8.1 Return the bead plate to its original position in the hamilton.
Place an empty hamilton reservoir in the correct position.
Place a hamilton reservoir filled with STOP-25 in the correct position.

10m

8.2 Continue with the Hamilton program. The robot will now collect the beads into the reservoir.
Pool the beads into a 50 mL tube. Clean the reservoir with STOP-25 and pool it in the 50 mL tube.

20m

8.3 Keep the 50 mL tube at room temperature for 30 min., flipping it intermittently so that the beads never settle.

35m

8.4 Centrifuge the pooled beads 3 min 800 rcf with braking speed reduced to 3 (to not resuspend beads during the braking in the 50 mL tube).
Remove supernatant and transfer pellet to a 15 mL falcon tube.
Wash the 50 mL tube with STOP-10.
Wash beads in STOP-10 for a total of two times as described above.

20m

8.5 Mix fresh denaturation buffer:

20m

A	B	C	D	E
7a. Denaturation - Soln 1070a - 60 ml				
Component	Volume	Stock	Final	
Water	60.5	-	-	
NaOH	0.938	10	0.150108821	M
Brij-35	1.05	5	0.084016131	M
	62.488			

Resuspend pellet in fresh denaturation buffer and incubate with rotation for 10 minutes.
Wash beads three additional times in denaturation buffer, with a 1 minute incubation inbetween every wash. Use 300 rcf centrifugation to prevent bead clumping.

8.6 Wash beads twice in neutralisation buffer. If bead clumping occurs, vortex vigorously and use a P1000 to resuspend the clumps.

15m

8.7 Wash beads twice with TET and filter through a 70 um strainer until no clumps are left. Invert strainer 15m
and centrifuge to reduce yield losses.

9   Go back to pre-PCR washes until the barcoding is finished. After the last step, filter beads three successive times to 1h 30m
eliminate dust, perform QC as described in Zilionis 2017.

1. Take 10 uL of packed bead stock
2. Wash twice with QC buffer
3. Remove supernatant, leaving about 40 uL left
4. Add 2 uL of 200 uM specific FAM probe
5. Vortex and put on rotator for 30 min at room temperature
6. Wash beads three times with QC buffer
7. Visualise beads under Zeiss Axioplan
8. Settings: 5-FAM filter, 80% light source intensity, 300 ms exposure
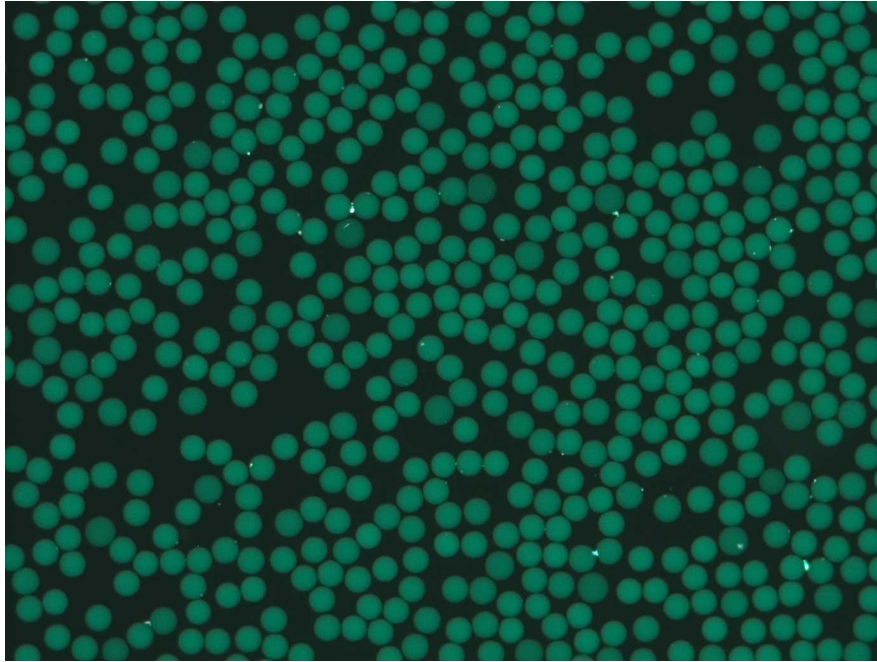
A good QC at this stage looks like so:
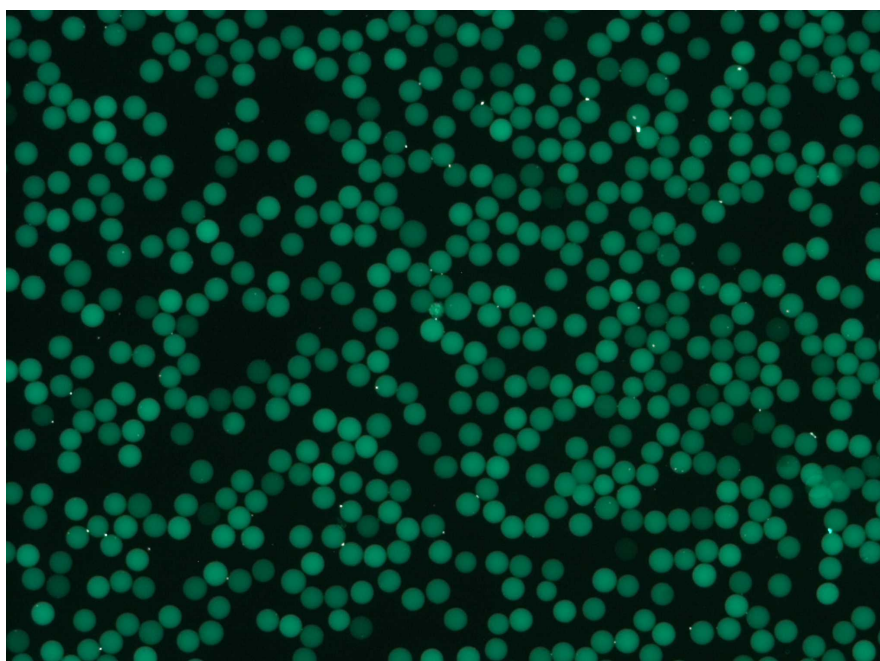
You want uniformity in size and intensity.

10x beads treated in the same manner look like so:

The 10x beads are clearly more uniform in intensity.

Proceed with bead freezing if QC is satisfactory.

Bead freezing	1h 10m

10 Bead freezing

We store the beads in a 30% glycerol freezing/lysis buffer in order to stabilise the weak disulfide bonds. The freezing buffer contains 30% glycerol to prevent freezing artifacts such as changing shape of the beads.

10.1 Prepare bead freezing buffer. This is the same for ATAC and RNA beads.   15m

A	B	C	D	E
BFB	Volume	Stock	Final	Final drop
Tris-HCl (pH 7)	3750	1000	125	25
NaCl	900	5000	150	30
MgCl2	300	1000	10	2
Tween-20	1200	100	4	0.80
TX-100	2250	10	0.75	0.15
Glycerol	9000	100	30	6.00
BSA	900	10	0.3	0.06
dH2O	11700	-		
	30000			

Importantly, filter this bead freezing buffer using a 0.2 um strainer to remove any dust.

10.2 Rinse two 15 mL falcon tubes and 3 50 mL falcon tubes with distilled water to remove possible dust   5m
particles. This can be skipped if you are confident your plasticware is sterile or dust-free. Keep in mind
that a single speck of dust may ruin an entire run.

10.3 Sequentialy filter the barcoded bead stock using 70 um strainers. To do this, put a strainer on one of   20m
your three 50 mL falcon tubes, pour your beads through (collect all remainders using TET buffer).

Perform this step a total of three times. If your beads have been freshly barcoded, you can count the two filtrations you performed right after barcoding.

Transfer the final filtrate to one of your clean 15 mL falcon tubes.

10.4 Add 5 mL of BFB to the beads and vortex well.   10m
Spin down at 500 xg for 2 minutes.
Remove and discard the supernatant.
Perform this wash a total of two times.

10.5 Fill the entire 15 mL falcon tube with beed freezing buffer.   5m
Incubate beads in freezing buffer at 4C for at least 3 hours or overnight.

10.6 Take clean/dust-free 8 strip PCR tubes   15m
Centrifuge beads 500 g for 2 minutes, remove supernatant and aliquot packed bead pellet to strip tubes. We recommend volumes between 30 and 60 uL per tube. As a rule of thumb, you need 1/4th the volume of beads per volume of PCR or RT mix during the hydrop experiment. So, 30 uL of beads is good to barcode 120 uL of PCR/RT + nuclei/cell mix - about 2 samples of 2-10k cells each. If you plan on doing large scale experiments, you can store larger aliquots.

Freeze and store beads in -80C.

10.7 When you need the beads, thaw the aliquot on room temperature for a few minutes until all ice disappears and, importantly, remove all supernatant.