**DropSynth bead barcoding protocol (version 5.0 July 2023)**

This protocol can be performed using 1x 384-well plate to generate 384 unique barcoded beads, or 4x 384-well plates to generate 1536 unique barcoded beads, or in a single 1536-well plate. Though the process can be done by hand, it is helpful to use high throughout pipettes or liquid handling robots, such as a Rainin Liquidator 96, Rainin BenchSmart 96, Integra Viaflo 96/384, Hamilton Vantage robot for liquid handling steps.

Reagents Required (384-plex):

1. 60 µL 100 µM anchor oligo (Integrated DNA Technologies) (6nmol)
2. 60 µL 100 µM ligation oligo (Integrated DNA Technologies) (6nmol)
3. 1,296 µL 10X T4 Ligase Buffer (New England Biolabs)
4. 1 µL of each 100 µM barcoded oligo (Integrated DNA Technologies)
5. 24 µL T4 Ligase (New England Biolabs)
6. 80 µL T4 PNK (New England Biolabs)
7. 1500 µL Streptavidin M270 Dynabeads (Invitrogen) (10 ug/uL, 15 mg, 3 nmol bin. cap.)
8. 30 uL 1 mM solution of biotin (made from biotin powder Sigma Aldrich B4639-500MG)
9. >10 mL UltraPure Distilled Water (Invitrogen)
10. >10 mL 2X B&W Buffer
11. >10 mL 2x B&W Buffer + 0.05%m Tween-20 (BWT)

Reagents Required (1536-plex):

1. 240 µL 100 µM anchor oligo (Integrated DNA Technologies) (24nmol)
2. 240 µL 100 µM ligation oligo (Integrated DNA Technologies) (24nmol)
3. 5,184 µL 10X T4 Ligase Buffer (New England Biolabs)
4. 1 µL of each 100 µM barcoded oligo (Integrated DNA Technologies)
5. 96 µL T4 Ligase (New England Biolabs)
6. 320 µL T4 PNK (New England Biolabs)
7. 6,000 µL Streptavidin M270 Dynabeads (Invitrogen)
8. 120 uL 1 mM solution of biotin (made from biotin powder Sigma Aldrich B4639-500MG)
9. >40 mL UltraPure Distilled Water (Invitrogen)
10. >40 mL 2X B&W Buffer
11. >40 mL 2x B&W Buffer + 0.05%m Tween-20 (BWT)

Prepare 50mL 2X B&W buffer (2M NaCl, 1mM EDTA, 10mM Tris):

- 4.675g NaCl salt
- 400 µL UltraPure 1M Tris, pH 7.5 (Invitrogen)
- 80 µL UltraPure 0.5 M EDTA, pH 8.0 (Invitrogen)
- UltraPure Distilled Water (Invitrogen) to 40 mL
- Adjust pH to 8 if needed

Prepare 50mL BWT Buffer

- To 50mL stock of B&W buffer add 0.05%m Tween-20

Prepare 10X Hybridization buffer:

- 500 mM NaCl
- 10 mM Tris-Cl, pH 8.0
- 1 mM EDTA, pH 8.0

For each 384well plate:

1. Hybridize the anchor and ligation oligos:
    - Add to a 1.5-mL tube:
        - 60 µL 100 µM anchor oligo (5 µM final)
        - 60 µL 100 µM ligation oligo (5 µM final)
        - 120 µL 10X Hybridization buffer
        - 960 µL UltraPure Distilled Water
        - TOTAL: 1,200 µL (duplex 5 µM final)
    - Anneal using the following conditions (in a Thermomixer C):
        - 3 min at 70ºC
        - Ramp down to 60ºC for 1 min, 0.1ºC/min
        - Ramp down to 50ºC for 1 min, 0.1ºC/min
        - Ramp down to 40ºC for 1 min, 0.1ºC/min
        - Ramp down to 30ºC for 1 min, 0.1ºC/min
        - Leave tube at RT
    - Total time 403min (6.7hr)

2. Bind to beads (per 384 well plate):
    - Prepare 1,500 µL stock Dynabeads M270 Streptavidin (10 mg/mL), BWT washed, and resuspended in 1,200 µL 2X B&W buffer. Split evenly into three tubes of 400 µL each.
    - Add the (3x 400 µL) beads to the (3x 400 µL) hybridized oligos.
    - Each tube has:
        i. 400 µL Dynabeads M270 Streptavidin (12.5 mg/mL, 5 mg total per tube, 1 nmol binding capacity)
        ii. 400 µL Duplexed anchor+ligation oligos (5 µM, 2 nmol per tube)
    - Mix overnight with shaking (>2000 RPM) at room temperature.

3. Add free biotin (optional):
    - Make 1 mM solution of biotin.
    - Add 10 uL of 1 mM biotin to each 800 µL tube (10 nmol biotin per tube).
    - This will saturate any unbound streptavidin on the bead surface.
    - Incubate with shaking for 30 min.

4. Combine the 3x tubes (2,400uL total) and wash the beads 2x with 5 mL BWT buffer
    - Resuspend in
        - 1000 µL 10X T4 Ligase Buffer
        - 9000 µL UltraPure Distilled Water
    - Bead concentration is 1.5 mg/mL (15mg total)

5. Aliquot beads into as many wells as desired (for example, 384):
    - Using a Rainin P200 12-channel pipette, add 86 µL of beads to all wells of a master 96-well plate.

- Using a Rainin Liquidator 96, distribute 19 µL of beads from the master 96-well plate to all wells of a new 384-well plate. The 384-well plate can be adjusted to 4 corners using a Rainin Plate Adapter 384, allowing all wells to be filled from the 96-well master plate.
- In the end every well has 19 µL of 1.5 mg/mL beads (28.5 µg beads total, 5.7 pmol binging capacity). (*changed to 12 uL of 1.5 mg/mL beads in 1536x plates making 18 µg beads total, 3.6 pmol binging capacity*)

6. Hybridize the barcoded oligos:
    - Using a Rainin Liquidator 96 or Viaflo96, transfer 1 µL from every well of the 100 uM barcoded oligo plate to every well of the 384-well plate. (100 pmol per well with total well volume of 20 uL) (*will be 13 uL in 1536x plates*)
    - Pipette mix (50% volume, 10x)
    - Anneal the mixed oligos on each plate using the following conditions (404 min total):
        - Mix at 1100RPM on thermomixer continuously throughout hybridization temperature sequence
        - 3 min at 70ºC
        - Ramp down to 60ºC for 1 min, 0.1ºC/min (101min)
        - Ramp down to 50ºC for 1 min, 0.1ºC/min (101min)
        - Ramp down to 40ºC for 1 min, 0.1ºC/min (101min)
        - Ramp down to 30ºC for 1 min, 0.1ºC/min (101min)
        - TOTAL 407min (just under 7 hours)
        - Put plate on ice

7. Ligate the barcoded oligo to the ligation oligo:
    - Ligation master mix (to make 12 wells of 80 uL total each)
        - 24 µL T4 Ligase (2,000,000 U/mL)
        - 96 µL 10X T4 Ligase Buffer
        - 840 µL UltraPure Distilled Water
        - 960 µL TOTAL
    - Add 80 uL to each well in the first row of a 96-well plate. This contains:
        - 2 µL T4 Ligase (2,000,000 U/mL)
        - 8 µL 10X T4 Ligase Buffer
        - 70 µL UltraPure Distilled Water
        - 80 µL TOTAL per well
    - Using a Rainin P20 12-channel pipette, add 10 µL of master mix to all wells of a master 96-well plate.
    - Using a Rainin Liquidator 96, distribute 2 µL master mix from the master 96-well plate to all wells of the 384-well plate. (22 uL total in each well in 384x plates) (*will be 15 uL in 1536x well plates*)
    - Pipette mix (40% volume, x10 before and after introduction of ligation master-mix)
    - Incubate plate at 16ºC for 1 hr or longer, followed by 65ºC for 20 min to heat inactivate the ligase - mixing at 1100RPM

**Old 384 well plate method - buffer summary**

| Step | Vol. Ini. | Vol. Final | Vol. Add | Input | Final |
|---|---|---|---|---|---|
| Add beads | 0 | 19 | +19 | Beads 1.5 mg/mL<br>1X T4 Ligase Buffer | Beads 1.5 mg/mL<br>1X T4 Ligase Buffer |
| Add oligos | 19 | 20 | 1 | 1uL 10mM Tris 0.1mM EDTA | Beads 1.425 mg/mL<br>0.95X T4 Ligase Buffer |
| Add ligase | 20 | 22 | 2 | 2uL 1x T4 Ligase buffer<br>T4 Ligase (4,000 U) | Beads 1.295 mg/mL<br>0.955X T4 Ligase Buffer |

**New 1536 well plate method - buffer summary**

| Step | Vol. Ini. | Vol. Final | Vol. Add | Input | Final |
|---|---|---|---|---|---|
| Add beads | 0 | 12 | +12 | Beads 1.5 mg/mL<br>1.07X T4 Ligase Buffer | Beads 1.5 mg/mL<br>1X T4 Ligase Buffer |
| Add oligos | 12 | 13 | 1 | 1uL 10mM Tris 0.1mM EDTA | Beads 1.385 mg/mL<br>0.988X T4 Ligase Buffer |
| Add ligase | 13 | 15 | 2 | 2uL 1x T4 Ligase buffer<br>T4 Ligase (4,000 U) | Beads 1.295 mg/mL<br>0.99X T4 Ligase Buffer |

8. Wash and pool beads:
    - Put plate on magnet.
    - Using a Rainin Liquidator 96, wash each well with 20 µL 2X B&W buffer 4 times.
    - Using a Rainin Liquidator 96, resuspend each well in 10 µL 2X B&W buffer.
    - Mix 10 µL of each well together, making a 3,840 µL mixed barcoded bead pool for each 384-well plate.
    - Distribute 1,280 µL into 3x 1.5 mL tubes (this can also be done in a single tube).
    - Wash another 3 times with 1mL 2X B&W buffer each.
    - Wash another time with 0.4mL 2X B&W buffer each.
    - Pool all tubes together and resuspend in 1720 µL UltraPure Distilled Water.
    - Modified wash method
        - Prepare 96 well-plate with 20uL BWT per well
        - Pipette mix 396 well plate mixture (40% volume, 10x) to suspend beads
        - Add 5uL BWT to each well and pipette mix
        - Harvest to 96 well plate
        - Wash pooled beads 2x in 8mL BWT

9. Phosphorylate the barcoded oligo:
    - Add to the tube of pooled beads:
        - 200 µL 10x T4 ligase Buffer
        - 80 µL T4 PNK
        - Mix well
    - Incubate the tube at 37ºC for 1 hr (or longer), followed by 65ºC for 20 min to heat inactivate the PNK - mixing at 1100RPM

10. Wash the beads with 2 mL 2X BWT buffer, resuspend in 2 mL 2X B&W buffer

11. Store beads at 4ºC when not in use. No drop in quality has been observed for up to 2 years after production when stored at 4ºC.