DNA Barcoded Hydrogel Bead Synthesis V.1

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SUBMIT TO PLOS ONE

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GUIDELINES

- Notes on Acrylamide Polymerization

MATERIALS TEXT

MATERIALS

Nuclease-free Water **Sigma Aldrich Catalog #W4502-1L**

Triton X-100 **Sigma Aldrich Catalog #T8787**

Potassium Chloride **Sigma Aldrich Catalog #P9541**

1M Tris-HCl (pH 8.0) **Thermo Fisher Scientific Catalog #15568025**

Perfluorooctanol **Sigma Aldrich Catalog #370533**

Tween-20 **Sigma Aldrich Catalog #P9416**

Sodium chloride **Sigma Aldrich Catalog #S3014**

10 N NaOH **Sigma Aldrich Catalog #72068**

Ammonium persulfate (APS) **Sigma Aldrich Catalog #A3678**

UltraPure™ 0.5 M EDTA pH 8.0 **Thermo Fisher Scientific Catalog #15575020**

HFE-7500 3M Novec Engineered fluid **Fluorochem Catalog #051243**

Hexane **Sigma Aldrich Catalog #227064**

Span 80 **Sigma Aldrich Catalog #S6760**

Acrylamide solution 40% **Sigma Aldrich Catalog #A4058**

Acrylamide/Bis-acrylamide 40% solution **Sigma Aldrich Catalog #A9926**

Tetramethylethylenediamine **Sigma Aldrich Catalog #411019**

Mineral oil **Sigma Aldrich Catalog #M5310**

Brij™-35 30% Solution **Thermo Fisher Scientific Catalog #20150**

10x Isothermal Amplification Buffer Pack **New England Biolabs Catalog #B0537S**

Deoxynucleotide (dNTP) Solution Mix **New England Biolabs Catalog #N0447L**

Bst 2.0 DNA Polymerase **New England Biolabs Catalog #M0537L**

Exonuclease I (E. coli) **New England Biolabs Catalog #M0293L**

Exonuclease I Reaction Buffer **New England Biolabs Catalog #B0293S**

FastDigest HinfI **Thermo Fisher Scientific Catalog #FD0804**

FastDigest Buffer (10X) **Thermo Fisher Scientific Catalog #B64**

Name	Recommended purchase amount
NaCl	500g-1Kg
KCl	500g-1Kg
Triton X-100	100 mL
Tween-20	100 mL
Perfluoro-1-octanol	25 g
HFE-7500	100 g
Tris-HCl	1 L
EDTA	4 x 100 mL
Hexane	1 L
Span 80	250 mL
NaOH	100 mL
Ammonium Persulphate	25 g
Acrylamide solution 40%	100 mL
Acrylamide/Bis-acrylamide 40% solution	100 mL
TEMED	100 mL
Mineral oil	100 mL
Brij-35	950 mL

SAFETY WARNINGS

COSHH sheet for this protocol can be found here:
https://docs.google.com/document/d/1uR8fdgWffDvPY2sY5ufCDKfpoQho4jAek9GcYnvBzvY/edit?usp=sharing

DISCLAIMER:

BEFORE STARTING

Glossary:

- Nuclease free water (NFW)
- Hydrogel Bead (HB)
- Tetramethylethylenediamine (TEMED)
- Microfluidic (MF)

NaCl

1 Prepare 5 **M NaCl**

Dissolve **291.65 g** of **NaCl** in **700 mL approx.** of **NFW**

Adjust final volume to **1 L**

Store at **Room temperature** for at least 1 year

KCl

2 Prepare 1 **M KCl**

Dissolve **74.55 g** of **NaCl** in **900 mL approx.** of **NFW**

Adjust final volume to **1 L**

Store at **Room temperature** for at least 1 year

Perfluorooctanol

3 

Prepare **20% (vol/vol) Perfluorooctanol in HFE-7500 oil (20% (vol/vol)) - 20% PFO**

Perform this step in a **fume-hood** with appropriate personal protective equipment and avoid contact with skin and eyes when handling this reagent.

Store the resulting mixture in a fume cupboard.

Combine **8 mL** of **HFE-7500 oil** with **2 mL** of **Perfluorooctanol**

Store at **Room temperature** for at least 1 year

10% (vol/vol) Tween-20

4 Prepare **10% (vol/vol) Tween-20**

Combine **90 mL** of **NFW** with **10 mL** of **Tween-20**

Store at **Room temperature** for at least 6 months

10% (vol/vol) Triton X-100

5 Prepare **10% (vol/vol) Triton X-100**

Combine **90 mL** of **NFW** with **10 mL** of **Triton X-100**

Store at **Room temperature** for at least 6 months

DNA elution buffer

6 Prepare **50 mL approx.** of **DNA elution buffer**

Reagent	Starting conc.	Reaction conc.	Volume to add
Tris-HCl	1 M	10 mM	500 µl
EDTA	0.5 M	100 µM	10 µl
NFW			49.5 mL
Total			**50 mL**

Make into **1 mL** aliquots and store them at **-20 °C** for up to 1 year.

Tris–EDTA–Tween (TET) buffer

7 Prepare **500 mL** of **TET buffer**

Reagent	Starting conc.	Reaction conc.	Volume to add
Tris-HCl	1 M	10 mM	5 mL
EDTA	0.5 M	10 mM	10 mL
Tween-20	10%	0.1 %	5 mL
NFW			480 mL
Total			**500 mL**

Filter solution through a **0.2 µm** membrane.

Store at **Room temperature** for up to 1 year

Tris-buffered saline–EDTA–Triton (TBSET) buffer

8 Prepare **1 L** of **TBSET buffer**

Reagent	Starting conc.	Reaction conc.	Volume to add
Tris-HCl	1 M	10 mM	10 mL
EDTA	0.5 M	10 mM	20 mL
NaCl	5 M	137 mM	27.4 mL
KCl	1 M	2.7 mM	2.7 mL
Triton X-100	10 %	0.1 %	10 mL
NFW			822 mL
Total			**1 L**

Filter solution through a **0.2 µm** membrane.

Store at **Room temperature** at least 6 months

1% (vol/vol) Span-80 in hexane

9

Prepare **100 mL** of **1% (vol/vol) Span-80 in hexane**

Perform this step in a **fume-hood** with appropriate personal protective equipment and avoid contact with skin when handling this reagent.

Store the resulting mixture in a fume cupboard.

Combine **99 mL** of **hexane** with **1 mL** of **Span-80**

Store at **Room temperature** in a fume cupboard for at least 6 months

Hydrogel bead wash buffer

10 Prepare **1 L** of **HB wash buffer**

Reagent	Starting conc.	Reaction conc.	Volume to add
Tris-HCl	1 M	10 mM	10 mL
EDTA	0.5 M	100 µM	200 µl
Tween-20	10 %	0.05 %	5 mL
NFW			980 mL
Total			**~ 1 L**

Filter solution through a **0.2 µm** membrane.

Store at **Room temperature** at least 6 months

STOP-25 buffer

11 Prepare **1 L** of **STOP-25** buffers

Reagent	Starting conc.	Reaction conc.	Volume to add
Tris-HCl	1 M	10 mM	10 mL
EDTA	0.5 M	25 mM	50 mL
Tween-20	10 %	0.1 %	10 mL
KCl	2 M	100 mM	50 mL
NFW			880 mL
Total			**1 L**

Filter solution through a **0.2 µm** membrane.

Store at **Room temperature** at least 6 months

Prechill to **4 °C** before use.

STOP-10 buffer

12 Prepare **1 L** of **STOP-10** buffer

Reagent	Starting conc.	Reaction conc.	Volume to add
Tris-HCl	1 M	10 mM	10 mL
EDTA	0.5 M	10 mM	20 mL
Tween-20	10 %	0.1 %	10 mL
KCl	2 M	100 mM	50 mL
NFW			910 mL
Total			**1 L**

Filter solution through a **0.2 µm** membrane.

Store at **Room temperature** at least 6 months

Hybridization buffer

13 Prepare **500 mL** of **Hybridization buffer**

Reagent	Starting conc.	Reaction conc.	Volume to add
Tris-HCl	1 M	10 mM	5 mL
EDTA	0.5 M	100 µM	100 µl
Tween-20	10 %	0.1 %	5 mL
KCl	2 M	330 mM	82.5 mL
NFW			407 mL
Total			**500 mL**

Filter solution through a **0.2 µm** membrane.

Store at **Room temperature** at least 6 months

QC Buffer

14

A	B	C	D
Reagent	**Starting conc.**	**Reaction conc.**	**Volume to add**
Tris-HCl	1 M	?	250 µl
EDTA	0.5 M	?	500 µl
Tween-20	10% (vol/vol)	?	250 µl
KCl	1 M	1 M	49 mL
NFW			0 mL
Total			50 mL

4× Acrylamide/bis-acrylamide (AB) solution

15 

Prepare 4x of **10 mL** of **Acrylamide/bis-acrylamide (AB) solution**

Acrylamide and **bis-acrylamide** are potent neurotoxins.

Perform this step in a **fume-hood** with appropriate personal protective equipment and avoid contact with skin and eyes when handling this reagent.

Reagent	Starting conc.	Reaction conc.	Volume to add
Acrylamide solution	40 %	-	2.58 mL
Acrylamide/bis-acrylamide solution	40 %	-	3.6 mL
NFW			3.82 mL
Total			**10 mL**

Filter solution through a **0.2 µm** membrane.

Store at **4 °C** at least 6 months

10% (wt/vol) ammonium persulfate (APS)

16 

Prepare **5 mL** of **10% (wt/vol) ammonium persulfate (APS)**

Perform this step in a **fume-hood** with appropriate personal protective equipment and avoid contact with skin when handling this reagent.

Combine **500 mg** of **ammonium persulfate** with **4.5 mL** of **NFW**

Adjust final volume to **5 mL**

Filter solution through a **0.2 µm** membrane

Aliquots of **100 µl** can be stored at **-20 °C** for up to 6 months

Acrydite-modified primer

17 **5'-/acrydite/photocleavable spacer/CGATGACGTAATACGACTCACTATAG GGATACCACCATGGCTCTTTCCCTACA CGACGCTCTTC-3'**

Resuspend and dilute to **250 Micromolar (µM)** in **DNA Elution Buffer**

Acrydite-modified DNA primers are light-sensitive, as they contain a photocleavable (1-(2-nitrophenyl)-ethyl-based) moiety.

At all times protect the DNA primers from exposure to UV light (<400 nm). Noticeable primer deactivation may occur at prolonged (≥20 min) exposure to ambient white light illumination.

Perform in dim illumination and away from UV light sources.

Store at **-20 °C** for at least 1 year

Carrier Oil (1% surfactant)

18 Prepare **2.5 mL** of **carrier oil mix**

Combine **1.25 mL** of **HFE-7500 oil** with **1.25 mL** of **dSurf (2%)**

Vortex well

Store at **Room temperature** and prepare freshly for the experiment run

Carrier oil / TEMED mix

19 

Prepare **2.5 mL** of **TEMED mix**

Perform this step in a **fume-hood** with appropriate personal protective equipment and avoid contact with skin when handling this reagent.

Combine **2.5 mL** of **carrier oil** with **10 µl** of **TEMED**

Vortex well

Store at **Room temperature** and prepare freshly for the experiment run

Neutralization buffer

20 Prepare **500 mL** of **Neutralization buffer**

Reagent	Starting conc.	Reaction conc.	Volume to add
Tris-HCl	1 M	100 mM	50 mL
EDTA	0.5 M	10 mM	10 mL
Tween-20	10 %	0.1 %	5 mL
NaCl	1 M	100 mM	50 mL
NFW			385 mL
Total			**500 mL**

Filter solution through a **0.2 µm** membrane.

Store at **Room temperature** at least 6 months

Prepare MF Equipment

21 Cut tubing for the following lengths using the template guides found in the template bag:

- 2 x lengths of each size of 1/32
- 3 x lengths of the 1/16 tube

Collect MF equipment:

22

- 2 x p-valve head block
- 2 x long 1/32 tubing
- 2 x p-valve attachment kit

Clean heads and o-rings and bring to fumehood

Acrylamide–primer mix

23

Prepare **1 mL** of **Acrylamide–primer mix** in a **2 mL** tube

Acrylamide and **bis-acrylamide** are potent neurotoxins.

Perform this step in a **fume-hood** with appropriate personal protective equipment and avoid contact with skin and eyes when handling reagents in this list.

Acrydite-modified DNA primers are light-sensitive, as they contain a photocleavable (1-(2-nitrophenyl)-ethyl-based) moiety.

At all times protect the DNA primers from exposure to UV light (<400 nm). Noticeable primer deactivation may occur at prolonged (≥20 min) exposure to ambient white light illumination.

Perform in dim illumination and away from UV light sources.

Reagent	Starting conc.	Reaction conc.	Volume to add
TBSET buffer	?	?	100 µl
AB Solution	?	?	250 µl
Acrydite-modified DNA primer	250 µM	50 µM	200 µl
10% (wt/vol) APS	10 %	0.3 %	30 µl
NFW			420 µl
Total			**1000 µl**

24

Vortex solution to mix and centrifuge - **1000 x g, Room temperature , 00:00:05**

25 Place **500 µl** of mineral oil on top of the solution

Prepare MF run

26 Place **Carrier oil/TEMED mix** and **Acrylamide–primer mix** into p-valve system.

- Connect the sample tube to p-valve head
- Cover tube with foil

- Slide green spacer over 1/32 tube to center
- Thread green space and tube through the cream seal and place the blue plug in reverse over the green spacer facing the cream seal thread
- Place 1/32 tubing through the aluminium seal in top until it hits the bottom of tube
- Slide the green spacer and seals down until they hit the aluminium thread
- Screw seal thread until the 1/32 tubing does not move anymore
- Place both into tube stand

27 Move tube stand from fume-hood to the MF bench

28 Attach flow units and connect conversion adapter to 1/32 tubing coming from each p-valve

- Slide flow-unit seal over the 1/32 tube coming from p-valve and connect to LHS of flow unit. Tighten until the tube does not move anymore
- Attach the short 1/32 tubing to the RHS of the flow unit using the same method
- Attach red conversion adapter to the 1/16 tubing
- Attach 1/32 from RHS flow-unit to the other side of the conversion adaptor

29 Place MF bead device onto microscope mount upside down and secure

30 Connecting tubing to MF device including output

31 Attach blue pressure tubing to p-valves

32 Place p-valve head unit over collection tube and place into tube rack.

Slide output tubing into top of p-valve but do not seal.

Wrap output tube in foil.

33 Turn on power
Turn on microscope lighting
Connect the camera to a laptop and get a live picture

34 Adjust lighting, slide position and focus until droplet junction is in ROI and focused/lit properly

MF run start-up

35 TODO

MF run shutdown

36 TODO

Polymerize beads

37

Transfer HB tubes to an incubator overnight at **65 °C** protected from light.

MF Clean

38 TODO

HB Clean-up

39 Remove the top (mineral oil) and bottom (carrier oil) phases from the 2-ml collection tubes, leaving **500 µl approx.** of polymerized HBs per tube.

TODO: PICTURE

40 Add **500 µl** of **TBSET buffer** on top of the creamy-looking emulsion.

TODO: PICTURE

41

Release HBs from the emulsion by adding **1 mL** of **20% PFO**

Vortex well then centrifuge at **5000 x g, Room temperature , 00:00:30**

HBs should appear as a milky (or semitransparent) phase.

TODO: PICTURE

42 Remove the bottom **20% PFO** phase.

Repeat **go to step #41** until the milky phase is transformed into a solid, well-packed, translucent mass on top of the **20% PFO** phase.

Remove the final bottom **20% PFO** phase.

43

Add **1 mL** of **1% (vol/vol) Span-80 in hexane**

Vortex well then centrifuge at **5000 x g, Room temperature , 00:00:30**

This time, the HBs will appear as a solid mass at the bottom of the tube.

Remove the hexane layer.

Repeat this step.

44 Add **1 mL** of **TBSET buffer** and resuspend HBs by vortexing.

45 Transfer HBs to **15 mL** tubes.

46

Fill tubes with **TBSET buffer.**

Vortex then centrifuge at **3000 x g, Room temperature , 00:03:00**

The HBs will sediment whereas traces of hexane will appear as a milky layer on the top. Carefully aspirate the supernatant, keeping the tip of the serological pipette at the liquid-air interface.

If the HBs do not sediment, extend the centrifuge time.

47 Repeat **go to step #46** until the top milky layer disappears.

48 TODO: QC the bead size

Put 20 microlitre on slide and check in microscope, take picture and use imageJ to check measurement - using python script for size of particles etc

get a histogram

49

Pass the HBs through a **70 µm** cell strainer on top of a **50 mL** tube.

Use ice-cold **TBSET buffer** to facilitate the passage of the beads through the mesh.

50

Centrifuge at **3000 x g, Room temperature , 00:10:00**

Remove supernatant.

Transfer batches of HBs into **15 mL** tubes and collect remaining into **50 mL** tube. Wash the sides of the tube with **1 mL** of **TBSET buffer** to release HBs stuck to the side for the **50 mL** tube.

51

Store at **4 °C** for up to 6 months protected from light.

Bead QC

52 Prepare **FAM-PE1 probe** 5'-/6-FAM/AGATCGGAAGAGCGTCGTGTAGGGAAAGAG-3' (standard desalting)

Prepare a **100 Micromolar (µM)** solution in DNA elution buffer.

This solution is stable for at least 1 year at **-20 °C**

53 Spin down hbs

add **10 µl** to **1.5 mL** tubes and 1.4 mL of QC buffer

Vortex
Cent 1 min @ 1,000g
remove supermatent

adjust final QC buffer vol to **36 µl**

Add **4 µl** of probe

mix by pipetting up and down
incubate room temp for 20 mins
Wash x 3 - add 1.4ml of QC buffer, vortex, centrifuge 1 min @ 1000g - remove supernatant
should have ~40 left in each tube
Split into 2 tubes
Place on slide and image
expose other tube to UV
place on slide and image

Denaturation solution

54 Prepare **250 mL** of d**enaturation solution.**

NaOH is highly corrosive; wear appropriate personal protective equipment when handling this reagent.

Perform this step in a **fume-hood** with appropriate personal protective equipment and avoid contact with skin and eyes when handling reagents in this section.

Reagent	Starting conc.	Reaction conc.	Volume to add
NaOH	10 M	150 M	3.75 mL
Brij-35	30 %	0.5 %	4.2 mL
NFW			242 mL
Total			**250 mL**

Filter solution through a **0.2 µm** membrane.

Store at **Room temperature**

PREPARE FRESH