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BONCAT-FACS on river water and sewage effluent samples

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Protocol status: Working
We use this protocol and it's working

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Abstract

Bioorthogonal non-canonical amino acid tagging (BONCAT) is a method for detecting translational activity at the single cell level. Briefly, samples are incubated with a non-canonical amino acid which gets incorporated into newly synthesised proteins. Samples are fixed, then reagents are added to fluorescently tag the non-canonical amino acid in new proteins. BONCAT can then be combined with fluorescent activated-cell sorting (FACS) to sort the BONCAT active population from the total community. The sorted BONCAT active population can undergo DNA extraction for 16S rRNA sequencing.
This protocol is for river water and sewage effluent samples.

This protocol was developed by the UKCEH Molecular Ecology group.

Guidelines

Wear nitrile gloves and lab coat.

Materials

Reagents:

100 mg ml^{-1} *L*-homopropaglycine (HPG)
20 mM copper sulfate pentahydrate, $CuSO_4$. $5H_2O$
50 mM tris[(1-hydroxypropyl-1H-1,2,3-triazol-4-yl)methyl]amine (THPTA)
0.13 mM Alexa Fluor 488 picolyl azide
100 mM sodium ascorbate
100 mM aminoguanidine hydrochloride
sterile filtered 1X phosphate buffered saline (PBS)
50%, 80%, 96%, and 100% molecular-grade ethanol
MilliQ water
molecular-grade DMSO

Equipment:

25 mm diameter filter kits
PVDF filters (25 mm, 0.2 µm)
2 ml o-ring tubes (sterile)
tweezers
stainless steel ball bearings

Safety warnings

ethanol: H225 – highly flammable liquid and vapour; H319 – causes serious eye irritation

copper sulfate pentahydrate: H302- harmful if swallowed; H318 – causes serious eye damage; H410 – very toxic to aquatic life with long lasting effects

THPTA: H315 – causes skin irritation; H319 – causes serious eye irritation; H335 – may cause respiratory irritation

aminoguanidine hydrochloride: H317 – may cause an allergic skin reaction; H411 – toxic to aquatic life with long lasting effects

Advance reagent preparation

1 Prepare [M] 100 mg/mL HPG solution by dissolving 100 mg of HPG in 1 mL of MilliQ water.

Store solution at 4 °C in the dark.

2 To prepare [M] 20 millimolar (mM) $CuSO_4$. $5H_2O$ solution, dissolve 0.5 g of $CuSO_4$. $5H_2O$ in 100 mL of MilliQ water.

Store solution at 4 °C .

3 To prepare [M] 1.15 Molarity (M) THPTA stock solution, dissolve 500 mg of THPTA in 1 mL of MilliQ water.

To prepare [M] 50 millimolar (mM) THPTA working solution, dilute 10 µL of stock solution in 220 µL of MilliQ water.

Store solutions at -20 °C .

Note

Aliquot multiple working solutions to minimise the number of freeze/ thaw cycles of stock solution.

4 To prepare [M] 6.5 millimolar (mM) Alexa Fluor 488 picolyl azide stock solution, dissolve 5 mg of Alexa Fluor 488 picolyl azide in 1 mL of molecular grade DMSO.

To prepare [M] 0.13 millimolar (mM) Alexa Fluor 488 picolyl azide working solution, dilute 10 µL of stock solution in 490 µL of molecular-grade DMSO.

Store solutions at -20 °C in the dark.

Note

Aliquot multiple working solutions to minimise the number of freeze/ thaw cycles of stock solution.

5 Prepare [M] 50 % (v/v) , [M] 80 % (v/v) and [M] 96 % (v/v) ethanol solutions with MilliQ water.

Reagent preparation on the day

6 Make fresh [M] 100 millimolar (mM) sodium ascorbate solution.

To prepare, dissolve 0.8 g of sodium ascorbate in 40 mL of 1X PBS.

7 Make fresh [M] 100 millimolar (mM) aminoguanidine hydrochloride solution.

To prepare, dissolve 0.4 g of aminoguanidine hydrochloride in 40 mL of 1X PBS.

8 Before use, remove all reagents from fridge/ freezer and allow to come up to/ thaw at Room temperature .

Non-canonical amino acid incorporation 1h 30m

9 To 25 mL of river water, add 32 µL of [M] 100 mg/mL HPG. Final concentration of HPG in sample is [M] 1 millimolar (mM) . 15m

Incubate in the dark at Room temperature for 00:15:00 .

Note

It is recommended to include 3 to 4 replicates for each sample, and to include negative controls where no HPG is added. Negative controls are used to verify gating on a flow cytometer.

Note

To optimise HPG concentrations and incubation times, run samples in replicate over a range of final concentrations (e.g., 1 µM to 1 mM) and incubation times (e.g., 15 to 60 mins).

10 Fix sample with 25 mL of molecular-grade ethanol (final concentration [M] 50 % (v/v)). 1h

Incubate in the dark at Room temperature for 01:00:00.

Biomass filtration and dehydration 16m

11 Set up filter funnels (25 mm diameter) with PVDF filters (25 mm, 0.2 µm). Connect to vacuum pump or tap.

Label funnels with sample names.

12 Condition filters with approximately 5 mL of [M] 50 % (v/v) ethanol.

13 Filter the ethanol fixed samples onto the filters.

14 Release vacuum and cover filters with 0.5 mL of [M] 80 % (v/v) ethanol. 3m

Incubate for 00:03:00, then vacuum through the ethanol.

15 Release vacuum and cover filters with 0.5 mL of [M] 96 % (v/v) ethanol. 13m

Incubate for 00:03:00, then vacuum through the ethanol.

Vacuum dry the filter for approximately 00:10:00.

Cu catalysed azide-alkyne cycloaddtion (click reaction) 33m

16 The volumetric amounts of reagents in steps 16 and 17 are for one sample's worth. It is recommended to make enough for n + 1 samples (n = number of samples). 3m

To make dye premix, add together 2.5 µL of $CuSO_4$, 5 µL of THPTA and 19 µL of Alexa Fluor 488 picolyl azide. Invert tube.

Incubate in the dark at Room temperature for 00:03:00 .

17 To dye premix, add 25 µL of sodium ascorbate, 25 µL of aminoguanidine hydrochloride, and 423.5 µL of 1X PBS. Invert tube.

Final concentration of Alexa Fluor picolyl azide in reaction mixture is [M] 5 micromolar (µM) .

Note

To maintain reducing conditions of the reaction mixture, do not vortex the mixture to mix the reagents.

Note

To optimise click dye concentrations and incubations times, run samples in replicate over a range of concentrations (e.g., 1 to 5 µM) and incubation times (e.g., 10 to 30 mins)

18 Release vacuum on filter funnels, and cover filters with 0.5 mL of reaction mixture. 30m

Incubate in the dark (e.g., cover funnels with foil) at Room temperature for 00:30:00 .

Filter washes 22m

19 Vacuum through reaction mixture.

20 Release vacuum and cover filter with 0.5 mL of 1X PBS. 9m

Incubate for 00:03:00 , then vacuum through.

Repeat twice more.

21 Repeat step 20 once with [M] 50 % (v/v) ethanol. 3m

22 Vacuum dry filters for approximately 00:10:00 . 10m

Resuspension of cells 10m

23 With clean tweezers, place filters into 2 ml o-ring tubes with the top of the filter facing inwards.

Note

Wash tweezers with [M] 70 % (v/v) ethanol between samples.

24 Add six clean stainless steel ball bearings to each tube.

Note

To clean stainless steel ball bearings, sonicate in [M] 70 % (v/v) ethanol for 00:10:00 .
Dry in oven at 40 °C .

25 Add 1.5 mL of 1X PBS to each tube.

26 Vortex/shake tubes at max speed for 00:03:00 . 3m

Sample storage

27 Aliquot 1 mL of bacterial suspension into 2 ml o-ring tubes.

28 Add 1 mL of molecular-grade ethanol to suspension. Final ethanol concentration is [M] 50 % (v/v) .

Can be stored at -20 °C for up to 2 weeks.

Fluorescent activated-cell sorting and DNA extraction

29 Dilute 300 µL of ethanol-fixed sample in 200 µL of sterile-filtered 1X PBS

30 Run a negative control to determine the BONCAT positive gate on a SH800S cell sorter at sample pressure of 5. Draw the BONCAT positive gate to encompass less than 0.1% of the negative control population on a AF 488 vs. FSC density plot. Use a 488 nm laser for excitation, and set threshold to 2 on FSC.

31 Run HPG-tagged samples according to step 30. Sort 500,000 events from the BONCAT positive gate in semi-purity mode. Collect sorted events in 5 ml flow cytometry containing tubes containing 1 mL of sterile-filtered 1X PBS.

> **Note**
>
> Maintain events per second below 3000 to ensure accurate sorting by lowering the sample pressure if need be.

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32 Vacuum concentrate sorted populations to dryness using a vacuum concentrator.

33 Store pellets at -80 °C until DNA extraction.

34 For DNA extraction, follow the manufacturer's standard protocol for the Qiagen DNeasy UltraClean Microbial kit.