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BONCAT-FACS on river water and sewage effluent samples

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Katharine Moss^{1,2}, Tim Goodall¹, Daniel S Read¹

¹UK Centre for Ecology & Hydrology (UKCEH); ²University of Bristol



Katharine Moss

UKCEH

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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, CuSO₄ · 5H₂O
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

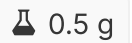
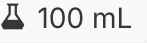
aminoguanidine hydrochloride: H317 – may cause an allergic skin reaction; H411 – toxic to aquatic life with long lasting effects



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) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ solution, dissolve  0.5 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in  100 mL of MilliQ water.

Store solution at  4 °C .

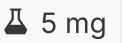
- 3 To prepare [M] 1.15 Molarity (M) THPTA stock solution, dissolve  500 mg of THPTA in  1 mL of MilliQ water.

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 μ L 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 μ M 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 μ m). 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 cycloaddition (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

 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  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  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  70 % (v/v) ethanol for

 00:10:00 .

Dry in oven at  40 °C .

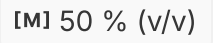
25 Add  1.5 mL of 1X PBS to each tube.

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  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.

- 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.

