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Bioorthogonal Noncanonical Amino Acid Tagging (BONCAT) incubation - ROCKS

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

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Abstract

BONCAT is a technique that allows for the visualization and isolation of metabolically active cells from complex environmental enrichments. This is achieved by incubating a sample with synthetic methionine analogs AHA (l-azidohomoalanine) or HPG (l-homopropargylglycine). Instead of the sulfhydryl side group of methionine , these analogs contain an azide or alkyne functional group, respectively, which can be chemically tagged with a fluorophore using a process termed “click-chemistry”. Throughout the incubation period actively growing cells incorporate these synthetic amino acids into newly synthesized proteins. After harvesting cells from a live enrichment, the cells are tagged with the fluorophore. This allows for microscopic visualization along with FACS-based isolation of the metabolically active cells in a community. Downstream analysis can include single-cell sequencing or metagenomics techniques to determine taxonomy and functions of isolated organisms.

Notes:

- Working with low-biomass samples has an inherent high-risk of contamination. Be mindful of sterility at all times. Perform all work in the biological safety cabinet and make sure to sterilize the hood with UV prior beginning work
- Some of the BONCAT reagents are light-sensitive, be careful to pay attention to if a particular step in the protocol needs to be performed in the dark
- The click-chemictry reaction is very sensitive to oxygen, so take care not to vortex/ introdroduce air unnecessarily
- Try to plan when you harvest incubations so that you are certain you will be able to perform the click-chemistry process the next morning

Guidelines

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Materials

- Equipment and Supplies:

o Dog bowl, mallet, large stainless steel mortar and pestle
o Sterile 50 ml Falcon tubes (1 per sample)
o Sterile 15 ml Falcon tubes
o 2 ml Eppendorf Tubes
o Eye Goggles, Isolation Headphones, Rubber Mat
o Sonicator

- Chemicals

o Basalt Salt Media (10 ml per sample)
o PBS made from 0.2 µl filtered and autoclaved Milliq Water
o PBS with 10 mM EDTA made from 0.2 µl filtered and autoclaved Milliq Water
o 20 mM CuSO4 (Stored at 4°C in refrigerator)
o 50 mM THPTA (in “BONCAT Reagents” box in freezer)
o Click-Chemistry Dye (in “BONCAT Reagents” box in freezer)
§ Carboxyrhodamine 110 Azide (For use with HPG)
§ Carboxyrhodamine 100 Alkyne (For use with AHA)
o 100 mM Aminoguanidine hydrochloride (Make Immediately before use, discard remainder)
§ 0.11 g in 10 ml PBS
o 100 mM Sodium Ascorbate (Make immediately before use, discard remainder)
§ 0.198 g in 10 ml PBS
o Propidium Iodide stock solution (0.75 mM - Aliquots stored in “Boncat Reagents” box in freezer)
§ To make new stocks dissolve 10.36 mg in 10 ml molecular grade H20 – Dilute 5x and freeze in 2 ml aliquots

Troubleshooting

Incubation Set Up (Time required: ~ 1.5 – 2.0 Hours)

1 02:00:00

Prior to Starting: Wipe down the interior of the BSC and other work areas with 70% ethanol. Run the UV light in the hood for 10 minutes.

getting things prepped pt. 2

2 On the bench, place the mortar base, cylinder and piston in the dog bowl. Cover with ethanol. WITH CAUTION, use a grill-lighter to set aflame. Allow all of the ethanol to burn off, while carefully using the blue chisel to occasionally move items around to ensure full sterilization. When the bowl is cool enough to handle, transfer it and all contents to the hood. Let cool in the hood under UV light (At this point, pull out enough weigh-boats for weighing your samples and sterilize under UV light while the other items are cooling, then leave in hood until needed)

getting things prepped pt. 3

3 When cool, carefully assembly base/cylinder or mortar(surface sterilize your gloves with ethanol before touching, and be careful to only touch exterior surfaces). DO NOT completely tighten the cylinder onto the base, leave approximately a half turn loose. Insert piston into cylinder, while being careful to only touch textured-region at the top.

weigh rock samples

4 Weigh out the amount of rock samples you will need for incubation (10g x N + 1, sterilized weigh-boats are not needed here.)

Sterilize outside of rocks (This may not be applicable to your experimental design!)

5 When you have your samples weighed, place them in the dog bowl and cover in ethanol and surface sterilize the same fashion as the mortar and pestle. When the bowl is cool, move to the hood. Flame sterilize tweezers and use them to transfer rocks into the mortar and pestle (Try your best to only remove the piston from the mortar and pestle when inside the hood).

Crush it.

6 Remove the mortar/pestle with your sample from the hood and place on top of the rubber mat on the floor (put on safety glasses and isolation headphones). Use the mallet to crush the rocks (rotate piston periodically between hits, but try not to remove it completely from the cylinder). Depending on the properties of the rock, this could take several rounds of 15 – 20 hits with the mallet.

Crush it pt. 2

7 Bring the mortar/pestle with sample back into the hood. Remove piston and stand it up on its end (top section near textured-handle on the surface of the hood, be careful to let nothing touch the area that comes into contact with rocks). Unscrew the cylinder from the base, the rock powder will be caked in the bottom of the cylinder (remove slowly in case it falls out). Use a flame sterilized spatula to chisel the powder out into a weigh-boat that has been UV sterilized. Using flame-sterilized tweezers or spatula, remove large uncrushed rock pieces to another sterilized weigh-boat – re-crush into sand-grain size as described above.

EASIER ALTERNATIVE: can weigh out into a falcontube (already sterile, can be closed and weighed)

Weigh crushed rock

8 Once your entire sample has been crushed, cover the weigh-boat with another weigh-boat and slowly remove from hood and bring to bench near scale. Weight out desired amount (usually 10g) into UV sterilized weigh-boat with ethanol and flame sterilized spatula. Work swiftly and carefully to avoid contamination as much as possible

ALTERNATIVE: See above for falcontube use.

Add media and non-canonical amino acid

9 In the hood, add the measured sample to a 50 ml Falcon Tube. Add 10 ml of BSM Media. Add desired BONCAT amino acid analog (typically HPG or AHA) for desired concentration. Cover the falcon tubes in tinfoil to keep sample dark

And wait.

10 Incubate for desired time period and at desired temperature. Experiemnt and sample dependant.