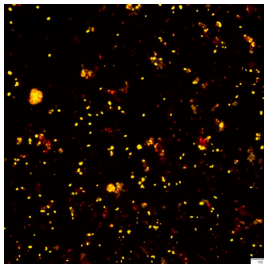
May 11, 2024 Version 1

AD BONCAT-FCM Screening & Coupled SYBR+ FCM Cell-Counting - 2022 V.1



DOI
dx.doi.org/10.17504/protocols.io.rm7vzyb75lx1/v1

Maxwell Madill[1]

[1]University of British Columbia

Max Madill
University of British Columbia

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.rm7vzyb75lx1/v1

Protocol Citation: Maxwell Madill 2024. AD BONCAT-FCM Screening & Coupled SYBR+ FCM Cell-Counting - 2022. **protocols.io https://dx.doi.org/10.17504/protocols.io.rm7vzyb75lx1/v1**

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

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

Created: February 18, 2022

Last Modified: May 11, 2024

Protocol Integer ID: 58450

Keywords: chemistry labeling of ad boncat microcosm sample, ad boncat microcosm sample, absolute cell count, cells within sample, cytometric analysis, original microcosm sample matrix, quantification of absolute cell density, cell fractions within these sample, boncat microcosm, absolute abundances of boncat, fcm cell, cell, chemistry labeling, cell fraction, absolute cell density, ad boncat

Abstract

This protocol provides a detailed description of all steps required to label, analyze and quantify the absolute abundances of BONCAT-labelled (i.e. translationally active) cells within samples collected from anaerobic-digester (AD) BONCAT microcosms. Specifically, this protocol overviews the click-chemistry labeling of AD BONCAT microcosm samples, followed the flow-cytometric analysis of BONCAT+ cell fractions within these samples, and the quantification of absolute cell densities in the bulk and post-clicked samples. The combined analyses of both BONCAT+ cell fractions with absolute cell counts allows the absolute abundance (i.e. cell#/volume) of translationally-active BONCAT+ cells within the original microcosm sample matrix to be calculated.

Troubleshooting

Reagent and Materials Preparation

1 **Long-Term Stored BONCAT Reagents**

20mM Copper sulfate solution:

Prepare 500 mL of stock by adding 2.4968 g of $CuSO_4*5H_2O$ to a 500 mL volumetric flask, rinsing the weigh boat and funnel thoroughly with DI water, and filling the flask to just above the neck with DI water. Sonicate to fully dissolve the reagent into solution, then fill flask up to 500 mL mark with DI water. Filter-sterilize 200 mL of the prepared stock through a 0.2um syringe filter into a clean and sterile 250 mL pyrex bottle. Cap bottle and store at 4 °C prior to use.

50 mM THPTA solution:

Dissolve 100 mg of THPTA into YYuL of DI water and vortex thoroughly to dissolve. Filter-sterilize through 0.2um PES syringe filter. Aliquot into 50 µL portions and store aliquots at -20 °C.

Glycerol + TE (GlyTE) Cryopreservation Buffer:

Working by a flame, transfer 100 mL of molecular-biology grade glycerol to a clean and sterile 250mL graduated cylinder - prior to reaching 100mL mark, warm the cylinder by passing over a flame to remove air bubbles and achieve an accurate volumetric measurement. Transfer 40 mL of sterile 50X TE buffer and 40 mL of sterile DI water to the graduated cylinder by pipetting with a calibrated 10mL pipette. Cap the graduated cylinder with flame-sterilized aluminum foil. Mix the solution thoroughly by capping the foil-covered end of the graduated cylinder with palm of hand and shaking while intermittently passing the cylinder over the flame to gently heat. Working by the flame, filter-sterilize the prepared buffer through a 0.2um syringe filter into a clean and sterile 250mL Pyrex bottle. Aliquot into 40 mL portions by transfering each portion into a sterile 50 mL Falcon tube. Cap tubes and/or bottle and store at -20 °C prior to use.

Fluorescent Probes/Dyes:

***Minimize light exposure during preparation and storage of all fluorescent probes*

1.5mM Calfluor 488 Azide - BONCAT labeling fluorogenic probe:

Remove tube containing 1mg portion of Calfluor 488 Azide from -20 °C freezer and wrap in a small piece of aluminum foil. Add 797.505 µL of 0.2um filter-sterilized DMSO directly to the tube, cap the tube, and vortex at maximum speed until fully dissolved (2-3min). Aliquot by pipetting out 5X 160 µL portions each into a sterile 1.5mL microtube. Wrap each microtube with aluminum foil and store at -20 °C .

100X Diluted SYTO 59 (50uM) - DNA probe for BONCAT Counterstaining:

Prepare 1X 1000 µL portion of 100X diluted, or [M] 50 micromolar (µM) , SYTO59 by adding 10 µL of Invitrogen [M] 5 millimolar (mM) SYTO59 stock into 990 µL of filter-sterilized and UV-treated 1X TE buffer. Prepare at least 5X portions during each preparation to reduce freeze-thaw and contamination of the original [M] 5 millimolar (mM) SYTO59 stock.

100X SYBR Green - DNA probe for absolute cell counting:

Prepare 1X 1000 µL portion of 100X SYBR Green by adding 10 µL of Invitrogen 10,000X SYBR Green stock into 990 µL of filter-sterilized and UV-treated 1X TE buffer. Prepare at least 5X portions during each preparation to reduce freeze-thaw and contamination of the original 10,000X SYBR Green stock.

1.1 Freshly-Prepared BONCAT Reagents

2m

Phosphate Buffered Saline (PBS):

To prepare 1 L or 2 L of 1X PBS, first weigh out the respective portions of each of the four salts following the table below.

	Reagent (Salt)	Reagent Mass (g) / 1L PBS	Reagent Mass (g) / 2L PBS
	NaCl	8.01	16.02
	Na2HPO4 * 7H20	2.6807	5.3614
	KH2PO4	0.245	0.490
	KCl	0.201	0.402

Add the weighed portions of all salt reagents to the respectively sized volumetric flask (1 L or 2 L), rinsing each weigh boat and the funnel thoroughly with DI water. Fill the volumetric flask up to the neck with DI water and cap the flask. Mix to dissolve all salts by placing flask into sonicating water bath and removing every 00:02:00 to invert and shake.

Once all salts are dissolved, adjust buffer pH to pH 7.4 by adding [M] 0.5 Molarity (M) NaOH and HCl solutions drop-wise while continuously monitoring pH with pH-probe. Add DI water to reach the final 1L or 2L volume mark and mix thoroughly by inverting. Filter-sterilize through a 0.2um syringe filter into clean and sterile 1L Pyrex bottles. Cap bottles and store at 4 °C prior to use.

100 mM Aminoguanidine HCl in 1X PBS:

To prepare 5 mL of [M] 100 millimolar (mM) solution weigh out 0.0553 g of aminoguanidine HCl into a 15mL Falcon tube - cap tube and store in the dark. Just prior to starting the click-chemistry procedure, add 5mL of 1X PBS to the tube, vortex thoroughly to mix, and filter sterilize through a 0.2um syringe filter into a sterile 15mL Falcon tube or into multiple smaller aliquots in sterile 1.5-2.0mL microtubes.

100 mM sodium ascorbate in 1X PBS:

To prepare 5 mL of [M] 100 millimolar (mM) solution weigh out 0.0991 g of sodium ascorbate into a 15mL Falcon tube - cap tube and store in the dark. Just prior to starting the click-chemistry procedure, add 5mL of 1X PBS to the tube, vortex thoroughly to mix, and filter sterilize through a 0.2um syringe filter into a sterile 15mL Falcon tube or into multiple smaller aliquots in sterile 1.5-2.0mL microtubes.

Preparation of Samples, Materials and Reagents for BONCAT Labelling

2 Remove selected microcosm samples from -80 °C freezer and thaw On ice at 4 °C .

2.1 **Pre-labelling sample processing tubes:**

For each sample to be processed for BONCAT-FC analysis, pre-label three sterile 2.0mL microtubes as follows to be used throughout the sample preparation procedure.

	BONCAT (B) Tube Label	Tube Purpose
	"Sample Code" - BA	*HPG removal wash tube*
	"Sample Code" - BB	*Click-chemistry reaction tube*
	"Sample Code" - BC	*Final collection tube for clicked, SYTO-stained and mesh-filtered cell-suspension*

For each sample to be processed for SYBR-stained absolute cell counting, pre-label two sterile 1.5mL or 2.0mL microtubes as follows to be used throughout the sample preparation procedure.

	SYBR (S) Cell-Count Tube Label	Tube Purpose
	"Sample Code" - SA	*Sample dilution and SYBR staining tube*
	"Sample Code" - SB	*Final collection tube for SYBR-stained and mesh-filtered cell-suspension*

Transfer each thawed microcosm sample into a 4 °C cooled microtube rack and place into 4 °C fridge to store temporarily. Following the same sample layout, transfer the BA, BB and SA tubes for each respective sample into a second microtube rack, and transfer the BC and SB tubes for each respective sample into a third microtube rack. Transfer the second and third microtube racks also into 4 °C fridge to store temporarily.

2.2 Pre-load sample tubes BA and SA with PBS

To streamline the sample preparation procedure, pre-load sample tubes ***BA*** and ***SA*** with the following volumes of sterile 1X PBS:

BA Tubes: Pre-load with 1500 µL PBS

SA Tubes: Pre-load with 980 µL PBS

Transfer pre-loaded sample tubes back into the microtube rack and store in 4 °C fridge prior to use.

2.3 **Prepare freshly-made BONCAT click-chemistry reagents:**

Prepare the 100mM sodium ascorbate and 100mM aminoguanidine hydrochloride stock solutions before starting the click-chemistry procedure following the instructions overviewed above. Store prepared filter-sterilized solutions at Room temperature prior to use.

Preliminary Sample Preparation Prior to Click-Chemistry Labeling

3 **Sample Homogenization** 1m

Remove microtube rack containing original microcosm samples from 4 °C and transfer to laminar flow hood.

Conduct sample homogenization to disperse microbial cell aggregates into single-cells by vortexing samples at 3200 rpm for 00:01:00. After vortexing, transfer samples back into 4 °C cooled microtube rack.

3.1 **Sediment Removal Centrifugation** 5m

Centrifuge samples at 500 x g, 4°C, 00:05:00 to sediment large particulates out of the sample matrix. After completing the centrifugation, carefully remove samples from rotor and place into 4 °C cooled microtube rack containing the ***BA***, ***BB*** and ***SA*** sample tubes.

3.2 10s

Sample distribution for Processing

Carefully unscrew cap of original sample tube containing homogenized and sediment-free cell-suspension and distribute the following aliquots of supernatant for BONCAT and SYBR cell-counting analyses.

Distribute sample for BONCAT Click-Chemistry Labeling

Using a wide-bore pipette tip, transfer 200 µL of cell-suspension into the respective ***BA*** tube containing 1500 µL PBS.

Distribute sample for SYBR-stained absolute cell counting

If conducting SYBR-stained absolute cell-counting on the respective sample, transfer 10 µL of cell-suspension into the respective ***SA*** tube containing 980 µL PBS.

Vortex samples at 3200 rpm for 00:00:10 to thoroughly disperse the cell-suspension. Transfer ***SA*** sample tubes into microtube rack containing ***BC*** and ***SB*** tubes and store in 4 °C fridge.

3.3 **HPG Removal Wash** 20m

Load ***BA*** sample tubes into microcentrifuge and centrifuge at 5000 x g, 4°C, 00:10:00 to pellet cells.

Carefully transfer microcentrifuge rotor into laminar flow hood. Carefully remove each sample tube from the rotor and remove supernatant by pipetting with 1000uL pipette. Recap sample tube after removing supernatant and place back into 4 °C cooled microtube rack.

After removing supernatants from all ***BA*** tubes, transfer another 1500 µL volume of PBS to each tube, cap tubes, and vortex tubes at 3200 rpm for 10s to disperse cell-pellets.

Re-pellet cell-suspensions using second centrifugation at 5000 x g, 4°C, 00:10:00 and remove supernatants by pipetting.

Re-suspend the washed cell-pellets to their original volume by transferring 200 µL of PBS to each tube and vortexing for 10s. Transfer washed cell-suspensions into microtube rack and store in 4 °C fridge.

Cu-Catalyzed Click-Chemistry Labeling with Calfluor 488 Azide

4 **Prepare dye premix** 3m

Prepare the dye premix by combining the respective volumes of each reagent listed in the table below in a sterile 1.5mL microcentrifuge tube. Centrifuge the tube briefly at 2000 x g, Room temperature to collect reagents. Leave the tube in the

microcentrifuge and allow the dye-premix to react in the dark at Room temperature for 00:03:00 .

	A	B	C	D
	Reagent	***1X Click RXN Volume (uL)***	***10X Click RXN Volume (uL)***	***18X Click RXN Volume (uL)***
	CuSO4 (Stock 20mM; Final 1mM)	25 uL	250 uL	450 uL
	THPTA (Stock 50mM; 500uM)	5 uL	50 uL	90 uL
	Calfluor 488 Azide (Stock 1.5mM; Final 10uM)	3.33 uL	33.3 uL	59.94 uL
	Total Volume (uL)	*33.33 uL*	*333.3 uL*	*599.94 uL*

**** Dye Premix for 5:1 THPTA:Cu Procedure**

	A	B	C	D
	Reagent	***1X Click RXN Volume (uL)***	***10X Click RXN Volume (uL)***	***20X Click RXN Volume (uL)***
	CuSO4 (Stock 20mM; Final 1mM)	25 uL	250 uL	500 uL
	THPTA (Stock 50mM; Final 5mM)	50 uL	500 uL	1000 uL
	Calfluor 488 Azide (Stock 1.5mM; Final 10uM)	3.33 uL	33.3 uL	66.6 uL
	Total Volume (uL)	*78.33 uL*	*783.3 uL*	*1566.6 uL*

4.1 **Prepare click-chemistry reaction buffer**

Prepare the reaction buffer by combining the respective volumes of each reagent listed in the table below in a sterile 1.5mL microcentrifuge tube or a 15mL Falcon tube.

	A	B	C	D
	Reagent	***1X Click RXN Volume (uL)***	***10X Click RXN Volume (uL)***	***18X Click RXN Volume (uL)***
	Aminoguanidine HCl (100mM; Made Fresh)	25 uL	250 uL	450 uL
	Sodium Ascorbate (100mM; Made Fresh)	25 uL	250 uL	450 uL
	PBS	396.67 uL	3966.7 uL	7140.06
	Total	*446.67 uL*	*4466.7 uL*	*8040.06*

****Reaction Buffer for 5:1 THPTA:Cu Procedure**

	A	B	C	D
	Reagent	***1X Click RXN Volume (uL)***	***10X Click RXN Volume (uL)***	***20X Click RXN Volume (uL)***
	Aminoguanidine HCl (100mM; Made Fresh)	25 uL	250 uL	450 uL
	Sodium Ascorbate (100mM; Made Fresh)	25 uL	250 uL	450 uL
	PBS	351.67 uL	3516.7 uL	6330.06
	Total	*401.67 uL*	*4016.7 uL*	*7230.06*

4.2 Prepare click-chemistry labeling solution and pre-load reaction tubes

Prepare the final click-chemistry labeling solution by adding the dye premix into the reaction buffer by pipetting. Mix gently by inverting 3X. Store in the dark at Room temperature prior to use.

Prepare the *BB* tubes for each respective sample for click-chemistry labelling by aliquoting 480 µL of the prepared labelling solution into each tube.

4.3 Perform click-chemistry labeling reaction

1h

Working under low-light, transfer 20 µL of homogenized and washed sample matrix from each ***BA*** tube into its respective ***BB*** tube containing the 480 µL of prepared click-chemistry labeling solution.

Cover microtube rack in aluminum foil and invert 1X to mix the cell-suspension and click-chemistry labeling solution to initiate the click-chemistry labeling reaction. After inverting, gently swirl microtube rack to collect click solution from the top and sides of microtubes.

Incubate samples in the dark at Room temperature for 01:00:00 to allow the click-reaction to occur.

Post Click-Chemistry Sample Preparation for Flow-Cytometry Analysis

5 Post Click-Chemistry Calfluor 488 removal wash

20m

Working under low-light, remove foil covering the microtube rack containing post-clicked ***BB*** tubes.

Open each ***BB*** tube, transfer 1250 µL of PBS to each tube, cap tubes and vortex briefly at 3200 rpm to mix. Pellet the post-clicked cell-suspension by centrifuging sample tubes at 5000 x g, 4°C, 00:10:00 .

Carefully transfer microcentrifuge rotor into laminar flow hood. Carefully remove each sample tube from the rotor and remove supernatant by pipetting with 1000uL pipette and place processed tubes into a 4 °C cooled microtube rack.

Transfer another 1500 µL volume of PBS to each tube, cap tubes, and vortex at 3200 rpm for 10s to disperse cell-pellets. Re-pellet cell-suspensions using second centrifugation at 5000 x g, 4°C, 00:10:00 and remove supernatants by pipetting. Remove sample supernatants by pipetting, and re-suspend the washed post-clicked cell pellet into 1000 µL PBS. Transfer washed sample tubes into 4 °C cooled microtube rack, cover with aluminum foil and store at 4 °C.

5.1 **SYTO 59 Counterstaining and mesh-filtration of click-chemistry labelled samples**

***Work under low-light conditions**

Approximately 30min prior to running flow-cytometry, counterstain samples by adding 20 µL of 100X diluted (50uM; in TE buffer) SYTO59 DNA stain to each ***BB*** tube. Vortex samples briefly at 3200 rpm to mix.

Prepare for the final filtration step by opening the respective ***BC*** sample tube and placing a sterile 40um nylon cell strainer on top of the sample tube.

Using a large-bore 1000uL pipette tip, mix the sample 5X by pipetting and then pass the SYTO-stained cell matrix through the 40um cell strainer into its respective ***BC*** tube while holding the cell strainer tight against the tube opening.

***Note, a single cell-strainer can be used for 3X replicate samples by pipetting through different locations of the mesh.**

After processing all samples, cover microtube rack with aluminum foil and store at 4 °C prior to conducting flow-cytometry analysis.

5.2 **SYBR Green staining and mesh-filtration of samples for absolute cell-counts**

***Work under low-light conditions**

Approximately 30min prior to running flow-cytometry,

counterstain samples by adding 20 µL of 100X diluted (50uM; in TE buffer) SYTO59 DNA stain to each ***BB*** tube. Vortex samples briefly at 3200 rpm to mix.

5.3

6