Calcium release from calcium pretreated S. mutans at low pHs

Livia Tenuta,Tarcísio Leitão,Jaime A. Cury

Abstract

Citation: Livia Tenuta,Tarcísio Leitão,Jaime A. Cury Calcium release from calcium pretreated S. mutans at low pHs. **protocols.io**
dx.doi.org/10.17504/protocols.io.mapc2dn
Published: 17 Dec 2017

Protocol

Bacterial growth

Step 1.

1. Reactivate *Streptococcus mutans* Ingbritt 1600 cells by inoculating 20 uL of a glycerol-frozen culture in 10 mL of Brain-Heart Infusion (BHI) broth.
2. Allow the BHI broth to grow at 37°C, 10% CO_2, for 18 h.
3. Streak the BHI culture on blood agar to obtain isolated colonies. Incubate at 37°C, 10% CO_2, for 18 h. Observe the purity of the culture by colony morphology. Store the plate at 4°C for up to one month.
4. Tranfer 6-7 isolated colonies from the blood agar to 100 mL of thioglycolate-based medium (2.4% of thioglycolate, 1.2% of meat extract, 1.5% of calcium carbonate and 0.5% of glucose).
5. Allow the thioglycolate medium to grow for 18h.
6. Check the purity of the culture by examining a 10 µL aliquot of the fresh culture at a microscope and also by Gram staining.
7. Store the thioglycolate medium at 4°C for up to 7 days.
8. Transfer 8 mL of the thioglycolate medium to 500 mL of a Tryptone-yeast extract (TYB) broth (2.5% of tryptone, 1.5% of yeast extract, 0.43% of potassium hydrogen phosphate and 0.1% of magnesium sulfate heptahydrate), pH 7.0 supplemented with 0.25% glucose.
9. Allow the TYB broth to grow at 37°C, 10% CO_2, for 18 h.

Ca pretreatment and harvesting of experimental bacterial pellets

Step 2.

1. After TYB growth for 18 h, centrifuge all content of the culture at 10,000 x g, 4°C, for 10 min.
2. Discard the supernatant broth and resuspend the whole bacterial pellets in 100 mL of 0.05 M PIPES buffer, pH 7.0, at 4°C (use 2 x 50-mL pre-weighted tubes).
3. Repeat all the steps below for each 50 mL tube.
4. Vortex mix for 30 s and sonicate the suspension at 7 W for 1 min.
5. Centrifuge at 10,000 x g, 4ºC for 10 min.
6. Discard the supernatant and resuspend in 50 mL of 0.01 M EDTA solution, 4°C (in order to chelate calcium).
7. Vortex mix for 30 s and sonicate the suspension at 7 W for 1 min.

8. Centrifuge at 10,000 x g, 4ºC for 10 min.
9. Discard the supernatant and resuspend in 50 mL of 0.05 M PIPES buffer, pH 7.0, at 4°C.
10. Vortex mix for 30 s and sonicate the suspension at 7 W for 1 min.
11. Centrifuge at 10,000 g, 4°C for 10 min and discard the supernatant.
12. Repeat the centrifugation at 10,000 g, 4ºC for 10 min and discard any remnants of the supenatant using a 20 µL pipette.
13. Weigh the 50 mL tubes to obtain the weight of the bacterial pellets.
14. To each 50 mL tube, add 1 or 10 mM Ca (from $CaCl_2$)-containing 0.05 M PIPES buffer, pH 7.0, at 37°C as the pretreatment solution, at a proportion of 150 µL/mg bacteria.
15. Vortex mix for 30 s and maintain the tubes at 37°C for 10 min to allow calcium binding to *S. mutans*.
16. Centrifuge at 10,000 g, 4°C for 10 min and discard the supernatant.
17. Resuspend in about 10 mL of 0.05 M PIPES buffer with the same pretreatment solution (1 or 10 mM Ca), pH 7.0, at 37 °C.
18. Vortex mix for 30 s and transfer aliquots of 0.9 mL to pre-weighted 1.5-mL microcentrifuge tubes to harvest bacterial pellets of aproximately 80 mg.
19. Centrifuge the microcentrifuge tubes at 21,000 x g, 4 °C for 5 min to expel all the liquid from the bacterial pellet.
20. Carefully discard the supernatant under microscope using a vaccum aspiration system and glass micropipettes.
21. Repeat steps 19 and 20.
22. Weigh the microcentrifuge tubes.

NOTES

Livia Tenuta 17 Dec 2017

In order to obtain about 80 mg of bacterial pellets, the volume of PIPES buffer mentioned in step 17 and the volume to be aliquoted in microcentrifuge tubes mentioned in step 18 should be previously determined in pilot studies, according to the bacterial yield.

Calcium release at decreasing pHs

Step 3.

1. Obtain the weight of the *S. mutans* pellet in each microcentrifuge tube.
2. To each tube, add 0.05 M PIPES buffer pH 7.0, 0.5 M acetate buffer pH 5.0 or 0.5 M HCl (pH 1.86) at 37 °C as treatment solution (all these solutions should containing the same calcium concentration of the pretreatment condition: 1 or 10 mM), at a proportion of 30% of the treatment solution per bacterial weight.
3. Vortex mix for 30 s and maintain the tubes for 10 min in a dry block at 37°C to study the Ca release at low pHs.
4. Centrifuge the microcentrifuge tubes at 21,000 x g, 4°C for 5 min.
5. Collect the supernatant to determine calcium release to treatment solution.

Calcium determination in the treatment solution

Step 4.

1. Determine calcium concentration using the Arsenazo III reaction (Vogel et al., 1983) in 96-well microplates.
2. Prepare a standard curve for each treatment solution (pH 7.0, 5.0 or HCl), as described in the tables below.

3. Prepare the samples as described in tables below, according to the tested pH.
4. Read the absorbance of the mixtures in a microplate reader at 650 nm.
5. Calculate calcium concentration using linear regression.

Table 1: Standard curve for calcium determination in PIPES buffer, pH 7.0.

	Blanck	0.025 mM Ca std	0.05 mM Ca std	0.1 mM Ca std	0.2 mM Ca std	0.3 mM Ca std
0.05 M PIPES buffer	150 µL	137.5 µL	125 µL	100 µL	50 µL	0 µL
0.3 mM Ca stock solution	0 µL	12.5 µL	25 µL	50 µL	100 µL	150 µL
Arsenazo III reagent	150 µL					

Table 2: Calcium determination in PIPES buffer pH 7.0 samples.

	Samples originally containing 1 mM Ca	Samples originally containing 10 mM Ca
Pre-dilution in 0.05 M PIPES buffer	-	10 x
PIPES 0.05 M	135 µL	135 µL
Sample	15 µL	15 µL
Arsenazo III reagent	150 µL	

Table 3: Standard curve for calcium determination in 0.5 M acetate buffer.

	Blanck	0.075 mM Ca std	0.15 mM Ca std	0.3 mM Ca std	0.6 mM Ca std	1.2 mM Ca std
Purified water	130 µL					
Respective Ca std prepared in 0.5 M acetate buffer	20 µL					
Arsenazo III reagent	150 µL					

Table 4: Calcium determination in acetate buffer pH 5.0 samples.

	Samples originally containing 1 mM Ca	Samples originally containing 10 mM Ca
Pre-dilution in 0.5 M acetate buffer	-	20 x
Acetate 0.5 M	135 µL	135 µL
Sample	15 µL	15 µL
Arsenazo III reagent	150 µL	

Table 5: Standard curve for calcium determination in 0.5 M HCl.

	Blanck	0.075 mM Ca std	0.15 mM Ca std	0.3 mM Ca std	0.6 mM Ca std	1.2 mM Ca std
Purified water	100 µL					
0.5 M NaOH	25 µL					
Respective Ca std prepared in 0.5 M HCl	25 µL					
Arsenazo III reagent	150 µL					

Table 6: Calcium determination in 0.5 M HCl samples.

	Samples treated with 1 mM Ca	Samples treated with 10 mM Ca
Pre-dilution in 0.5 M HCl	-	20 x
Purified water	100 µL	100 µL
0.5 M NaOH	25 µL	25 µL
0.5 M HCl	20 µL	20 µL
Sample	5 µL	5 µL
Arsenazo III reagent	150 µL	