Kinetics of calcium binding and release from S. mutans

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Abstract

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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 uL 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.

Obtaining 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 ressuspend the whole bacterial pellets in 100 mL of 0.05 M PIPES buffer, pH 7.0, at 4°C (use 2 x 50-mL 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, 4°C for 10 min.
6. Discard the supernatant and ressuspend 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, 4 °C 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 x g, 4 °C for 10 min and discard the supernatant.
12. Resuspend in 20 mL of 0.05 M PIPES buffer, pH 7.0, at 4°C.
13. Vortex mix for 30 s and transfer aliquots of 0.4 mL to pre-weighted 1.5-mL microcentrifuge tubes to harvest bacterial pellets of aproximately 10 mg.
14. Centrifuge the microcentrifuge tubes at 21,000 x g, 4 °C for 5 min to expel all the liquid from the bacterial pellet.
15. Carefully discard the supernatant under microscope using a vaccum aspiration system and glass micropipettes.
16. Repeat steps 14 and 15.
17. Weigh the microcentrifuge tubes.

NOTES

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In order to obtain about 10 mg of bacterial pellets, the volume of PIPES buffer mentioned in step 12 and the volume to be aliquoted in microcentrifuge tubes mentioned in step 13 should be previously determined in pilot studies, according to the bacterial yield.

Kinetics of calcium binding to S. mutans

Step 3.

1. Obtain the weight of the *S. mutans* pellet in each microcentrifuge tube.
2. To each tube, add 1 or 10 mM Ca (from $CaCl_2$)-containing 0.05 M PIPES buffer, pH 7.0, at 37°C as the treatment solution, at a proportion of 150 µL/mg bacteria.
3. Vortex mix for 30 s and maintain the tubes at 37°C for 5, 10, 30 or 60 min in a dry block to study the kinetics of binding as a function of time.
4. As a control, treat additional tubes with calcium-free 0.05 M PIPES buffer, pH 7.0, at 37°C, as described in steps 2 and 3 above, for 60 min.
5. After each specific time, centrifuge the microcentrifuge tubes at 21,000 x g, 4°C for 5 min.
6. Carefully discard the supernatant under microscope using a vaccum aspiration system and glass micropipettes.
7. Centrifuge the microcentrifuge tubes again at 21,000 x g, 4 °C for 5 min.
8. Repeat step 5.
9. Determine calcium bound to the bacterial pellets.

NOTES

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Pellets can be stored at -20 ºC before acid extraction to determine bound calcium.

Kinetics of calcium release from S. mutans

Step 4.

1. Obtain the weight of the *S. mutans* pellet in each microcentrifuge tube.
2. To each tube, add 1 or 10 mM Ca (from $CaCl_2$) containing 0.05 M PIPES buffer, pH 7.0, at 37 °C as the treatment solution, at a proportion of 150 µL/mg bacteria.
3. Vortex mix for 30 s and maintain the tubes for 10 min in a dry block at 37 °C to allow calcium binding to *S. mutans*.
4. Centrifuge the microcentrifuge tubes at 21,000 x g, 4°C for 5 min.

5. Carefully discard the supernatant under microscope using a vaccum aspiration system and glass micropipettes.
6. Repeat steps 4 and 5.
7. Add calcium-free PIPES buffer at 37°C to each pellet at proportion of 150 µL/mg of bacteria.
8. Vortex mix and maintain the tubes in a dry block at 37°C for 10, 30 or 60 min, to study the kinetics of calcium release as a function of time.
9. In half of the bacterial pellets previously treated with 10 mM Ca, add 1 mM Ca containing 0.05 M PIPES buffer, pH 7.0, 37°C at proportion of 150 µL/mg of bacteria, vortex mix, and also maintain in a dry block at 37°C for 10, 30 or 60 min.
10. After each specific time, centrifuge the microcentrifuge tubes at 21,000 x g, 4°C for 5 min.
11. Carefully discard the supernatant under microscope using a vaccum aspiration system and glass micropipettes.
12. Centrifuge the microcentrifuge tubes again at 21,000 x g, 4°C for 5 min.
13. Repeat step 11.
14. Determine calcium bound to the bacterial pellets.

NOTES

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Pellets can be stored at -20 ºC before acid extraction to determine bound calcium.

Extraction of calcium bound to S. mutans

Step 5.

1. Add 0.5 M HCl to each bacterial pellet at a proportion of 0.1 mL/10 mg.
2. Vortex mix until the pellet is completelly suspended in the acid.
3. Maintain under agitation in a rotating mixer for 3 h at room temperature; at frequent intervals (approximately 30 min), vortex mix the samples.
4. Centrifuge the microcentrifuge tubes at 10,000 x g, for 3 min, at room temperature.
5. Collect the supernatant to mensure calcium in the acid extract.

Calcium determination in the acid extract

Step 6.

1. Determine calcium concentration using the Arsenazo III reaction (Vogel et al., 1983) in 96-well microplates.
2. The standard curve can be prepared as described in the table 1 below.
3. Samples can be prepared as described in table 2 below.
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 0.5 M HCl extract.

	Blanck (purified water)	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
Arseanzo III reagent	150 µL

Table 2: Calcium determination in 0.5 M HCl extract.

	Samples treated with 0 mM Ca (control)	Samples treated with 1 mM Ca	Samples treated with 10 mM Ca
Purified water	100 µL	100 µL	100 µL
0.5 M NaOH	25 µL	25 µL	25 µL
0.5 M HCl	0 µL	0 µL	15 µL
Sample	25 µL	25 µL	10 µL
Arsenazo III reagent		150 µL	