Validation of protocol to assess calcium binding to S. mutans

Livia Tenuta,Tarcísio Leitão,Jaime A. Cury

Abstract

Citation: Livia Tenuta,Tarcísio Leitão,Jaime A. Cury Validation of protocol to assess calcium binding to S. mutans. **protocols.io**
dx.doi.org/10.17504/protocols.io.kuqcwvw
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 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 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 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 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, 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 g, 4°C for 10 min and discard the supernatant.
12. Resuspend in about 10 mL of 0.05 M PIPES buffer, pH 7.0, at 4 °C.
13. Vortex mix for 30 s and transfer aliquots of about 1.4 mL and 0.9 mL to pre-weighted 1.5-mL microcentrifuge tubes to harvest bacterial pellets of aproximately 120 mg and 80 mg, respectively.
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

Livia Tenuta 16 Dec 2017

In order to obtain about 120 mg or 80 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.

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. In the 1 mM Ca treatments, use 15 µL/mg bacteria; in the 10 mM Ca treatments, use 7.5 µL/mg bacteria.
3. Vortex mix for 30 s and maintain the tubes at 37°C for 60 min in a dry block to allow complete calcium binding to S. mutans.
4. Centrifuge the microcentrifuge tubes at 21,000 x g, 4°C for 5 min.
5. Collect the supernatant to a new tube to determine calcium concentration.
6. Repeat step 4.
7. Carefully discard remnants of the supernatant under microscope using a vaccum aspiration system and glass micropipettes.
8. Determine calcium bound to the bacterial pellets after acid extraction.

NOTES

Livia Tenuta 16 Dec 2017

The final bacterial pellet obtained may be stored at -20ºC until the acid extraction of bound calcium. The supernatant may also be stored at -20ºC until determination of calcium concentration.

Calcium determination in the treatment supernatant

Step 4.

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 PIPES supernatant.

	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
Arseanzo III reagent	150 µL					

Table 2: Calcium determination in PIPES supernatant.

	Samples originally containing 0 mM Ca (control)	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	0	135 µL	135 µL
Sample	150 µL	15 µL	15 µL
Arsenazo III reagent	150 µL		

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 measure 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 microplate.
2. The standard curve can be prepared as described in the table 3 below.
3. Samples can be prepared as described in table 4 below.

4. Read the absorbance of the mixtures in a microplate reader at 650 nm.
5. Calculate calcium concentration using linear regression.

Table 3: 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 4: 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	