Biofilm growth with starch treatment V.1

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protocol .

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Protocol to grow mature, mineralised oral biofilm with starches.

Modified from protocol by Sissons et al.:

Sissons, C. H., Cutress, T. W., Hoffman, M. P., & Wakefield, J. S. J. (1991). A Multi-station Dental Plaque Microcosm (Artificial Mouth) for the Study of Plaque Growth, Metabolism, pH, and Mineralization: *Journal of Dental Research*. https://doi.org/10.1177/00220345910700110301

Bjorn Bartholdy, a.g.henry 2021. Biofilm growth with starch treatment. **protocols.io** https://protocols.io/view/biofilm-growth-with-starch-treatment-bu7jnzkn

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The preferred substratum for inoculation is glass or hydroxyapatite. Plastic substrata can be used but are less effective, so surface treatment of the plastic is recommeded (e.g. heated HCl or acetone).

Substrata should be autoclaved prior to use, both before and during the experiment.

Solutions
Artificial saliva

Artificial saliva
by Bjorn Bartholdy
PREVIEW RUN

CPMU

CPMU
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PREVIEW RUN

20% (v/v) sterile glycerol in dH_2O
5% (w/v) sucrose in dH_2O
0.25% (w/v) potato starch in dH_2O
0.25% (w/v) wheat starch in dH_2O
0.50% (w/v) equal parts wheat (0.25%) + potato (0.25%) in dH_2O

Equipment
24 deepwell polypropylene plates (w. lid containing pegs suspended from the lid)
Shaking incubator

Saliva donor criteria

- Must have no/limited history of dental caries
- Must not have used antibiotics in the past 6 months
- Abstain from oral hygiene 24 hours prior to donation
- Refrain from eating and drinking (except water) 2 hours before donation

For experiments involving starches, donors should avoid eating starch-containing foods on the day of donation. To make this more bearable, saliva donation should take place in the morning before breakfast.

Saliva collection

1 Saliva donors rinse their mouth with water for 30 seconds.

2 Stimulate saliva production by chewing tasteless gum or parafilm.

3 Collect the saliva by spitting into 50 ml plastic centrifuge tubes.

Make sure donors wear gloves to avoid contamination with non-oral bacteria.

4 Make a 2-fold dilution of saliva in **sterile** [M]**20 % (v/v) glycerol** and vortex the solution.

Day 0: Inoculation and feeding

5 Before inoculation, vortex the saliva solution again.

6 Pipette the saliva solution into the wells, so approx. 1-2 cm of the substratum is submerged.

7 Place the plate in the incubator at 36°C for 4 hours for static inoculation **04:00:00** 4h

8 After inoculation, transfer the samples to a new plate containing the artificial saliva, and place in a shaking incubator at **30 rpm, 36°C** for **04:00:00** 4h

Artificial saliva
by Bjorn Bartholdy

PREVIEW RUN

8.1 Add **300 mL** distilled (or deionized) dH_2O to a **1000 mL** beaker, with stirring and heat

60 °C .

8.2 Add:

Mucin from porcine stomach (Type III) **Sigma**
- **2.5 g** **Aldrich Catalog #M1778**

Trypticase™ Peptone **Thermo**
- **5 g** **Fisher Catalog #211921**

Oxoid™ Proteose Peptone **Thermo**
- **10 g** **Fisher Catalog #LP0085B**
- **5 g** Bacto Yeast Extract **Becton-Dickinson**

Let the reagents completely dissolve before continuing to the next step

8.3 Add:

- **2.5 g** KCl **Contributed by users**
- **0.35 g** NaCl **Contributed by users**
- **0.2 g** CaCl2 **Contributed by users**

Sodium phosphate dibasic **Sigma**
- **0.74 g** **Aldrich Catalog #7558-79-4**
- **0.54 g** NaHCO3 **Contributed by users**
- **2.5 mg** Hemin **Contributed by users**

8.4 Add the remaining **700 mL** distilled (or deionized) dH_2O

8.5 Adjust to pH **7** with NaOH **Contributed by users** and stirring

8.6 Transfer to two 1000 ml bottles, so half of each bottle is filled. 15m

Autoclave at **121 °C** , **1 Bar** for **00:15:00** minutes

Do NOT screw bottle caps on tightly.

Loosely screw the caps on the bottles or cover the tops with foil

8.7 Once the solution has cooled, add:

- **1 mg** Menadione **Contributed by users**
- **0.3 g** Urea **Contributed by users**
- **0.17 g** L-Arginine **Contributed by users Catalog #A5006**

8.8 Store in fridge at ca. **4 °C**

Occasionally test the pH to ensure it stays around pH **7**

9 6m
Transfer the samples to a plate containing a 5% (m/v) Sucrose **Contributed by users** solution for **00:06:00** , then transfer back to the artificial saliva and leave **Overnight** .

Day 1-2: Feeding 8h 12m

10 6m
First thing in the morning, transfer the samples to a new plate containing a 5% (m/v) Sucrose **Contributed by users** solution for **00:06:00** . While in the sucrose solution, add more artificial saliva to the wells on the original plate that have been partially depleted overnight.

11 8h
After the 6 mins. return the samples to the plate with **artificial saliva**, and cover up the sucrose plate and leave for **08:00:00** .

12 6m
After 8 hours, transfer the samples back to the plate with 5% (m/v) Sucrose **Contributed by users** solution for **00:06:00** . Transfer back to the artificial saliva and leave **Overnight** . Dispose of the sucrose.

Day 3: Inoculation and feeding 8h

13 Repeat steps from saliva collection and Day 0: Inoculation and feeding. 8h

A layer of clear plaque should be visible on the substrata

14 Prepare a new plate with artificial saliva. Transfer the samples from the inoculation plate to the **artificial saliva**.

Day 4: Feeding 8h

15 Repeat steps 10 through 12 8h

go to step #10

Prepare a new plate of artificial saliva every third day throughout the experiment. Every other morning, top up the wells with artificial saliva (so ca. 1-2 mm of the substratum is submerged).

Day 5: Inoculation

16 Repeat steps 1 through 9

go to step #1

Day 6-8: Feeding

17 Repeat steps 10 through 12

go to step #10

Day 9-14: Starch treatment 8h 6m

18 Transfer the samples to a plate with the starch treatment(s) for **00:06:00** at 6m

60 rpm, 36°C

If you are using multiple starch treatments within a single plate, take care to avoid cross-contamination.

19 Transfer the samples back to the artificial saliva plate for **08:00:00** at **30 rpm, 36°C** 8h

20 Transfer the samples to a plate with the starch treatment(s) for **00:06:00** at **60 rpm, 36°C** 6m

21 Transfer the samples back to the artificial saliva plate at **30 rpm, 36°C** and leave **Overnight**

Day 15-24: Mineralisation 8h 30m

22 Transfer the samples to a plate containing the calcium phosphate monofluorophosphate urea (CPMU) solution for **00:06:00** at **60 rpm, 36°C** 6m

CPMU
by Bjorn Bartholdy
PREVIEW RUN

22.1 Add **300 mL** distilled (or deionized) dH_2O to a **1000 mL** beaker, with stirring and heat **60 °C**

22.2 Add:

- **1.55 g** Calcium Chloride **Contributed by users**
- **1.44 g** Sodium Phosphate monobasic **Contributed by users**
- **0.72 g** Sodium Fluorophosphate **Sigma – Aldrich Catalog #344443**
- **0.08 g** Magnesium Chloride **Fisher Scientific Catalog #AC223210010**
- **30 g** Urea **P212121**

22.3 Add the remaining **700 mL** and keep stirring until precipitate has completely dissolved

Store in fridge at **4 °C**

23 Transfer the samples back to the **artificial saliva** for **02:00:00** at **30 rpm, 36°C** 2h

Put a lid on the plate with CPMU (or cover with foil) to prevent evaporation.

Repeat step 22 and 23, four more times every two hours.

24 Transfer the samples to a plate with the **starch treatment(s)** for **00:06:00** at **60 rpm, 36°C** 6m

25 Transfer the samples back to the **artificial saliva plate** at **30 rpm, 36°C** and leave **Overnight**

Analysis

26 Samples should be dried before sampling.

Transfer to a new plate with no liquid and leave in the incubator.

27 Once dried, samples are processed like archaeological samples.