Molecular Assays

Version 1.9

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PCR

Protocol adapted Macarena Toll-Riera and Promega

1. Harvest bacterial cells to run PCR on
   - Liquid culture:
     1. Transfer 20 µL of bacterial culture into a centrifuge tube containing 80 µL of sterile MilliQ
     2. Centrifuge at maximum speed for 3 minutes
     3. Discard supernatant and resuspend pellets in 50 µL sterile MilliQ
   - Colonies: Barely touch the colony with a pipette tip, place in 50 µL of sterile MilliQ and pipette up and down a few times
2. Incubate at 95°C for 10 minutes in heating block
3. Centrifuge at maximum speed for 5 minutes
4. Store at 4°C
5. Perform PCR using Promega's GoTaq reagents
   1. Prepare a master mix for n + 1 reactions of 25 µL each. This master mix will have a final primer concentration of 200 nM. Primer and water volume can be adjusted if necessary, also to accomodate more DNA template volume
      - 5.00 µL 5X GoTaq Green Buffer
      - 0.50 µL dNTP Mix (10 µM)
      - 0.125 µL GoTaq G2 Polymerase
      - 0.50 µL forward primer (10 µM)
      - 0.50 µL reverse primer (10 µM)
      - 18.00 µL nuclease-free water
   2. Add 24.00 µL of master mix to PCR tubes and add 1.00 µL DNA template to each
   3. Gently vortex and then briefly centrifuge reactions
   4. Start PCR program (cycle through steps 2-4 30 times; this will take just under 2 hours)
      1. Initial Denaturation for 2 min at 95°C
      2. Denaturation for 30 s at 95°C
      3. Annealing for 30 s at T_m – 5°C
         - Use lower T_m of each primer pair to calculate annealing temperature
         - Higher temperatures make annealing more specific
      4. Elongation for 1 min/kb at 72°C
      5. Final Elongation for 5 min at 72°C
      6. Hold at 4°C
6. Run gel as a control that amplification worked
   1. Prepare 1% agarose gel
      1. Add 1g of agarose to 100 mL 1x TAE buffer inside erlenmeyer flask and microwave at standard settings for 1 minute
      2. Add 10 µL of SYBR Safe to the gel chamber
      3. Pour gel in small gel chamber with the comb appropriate for the number of samples. Use the comb to ensure SYBR Safe is properly mixed with the liquid
   2. Load 10 µL of a 1 kb ladder and PCR reaction directly into the gel (or 5 µL if using small wells)
   3. Run gel at 120V for 30 minutes and image it
   4. If PCR did not work, the DNA might have been too concentrated. Retry after dilluting the sample

16S rRNA Sequencing

1. Perform PCR according to PCR
   - Use one of the following primer pairs. Melting temperatures according to the Promega calculator specifically for use with GoTaq G2 Green Master Mix and 200 nM primers

Primer Pair	Specificity	Source
16s_fw + 16s_rv	*Ph*TAC125 & *E. coli*	Toll-Riera et al. 2022
F27 + R1525	universal, two bands for *Ph*TAC125	Lane et al. 1991, Stackebrandt et al. 1993

Primer Pair	Annealing [°C]	Product [bp]	Elongation [s]
16s_fw + 16s_rv	60	1008	60
F27 + R1525	57	1528	90

Primer	Sequence	T_m [°C]
16s_fw	GATCATGGCTCAGATTGAACGC	65
16s_rv	AGGCACCAAACCATCTCTGG	65
F27	AGAGTTTGATCCTGGCTCAG	62
R1525	AAGGAGGTGATCCAGCCGCA	69

2. PCR cleanup according to one of the following options:
   - Use the Promega Wizard SV Gel and PCR Cleanup System or the Qiagen QIAquick PCR Purification Kit according to the respective manufacturer protocols. Elute with 30 µL of nuclease free water
   - According to protocol adapted from Macarena Toll-Riera
     1. Mix PCR cleanup reaction
        - 17 µL PCR product
        - 0.3 µL Exonuclease I
        - 0.3 µL Antarctic Phosphatase
        - 2.4 µL sterile MilliQ (to fill up volume)
        - Prepare a master mix for multiple reactions (n + 1) and add 3 µL to each tube
     2. Incubate samples in thermocycler or waterbath
        1. 15 minutes at 37°C
        2. 15 minutes at 80-85°C
        3. Hold at 4°C
3. Measure DNA concentration and purity using the GDC's NanoDrop. 260/280 (protein purity) and 260/230 (salt purity) should both be between 1.8 and 2.0. The latter often tends to be as small as 1.2, even after using a PCR cleanup kit
4. Calculate the necessary dilution factor to reach a final DNA concentration of 1.5 ng/µL per 100 bp of PCR product after adding primers. Taking this into account, the target DNA concentration before addition of primers should be:

$$\text{target concentration} = \frac{\text{product length } bp}{100\ bp} * 1.5 \frac{ng}{\mu L} * \frac{15\ \mu L}{12\ \mu L}$$

   E.g. for a 1.5 kb product the target concentration should 28.125 ng/µL
5. Prepare samples for sequencing by adding to each shipping tube
   - 12 µL purified PCR reaction at the appropriate DNA concentration
   - 3 µL 20 µM primer (just one, either forward or reverse)
6. Add Microsynth label to tube and register samples online. Print out order summary and package it in a clear plastic bag together with the samples
7. Bring samples to Microsynth pickup point at the main entrance of the LFV building

Additional Primers

Melting temperatures according to the Promega calculator specifically for use with GoTaq G2 Green Master Mix and 200 nM primers

Primer Pair	Specificity	Source
398078_fw + 398078_rv	*Ph*TAC125	Macarena Toll-Riera
Ml_rpoB_fw + Ml_rpoB_rv	*Micrococcus* spp.	Huang et al. 2019

Primer Pair	Annealing [°C]	Product [bp]	Elongation [s]
398078_fw + 398078_rv	58	346	30
Ml_rpoB_fw + Ml_rpoB_rv	58	891	60

Primer	Sequence	T_m [°C]
398078_fw	CATTTACTTACAGGGGCTCTGG	62.1
398078_rv	GCTCCGTGATTTCATCTCGC	60.5
Ml_rpoB_fw	ATGAACGCGACGAGGAGGTT	67
Ml_rpoB_rv	GTTCTTCGGCACCTC**S**C	63-64

References

- Huang, C.-H. et al. Reclassification of Micrococcus aloeverae and Micrococcus yunnanensis as later heterotypic synonyms of Micrococcus luteus. *Int J Syst Evol Microbiol* 69, 3512–3518 (2019).