**PBAD –FIMB PCR**

**Preparing template**

Procedure for preparing colony suspensions:

1. For each sample, pipette 250 μL sterile (ultrapure) dH2O into sterile eppendorf tube. Label tubes as appropriate
   a. *Use repeater pipette to speed up preparation*
2. Use a 200 μL pipette tip to pick a small piece of cryopreserved stock. Dip into sterile dH2O in eppendorf tube and resuspend. Vortex to mix. Repeat for each sample

**Need to prepare cell suspension for pBAD Freezer Location: iGEM Cryobox , cell #73**

**Preparing Materials**

1. Templates
   **a.** See above
2. Re-suspend the PCR primers to 50 μM concentration. The primers are dessicated and shipped in plastic vials, so an appropriate amount of ultrapure dH2O needs to be added to each vial to bring the final concentration to 50 μM Vials are labeled with the quantity of DNA they contain, in nanomoles (amount in nmoles x 20 = μL resuspension volume). Briefly vortex and touch centrifuge.
3.

| Primer | Nmoles | Resuspension volume (μL) |
|---|---|---|
| FIMB_fwd | 27.9 | 558 |
| FIMB_rev | 26.4 | 528 |

4. Gather other required PCR supplies. Buffers, dNTPs, Pfu polymerase are already pre-mixed and in the -20C freezer or 4C refrigerator. If frozen, thaw out at room temperature before use. Vortex after thawing. Spin down all materials prior to use (to collect small amounts of liquid at bottom of tubes)

Since we will be using the same reaction for each template, a PCR reaction master mix can be prepared as follows:

**FIMB master mix:**

| Component | Volume per 50 μL rxn | Master Mix (13x20μL rxns) | Final Concentration |
|---|---|---|---|
| H2O | 14.6 | 189.8 | - |
| 5x Phusion HF Buffer | 4 | 52 | 1x |
| 10 mM dNTPs | .4 | 5.2 | 200 μM each |

**Comment [a1]:** You might want to give a little background on what you are amplifying and what you plan on doing with it, if you have this earlier in your notes then it shouldn't matter

**Comment [a2]:** Are you using a cryopreserved stock or the isolated genomic DNA?

**Comment [a3]:** You want to make sure that the ultra pure water become just barely cloudy, that means you got the right amount of template

**Comment [a4]:** This reaction must be performed on ice since we do not have the hot start polymerase, you will need to chill 200 and 10 ul tips for further steps in the reaction along with the PCR tubes you will use for the reaction

**Comment [a5]:** Before you start mixing materials, turn on the PCR machine to instant incubate with a heated lid to your starting temperature so it is warmed up and ready to go, this is especially important when you have to start your pcr reaction on ice

**Comment [a6]:** Phusion

**Comment [a7]:** Do not thaw polymerase

**Comment [a8]:** You can vortex the buffers but you should only touch centrifuge the dntps and the primers, you do not want to shear the dna

**Comment [a9]:** Are you just checking to make sure the PCR works? You can lower this to a 20 uL volume and when you find the working PCR condition you can run a larger reaction volume for further genetic manipulation, this saves materials

| chb_fwd | 0.2 | 2.6 | 0.5 μM |
|---|---|---|---|
| miaA_hfq_rev | 0.2 | 2.6 | 0.5 μM |

5. Label PCR tubes using the following coding scheme:
    a. Color code: yellow tubes = FIMB rxn
    b. *Note: need 11 tubes total*
6. Carefully dispense .4 μL of appropriate cell suspension (K12 MG1655) into each PCR tube. Be sure to deposit cell suspension at bottom of tube. Put cap strips back on tubes as soon as possible after dispensing cell stocks (to prevent evaporation).
7. Add 19.4 μL of master mix to each PCR tube (to give 20 μL total volume).
8. Add 0.2 μL phusion high-fidelity DNA polymerase to each PCR tubes.
    a. ***Note: phusion polymerase has 3'->5' exonuclease activity; it must be added last to the PCR reaction, after template has been added. Add phusion polymerase to reaction mix w/o template can lead to primer degradation!***
9. Firmly cap all PCR tubes. Briefly vortex to mix, then spin down.
10. Setup the following program(s) on the thermal cycler:

| Cycle Step | Temperature | Time | Number of Cycles |
|---|---|---|---|
| Initial Lysis | 95C | 6:00 minutes | 1x |
| Initial Denaturation | 98C | 30s | 1x |
| Denaturation | 98C | 10s | 35x |
| Annealing | 66C (Tm+3C) | 15s | |
| Extension | 72C | 19s (25s/kb) | |
| Final Extension | 72C | 10 minutes | 1x |
| Hold | 4C | Hold | 1x |

11. Load PCR tubes into heating block of thermal cycler. Close lid and tighten down until snug (turn screw clockwise). Open lid, and then tighen further ½ turn clockwise, then close
    a. *Tightening the lid this way ensures that the lid is firmly pressing down on the PCR tubes – this prevents the tubes from popping open during the heating step*
12. Start the thermal cycling program. Choose 'yes' for heated lid, calculated temperature method, and enter 50 μL for reaction volume.

When all PCR reactions are finished, run gel to check results. Use 0.7% agarose, 98V TBE buffer for 45 minutes. **NOTE: MIX BLUEJUICE 1:20 OR LOWER! EG 0.5 μL BLUEJUICE + 9.5 μL PCR** (see electorphoresis protocol for more details).

**Comment [a10]:** If you are using the same template for every sample you can mix this in your master mix

**Comment [a11]:** After step 7 you need a step that chills the master mix before adding the polymerase for 15 minutes, this helps prevents unwanted amplification

**Comment [a12]:** Once again, if you are using the same template you can mix this in your master mix

**Comment [a13]:** Set up this program in the thermocycler before mixing reagents for the PCR so it is ready to go

PCR of FimB in K12 strain Gel 01 08-07-10 (Saturday)

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