Q (Sarah): What kind of wet lab deliverable is feasible at this late stage?
A (Isaac): We can digest the 6 library plasmids from the *B. subtilis* Protein Secretion Toolkit with BsaI and PmeI to prove we can cut the ~25 *B. subtilis* secretion tags out of each.

Received the *B. subtilis* Protein Secretion Toolkit from FreeGenes: https://stanford.freegenes.org/collections/all/products/b-subtilis-protein-secretion-toolkit.
Note: Purified plasmid DNA was in A1 - F1. A1 has a kanamycin resistance gene and B1 - F1 have ampicillin resistance genes.

Ordered *Bsa* I and *Pme* I from NEB.

October 6, 2021, Wednesday
BioBlaze lab work day, 4:00 pm, with Mike, Claudia, Shravan, Sakti and Sarah.
Transformed plasmids in A1 - F1 from FreeGenes into *E. coli* DH5α from NEB. Used 2 µl of A1, B1 and C1. No liquid in D1, E1 or F1, so added 5 µl water to these and then used 2 µl for transformation. Followed NEB protocol that came with competent cells. Plated 100 µl each: A1 on Nutrient Agar - (NA) with 100 ul kan 50 pipetted on top; B1 - F1 on LB agar + amp 100 (old plates at -4°C); untransformed DH5α on NA -. RT until 12 October, 2021.

**October 12, 2021, Tuesday**

(Sarah) Single colonies were obtained for A1 and lawns for B1 - F1 and untransformed DH5α. Therefore, the LB amp plates that were old seemed to not be selective.

**October 13, 2021, Wednesday**

BioBlaze lab work day, 4:00 pm, with Mike, Claudia, Shravan, Sakti, Mars and Sarah. Added 100 µl LB - to each plate, then scraped bacteria with pipette tips and inoculated: A1 into LB + kan 100, B1 - F1 into LB + amp and untransformed DH5α into LB - broth. Incubate/shake at 37°C overnight.

**October 14, 2021, Thursday**
(Sarah) Growth in all flasks. To confirm we are selecting only for cells transformed with the intended plasmids, inoculate 100 µl A1 - F6 onto both NA + amp and NA + kan plates.

October 15, 2021, Friday

(Sarah) A1 grew as a lawn as expected on LB + kan, but also grew unexpectedly as a lawn on LB + amp. Unclear whether there is also a gene for amp resistance on this plasmid?? B1 - B6 grew expectedly as a lawn on LB + amp but did not grow on LB + kan. Untransformed DH5α grew expectedly as a lawn on LB -. Added new NA medium with appropriate antibiotic to each flask. Incubate/shake at RT until the 17th.
October 17, 2021, Sunday

BioBlaze lab work day, 2:00 pm, with Shravan, Britney, Bruce and Sarah. Plan: Do plasmid DNA miniprep/Miraprep from A1 - F6 growing in flasks. Participants were asked whether they wanted to try to standard miniprep protocol from Bioneer or adapt it to the Miraprep protocol. They chose to follow the Bioneer protocol. Participants were also given the option of doing just A1 and B1 or all six, and they chose to do A1 and B1. Started with 3 X 1.5 ml tubes for A1 and for B1, each with 1,400 ul bacterial culture from flasks, then combined all three in the resuspension stage. Final elution in P5: 50 µl, rest one minute, spin 1 min., then repeat for a total of 100 µl.

Prepared 1 % agarose gel with Borax buffer. Loaded 8 ul plasmid prep + 3 ul 5X Orange g loading buffer + 1 ul Gel Red; 75 V for 15 mins. Note: saw no bands under UV. Either we didn’t load enough or the extraction was not successful or did not yield much.
Plan moving forward:

1) Even though no bands were visualized, go ahead and do the restriction digestion with Bsai and Pmel to prove we can cut the ~25 B. subtilis secretion tags out of each. See below.

2) Do another plasmid DNA extraction, this time modifying with the Miraprep protocol.

Restriction Digestion of A1 and B1 with Bsa I and Pme I.

Plasmid DNA 43 µl (should be 1 µg but we have no way to quantify this)
Pmel 1 µl (10 units)
Bsai-HFv2 1 µl (10 units)
10X rCutSmart Buffer 5 µl (1X)
Nuclease-free Water 0 µl

37°C for 15 mins.

October 20, 2021, Wednesday

BioBlaze lab work day, 4:00 pm, with Claudia, Sakti, Mike and Sarah. Did above restriction digestion using A1 and B1 plasmid preps from 17 October. Loaded 20 µl each mixed with 5 µl 5X orange G buffer plus Gel Red (100 µl Orange G + 1 µl 10,000X Gel Red). Result after 1 hr at 75 V: Very faint band for A1 digestion, but very strong detection for B1 digestion. See more of a smear than separation, but proof of concept in isolating plasmid DNA from B1.
Also tried Miraprep modifications for Bioneer kit in new plasmid extraction to hopefully yield more. Began with liquid LB + amp or kan for A1 and B1. 3 x 15 ml each. Centrifuged at ~3,000 rpm for 20 mins. at 4°C. Note: Followed Scott Pownall’s simplified protocol. Stored at -20°C.

October 21, 2021, Thursday

(Sarah) Loaded 8 ul A1 and B1 Mirapreps + 3 ul orange G + Gel Red onto 0.8% agarose gel. Result: Bright bands for both! Therefore, Miraprep was successful and yielded more than the Bioneer miniprep kit when following the kit’s instructions.

Digestion of Miraprep plasmid preps...

**Restriction Digestion of A1 and B1 with Bsa I and Pme I.**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA</td>
<td>20 µl (should be 1 µg but we have no way to quantify this)</td>
</tr>
<tr>
<td>PmeI</td>
<td>2 µl (10 units)</td>
</tr>
<tr>
<td>BsaI-HFv2</td>
<td>2 µl (10 units)</td>
</tr>
<tr>
<td>10X rCutSmart Buffer</td>
<td>5 µl (1X)</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>21 µl</td>
</tr>
</tbody>
</table>

<p>37°C for 30 mins.</p>

<p>Result after 15 mins. at 75 V.</p>
Result after 45 mins. at 75 V.

Result after 1 hr 15 mins. at 75 V.
It seems digestion has occurred, but it is not possible to see defined bands representing individual secretion tags.