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Version 2

Automated combinatorial media preparation V.2

Forked from a private protocol

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Protocol status: Working

We use this protocol and it's working

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Abstract

Although synthetic biology can produce valuable chemicals in a renewable manner, its progress is still hindered by a lack of predictive capabilities. Media optimization is a critical, and often overlooked, process which is essential to obtain the titers, rates and yields needed for commercial viability. Here, we present a molecule- and host-agnostic active learning process for media optimization that is enabled by a fast and highly repeatable semi-automated pipeline. Its application yielded 60% and 70% increases in titer, and 350% increase in process yield in three different campaigns for flaviolin production in *Pseudomonas putida* KT2440. Explainable Artificial Intelligence techniques pinpointed that, surprisingly, common salt (NaCl) is the most important component influencing production. The optimal salt concentration is very high, comparable to seawater and close to the limits that *P. putida* can tolerate. The availability of fast Design-Build-Test-Learn (DBTL) cycles allowed us to show that performance improvements for active learning are rarely monotonous. This work illustrates how machine learning and automation can change the paradigm of current synthetic biology research to make it more effective and informative, and suggests a cost-effective and underexploited strategy to facilitate the high titers, rates and yields essential for commercial viability.

Here we provide the protocol for automated media optimization on a flaviolin producing *Pseudomonas putida* KT2440 strain.



Protocol materials

⊗ Filter-sterilized HPLC-grade water

⊗ Iron (II) sulfate **Fisher Scientific Catalog #7782-63-0**

Safety warnings

! Wear PPE.

Before start

Make sure you booked the equipment in the calendar:

- 978-PRO-BIOLECTOR (4148) EQ and
- 978-4-BIOMEK-NX-S8_ SPECTRAMAX (4148) EQ (1) or
- 978-4-BIOMEK-NXP (4148) EQ (1)

Make sure you received proper training before operating on the Biomeks and Biolector.

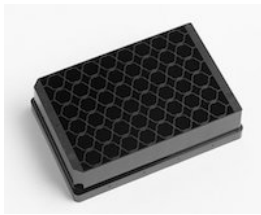
Have an overnight of your culture ready.

Required equipment/labware

1 Destination plate:



m2p flower plate
(for running in the Biolector)



m2p black bottom flower plate
(for running off the Biolector)

Water plate:



deep reservoir (available at Robotics lab)

Source plate (with stock components):



24-deep well x3

2 Liquid handler:

Equipment

new equipment

NAME

Beckman Coulter

BRAND

Biomek NXp

SKU

Pipette tips needed (available at Robotics lab):

- tips f200 (light green box)
- tips f20 (light blue box)

3 Fermentation platforms available:

- [Biolector]
- Biolector Pro or
- Multitron

4 Additional components:

- ☒ Filter-sterilized HPLC-grade water
- Kanamycin

To be prepared fresh for every cycle:

- ☒ Iron (II) sulfate **Fisher Scientific Catalog #7782-63-0**
- Overnight culture of *P. putida* **PP_5404::attB::pIS100**. (The base strain for this assay was simply KT2440 modified for PhiC31 integrations.)



Additional labware:

- 5mL, 10mL pipettes
- 3× 50mL tubes for FeSO₄
- filter, syringe for FeSO₄ sterilization
- 2× 2mL tubes for culture

Additional equipment:

- vortex
- centrifuge
- flame

Calculate stock concentrations

5 Input:

- total volume of the culture (we use  1500 µL as total volume)
- standard media recipe (e.g.  standard_recipe_concentrations.csv)

Run the notebook [A_Find_Stock_Concentrations.ipynb](#).

Prepare stock solutions

6 Use the list of components concentrations created in step 5 and prepare stock solutions. Ideally, you should prepare enough volume for all DBTL cycles.

Note

Steps 5 and 6 should be performed only once during a study and not for every DBTL cycle.

Create stock plates definitions

7 Run the notebook [B_Create_Stock_Plates.ipynb](#). Prepare the stock plates following the instructions resulting from the notebook by aliquoting the components into the appropriate wells of the source plate. A stock plate with culture and FeSO₄ should be prepared fresh for every cycle. The other two, with high and low level concentrations, can be used and refilled if needed for consecutive cycles.

Example output files:



- source plate instructions  24-well_stock_plate_high.csv
-  24-well_stock_plate_low.csv
-  24-well_stock_plate_fresh.csv

An example of how to prepare a stock of **Kanamycin**:

- Take 1000x concentration from the freezer
- If we need e.g.  1 mL of the 300x concentration in the stock plate, we need a 3.33 dilution, so  300 μ L of Kan and $1000-300=$  700 μ L of H₂O

Choose target media composition

- 8 E.g. by using ART. For the initial cycle you may run the notebook **C_Initial_Media_Designs.ipynb**. Once there is a training data set you may run the notebook **C_ART_Media_Designs.ipynb**.

Example output file:  target_concentrations.csv

Create files for biomek transfers and source plate instructions

- 9 Run the notebook **D_Create_Transfers.ipynb**. It will generate a file with volumes for all components for all wells, files for biomek that are used to define transfers of those volumes, and stock plate definitions with additional columns with required volumes for each stock component, for this particular run.

Example output files:

- destination volumes  dest_volumes.csv
- source plate instructions  24-well_stock_plate_high.csv
- biomek files  P20_components.csv  P20_culture.csv  P20_kan.csv
-  P20_water.csv  P200_components.csv  P200_water.csv

This notebook will also provide a number of tip boxes needed to perform the transfers. Most likely, the number of boxes will be larger than the number of slots on the Biomek deck, so the method will need to pause, which gives you time to refill the deck with tips.

Prepare the source plates

- 10 Make sure the stock plates with low and high concentration levels, prepared at step 7, have at least volume levels as defined in step 9.

2m



Safety information

Perform this step in a sterile environment (use flame).

Prepare the plate with fresh stocks:

FeSO₄:

- To prepare  20 mL of  60 millimolar (mM) stock, find molar mass of the iron-sulfate available and use a calculator to find mass in mg
- *Typical stock is FeSO₄*7H₂O aka the heptahydrated salt. 333.612mg heptahydrate / 20mL sterile H₂O to make 60mM stock*
- Measure and add FeSO₄*XH₂O into a 50 mL falcon tube, add  20 mL of H₂O, vortex
- *The solution may be cloudy before the filtration. This is because of some oxidized FeSO₄ (new insoluble Fe(III) species) in the solution. Do not be alarmed. This is a small proportion of the stock iron sulfate that was measured.*
- Use a sterile 0.2µm luer-lock syringe filter (or SteriFlip if available) to filter sterilize the 60mM solution into a new 50mL conical tube
- Find volume of this solution needed to create 10 mL of solution with target concentration of  0.3 millimolar (mM) using the formula:
$$V_{\text{stock}} = V_{\text{target}} \cdot C_{\text{target}} / C_{\text{stock}}$$
i.e. 10mL * 0.3mM / 60mM = 0.05 mL of FeSO₄
- Add  9.95 mL of H₂O to another (falcon) tube and  0.05 mL of FeSO₄, vortex, filter sterilize

Culture:

- Take 2mL of overnight culture and place into the 2mL tube (2x)
- Centrifuge the 2 2mL tubes of culture at  10000 rcf, 00:02:00
- Decant the supernatant
- Re-suspend the overnight culture with an equal volume MOPS minimal medium without carbon
- Place the resuspended culture in the appropriate well of the source plate

Biomek setup

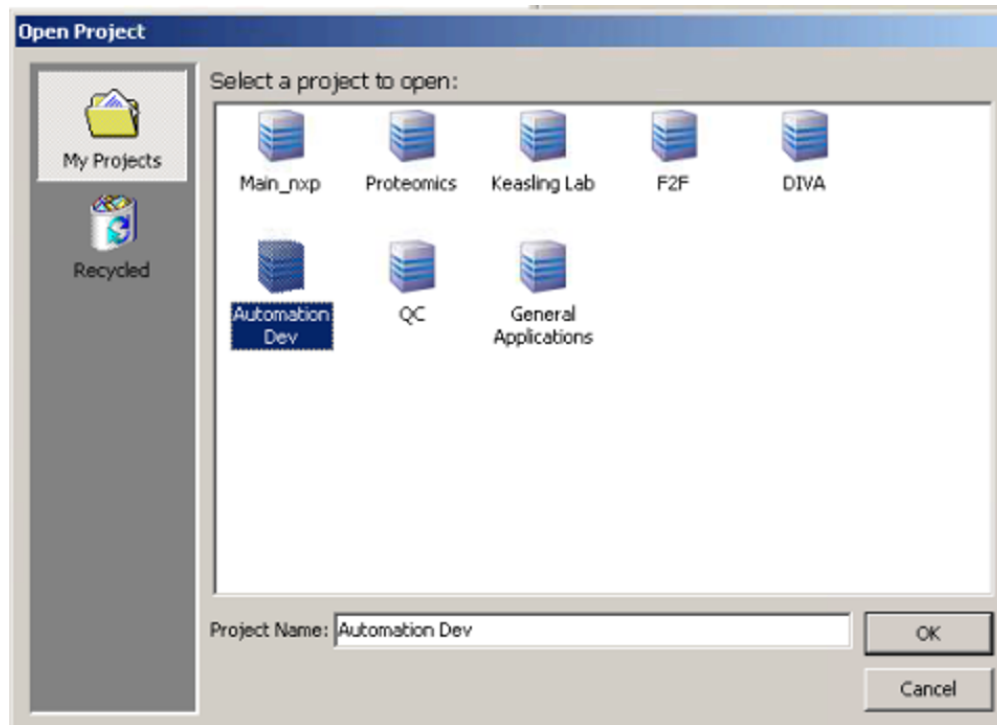
11

Note

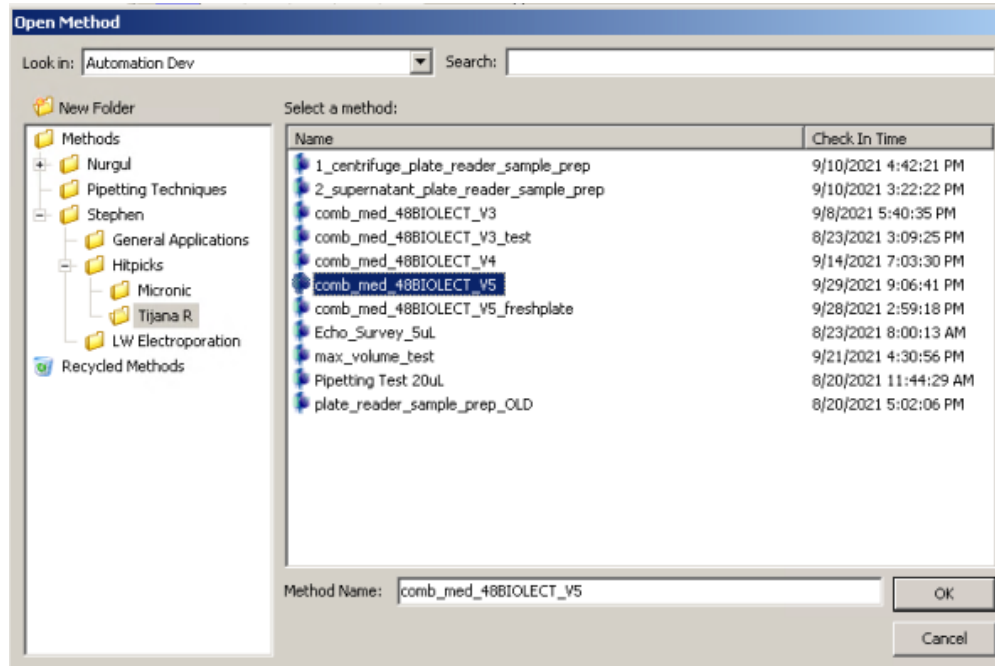
If you wish to set up a method in advance of a run, you may access the Biomek remotely.

- Follow the instruction provided in [this file](#).
- Request a password for a Windows Active Directory (AD) account from Arthur Panganiban (ahpanganiban@lbl.gov)
- Find your Biomek, use password **Robotp@ss978**

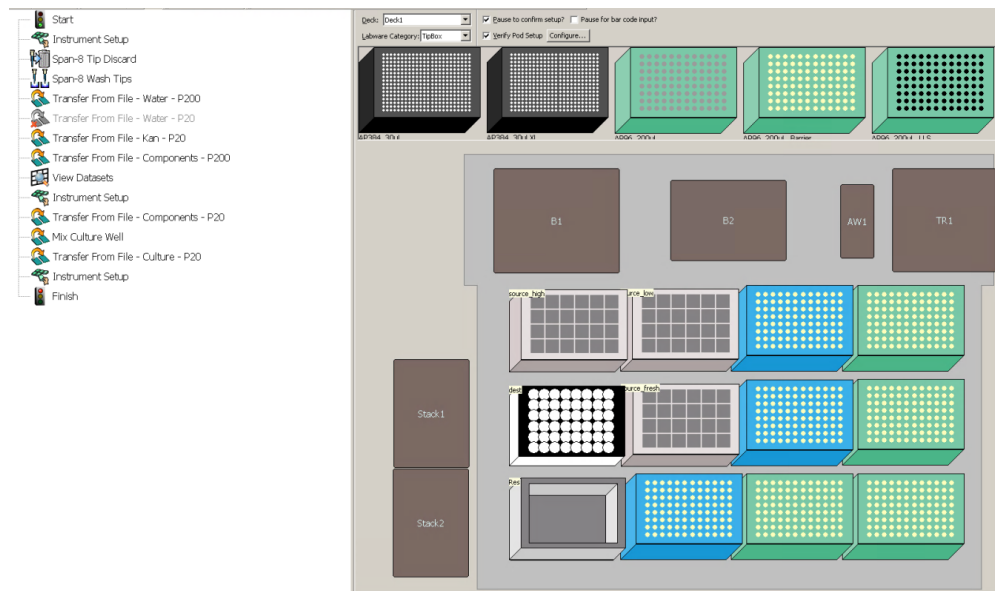
- 11.1 Open the Biomek Software application on the Biomek. At the top toolbar, select Project > Open Project to open the "Automation Dev" project.



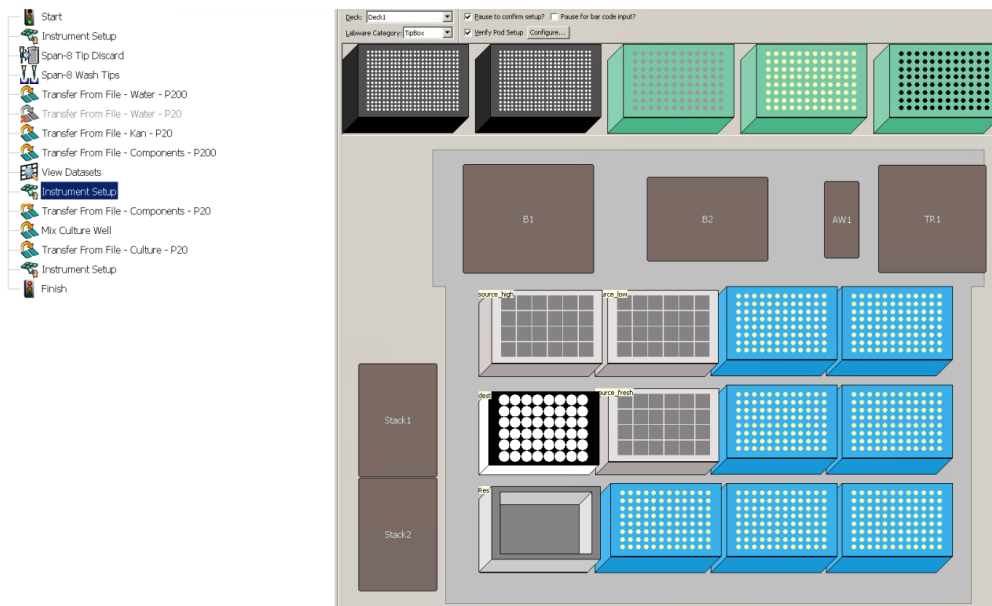
- 11.2 At the top toolbar of the Biomek Software, select File > Open to open the method "comb_media_48BIOLECT_V5"



- 11.3 Set up the deck as specified by the Instrument Setup pop-up. Check if the number of tip boxes corresponds to what was calculated in step 9, notebook **D_Create Transfers.ipynb**.

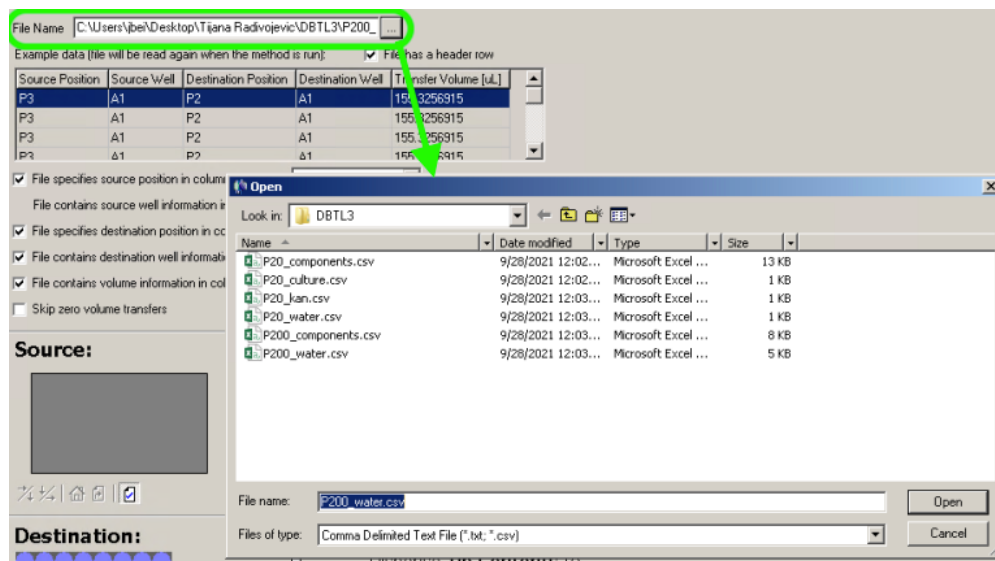


- 11.4 Check that the deck layout after the pause (second "Instrument Setup" step) corresponds to the number of tip boxes calculated in Step 9, notebook **D_Create Transfers.ipynb**.



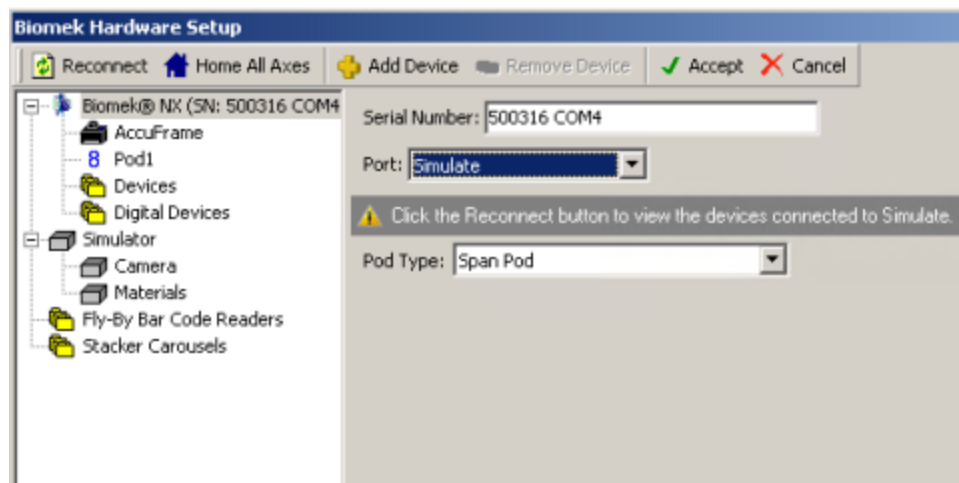
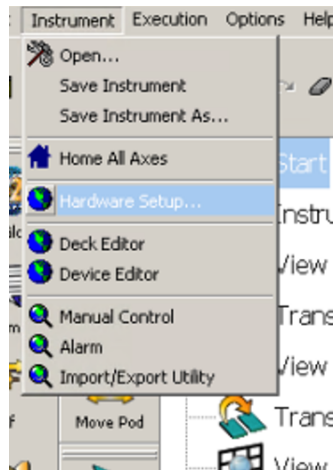
11.5 For each of the "Transfer From File" steps, upload the corresponding Biomek files created in Step 9.

For example:



Biomek simulation

12 To run the Biomek in the simulation mode choose the following:



Biomek run

13

Note

Do not start a Biomek run remotely if it is not in the Simulation mode.

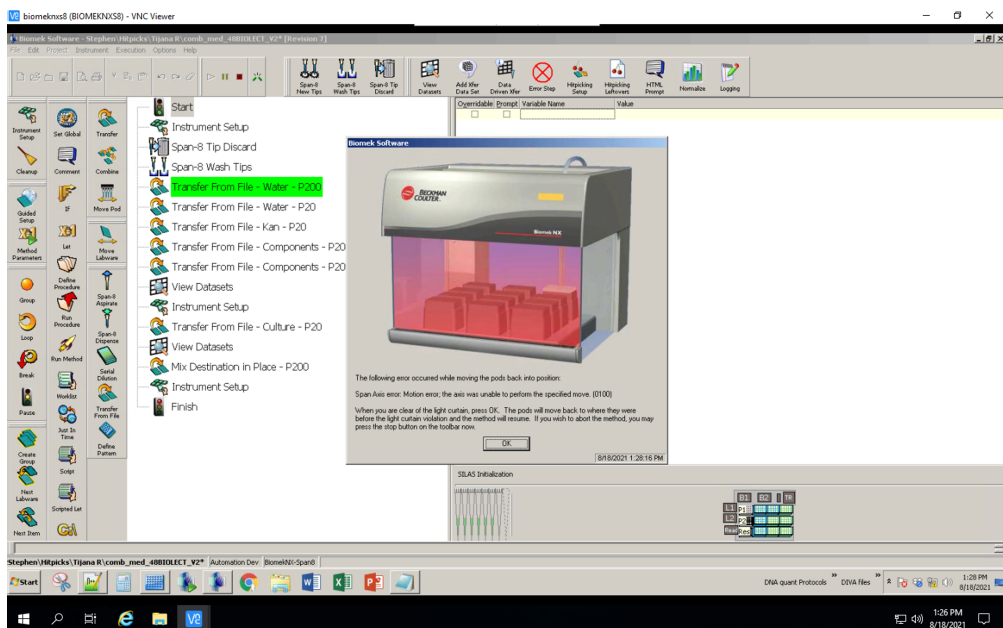
Hit the play button (green arrow) to run the method.



Note

Visually inspect if aspirations are happening and there is nothing suspicious. At the end inspect if all the wells have the same total volume.

- 13.1 During the run you may touch the light curtain to pause the Biomek. The following window will pop-up.



Biolector/Multitron run

14

2d

2d



Note

Before operating the BioLector get appropriate training.

On the Biolector, open the protocol labelled "matthew_flower".

Adjust the calibration "lot number" to that which corresponds to the sticker on the M2P biolector flower plate.

If the lot number is unavailable, import it and load it into the matthew_flower method.
(you should have been trained on this. if you can't remember how, email the current biolector superuser)

Place the plate inside the machine and start the method.

Temperature  30 °C

Humidity 80%

Length of the run  48:00:00

Shake Speed (800 rpm)

Filters:

- ID401 (620nm for biomass measurement)
- ID402 (pH measurement)
- ID403 (DO measurement)

15 On Multitron:

Temperature  30 °C

Shake speed 700RPM

Place plate in Infors Multitron

Absorbance assay

16 Follow a [protocol for absorbance assay](#) from an m2p plate.

Upload data into EDD

17 Run the notebook [E_Create_EDD_Study_Files.ipynb](#).



Once you create files for EDD import, follow a [protocol for EDD study creation](#) and a protocol for [importing data into EDD](#).