**STEP 3. Setting Up FBA Inputs**

1. Create a new folder within the “FBA-pipeline\Code + Models\fba\input” folder. For each FBA analysis you want to run, place a new copy of “\_TEMPLATE\_.xlsx” in this input folder, and provide each copy with a separate file name. The \_TEMPLATE\_ file provides instructions to the FBA code, and each tab in the file will be explained here.
2. Unless otherwise stated:
   * Options can be chosen by placing a capital “X” in yellow cells.
   * Custom reactions can be entered by inputting the reaction formula with Recon3D metabolite names.
     + Provide the cellular compartment for each metabolite (e.g. “nadph[c]”). To signify a reaction for every cellular compartment, wrap (“all( )”) around the reaction formula, and use empty compartment names for each metabolite (e.g. “nadph[]”).
     + Provide a numerical stoichiometry for each metabolite in the formula. Separate all stoichiometries and reaction names with one space.
     + Use “-->” for irreversible reactions, and “<=>” for reversible reactions.
3. **General** – Here you choose which type of analysis to perform and provide analysis parameters. Options for type of analysis are:
   * **Objective function value** – Only calculates the maximum objective function value for each sample
   * **Parsimonious FBA (pFBA)** – pFBA calculates the reactions fluxes which minimize the total sum of fluxes while maintaining some fraction of the maximum objective function value. Required options are:
     + Fraction of maximum objective value (between 0 and 1). Typically want 1, but may need to choose some value very close to 1 (e.g. 0.9999) for optimization software to find solution.
   * **Flux variability analysis (FVA)** – FVA calculates the minimum and maximum flux for each reaction while maintaining some fraction of the maximum objective function value. This can be performed for “All reactions” or “Select reactions”. Required options for both are:
     + Fraction of maximum objective value (between 0 and 1). Typically want 1, but may need to choose some value very close to 1 (e.g. 0.9999) for optimization software to find solution.

For select reactions, can choose any number of premade groups (e.g. all reactions which reduce NADP+ to NADPH), and can name individual Recon3D reactions of interest.

* **Uniform sampling** – Currently not implemented for Recon3D.
* **Media sensitivity** – Not completed.

1. **Constraints – Proliferation** – Custom constraints on the biomass production rate of samples relative to experimental values denoted in the sample “Clinical” files. Values here are the factor multiplied by experimental values.
2. **Constraints – Value** – Custom constraints on the value of select reactions.
3. **Constraints – Fraction** – Custom constraints on the value of select reactions, expressed as a fraction of maximum possible flux value (FVA is used to find this maximum value).
4. **Constraints – Max Multi** – Implement a custom constraint simultaneously maximizing multiple reaction fluxes. Relative weights for each reaction can be provided.
5. **Objective Function** –Choice of objective function, either a preset Recon3D reaction or new custom reaction. Multiple objective functions can be simultaneously maximized with provided relative weights.
6. **Samples – TCGA** –Choose TCGA samples to run based on cancer type, primary tumors versus normal tissue, and available sample information.
7. **Samples –** **CCLE** – Choose CCLE samples to run based on cancer type and available sample information.
8. **Samples – GTEx** – Choose GTEx samples to run based on tissue type.
9. **Samples – Other** – Choose individual samples to run, either within the TCGA/CCLE/GTEx datasets or from other datasets. “Folder Name” and “Sample Name” are the names of the folder and sample file within the ““FBA-pipeline\Code + Models\data\vmax\” folder, respectively.
10. **Drugs** – Restrict samples to only those with responses for particular drugs in the “Clinical” file.
11. **Mutations** – Decide whether sample mutation information should be used to alter reaction kcat values (based on Envision scores).
12. **Media** – Choose cell culture media formulation to simulate. Multiple formulations can be chosen (e.g. both “RPMI-1640” and “FBS”). Specific media uptake constraints can be set for individual metabolites.
13. **Concentrations** – Choose whether to use default thermodynamic parameters, or recalculate based on different default concentration ranges and sample metabolite concentration ranges.
14. **Modules** – Choose (a) whether to implement NADPH dependence on catalase reactions; and (b) whether to implement drug metabolism modules.
15. **Gene Knockdown** – Choose whether to knock down a particular metabolic gene for all samples. Knockdown fraction is the fractional protein abundance to use (e.g. “0.25” is 75% knockdown, “0” is complete knockout).
16. To implement a gene knockdown screen (test effect of knocking down each individual gene in Recon3D):
    * Create a \_TEMPLATE\_ file will all user inputs except for “Gene Knockdown”. Place file in the “\_create\_gene\_knockdown\_screen\_” folder.
    * Open “\_create\_gene\_knockdown\_screen\_.m” in MATLAB, enter user inputs:
      + Name of \_TEMPLATE\_ file
      + Desired output folder name
      + Desired output file name (separate \_TEMPLATE\_ files will be generated for each gene knockdown, with file name “\*CHOICE\*\_geneX.xlsx”, where X is the number.
      + Knockdown fraction
    * Run “\_create\_gene\_knockdown\_screen\_.m”. A new input folder will be created with separate \_TEMPLATE\_ files for each gene knockdown.
17. To implement a metabolome-wide metabolite production screen:
    * Create a \_TEMPLATE\_ file will all user inputs except for “Objective Function”. Place file in the “\_create\_objective\_function\_screen\_” folder.
    * Open “\_create\_objective\_function\_screen\_” in MATLAB, enter user inputs:
      + Name of \_TEMPLATE\_ file
      + Desired output folder name
      + Desired output file name (separate \_TEMPLATE\_ files will be generated for each metabolite, with file name “\*CHOICE\*\_X.xlsx”, where X is the metabolite.
    * Run “\_create\_objective\_function\_screen\_.m”. A new input folder will be created with separate \_TEMPLATE\_ files for each metabolite.
18. To implement an objective function screen for chemotherapy response:
    * Create a \_TEMPLATE\_ file will all user inputs except for “Objective Function”. Place file in the “\_create\_drug\_objective\_screen\_” folder.
    * Open “\_create\_drug\_objective\_screen\_” in MATLAB, enter user inputs:
      + Drug name (either “5fu”, “cis”, “cpa”, or “dox”)
    * Run “\_create\_drug\_objective\_screen\_.m”. A new input folder will be created with separate \_TEMPLATE\_ files for each objective function.