“Multi-Agent” Screening Improves Directed Enzyme Evolution by Identifying Epistatic Mutations

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Supplemental Figures

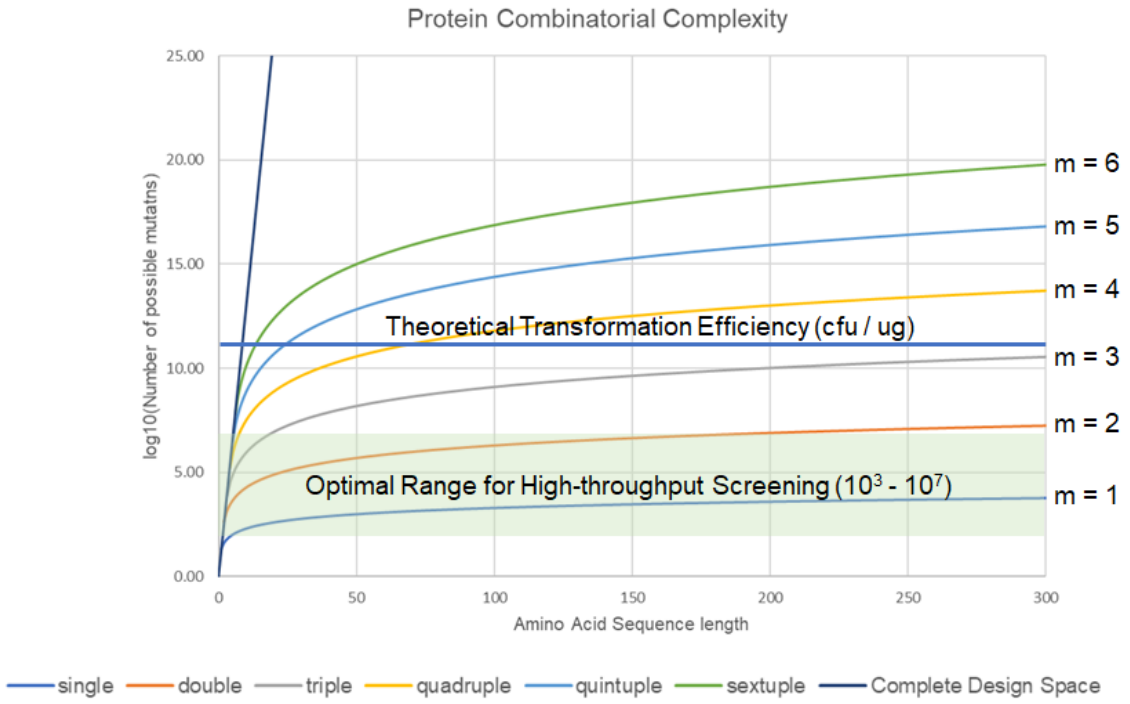


Figure S1: The Complexity of Protein Design Space with Different Criteria. The complexity of complete design space grows rapidly out of the range for high-throughput screening at very small sequence length. The curves designated with an m value represent the complexity of the m-mutant space at different sequence length, with only single- and double-mutant space manageable for the high-throughput screening capacity. The theoretical transformation efficiency is also labeled in the plot for reference.

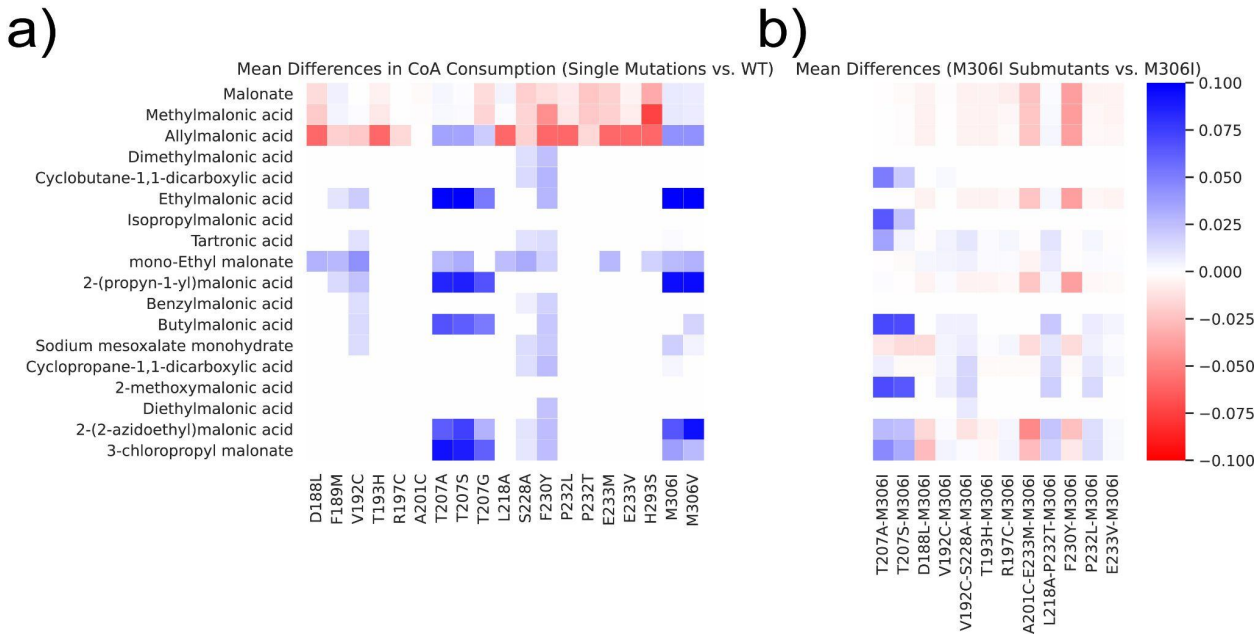


Figure S2. Mutation effects on different substrates given the **a)** WT sequence and **b)** M306I in the confirmatory activity assay. T207A/S/G all have a positive effect on some of the substrates given the WT sequence, and were missed in our initial screening, potentially due to sampling bias and/or assay variation. On average, 4.6 mutations would be found to have a positive effect given the WT sequence by screening only one substrate, ranging from 0 (for isopropylmalonic acid and 2-methoxymalonic acid) to 12 (for mono-ethyl malonate).

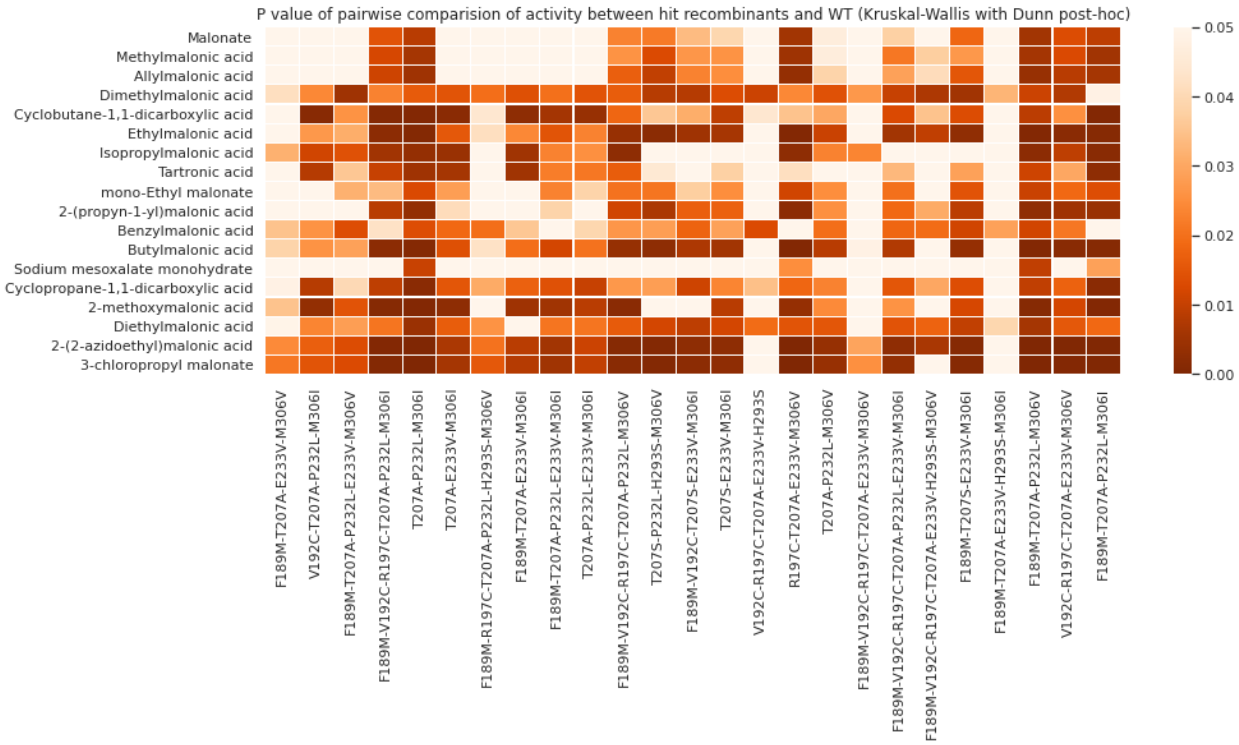


Figure S3. Statistical significance of comparison of activity between hit recombinants and WT. All hit recombinants show significantly improved activity on many substrates.

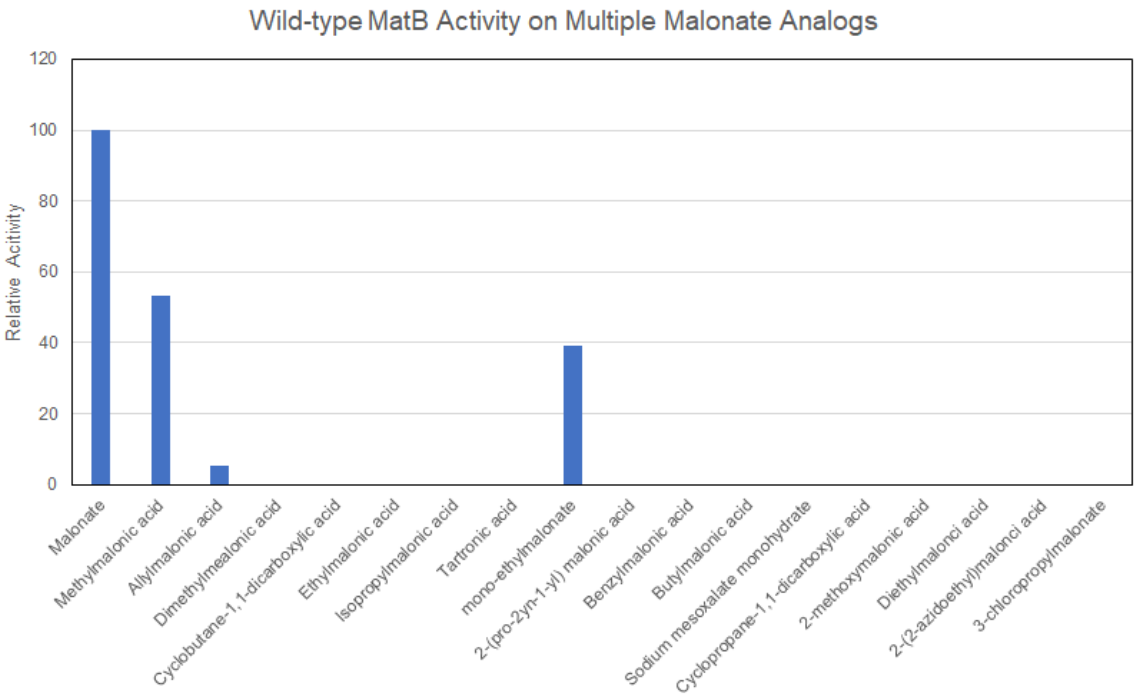


Figure S4. WT MatB Screening Activity Profile on the Selected Panel of Substrate Analogues.

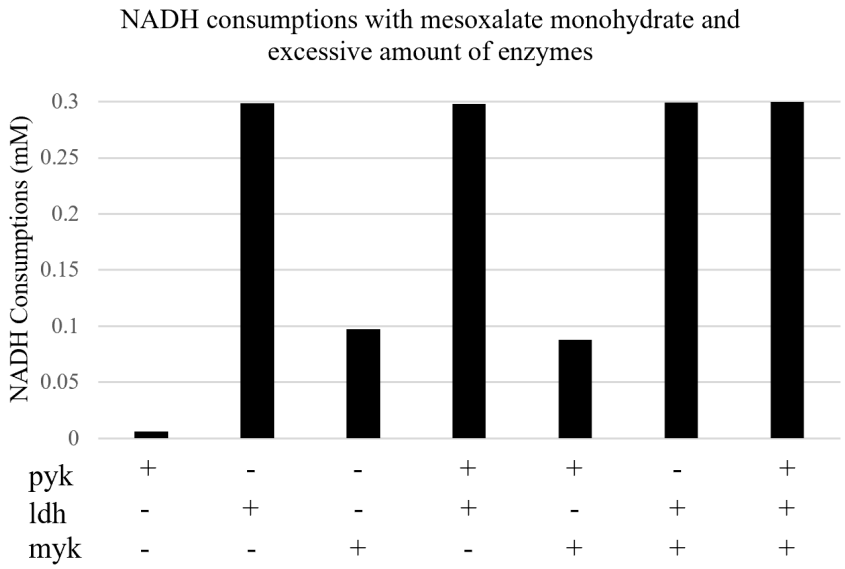
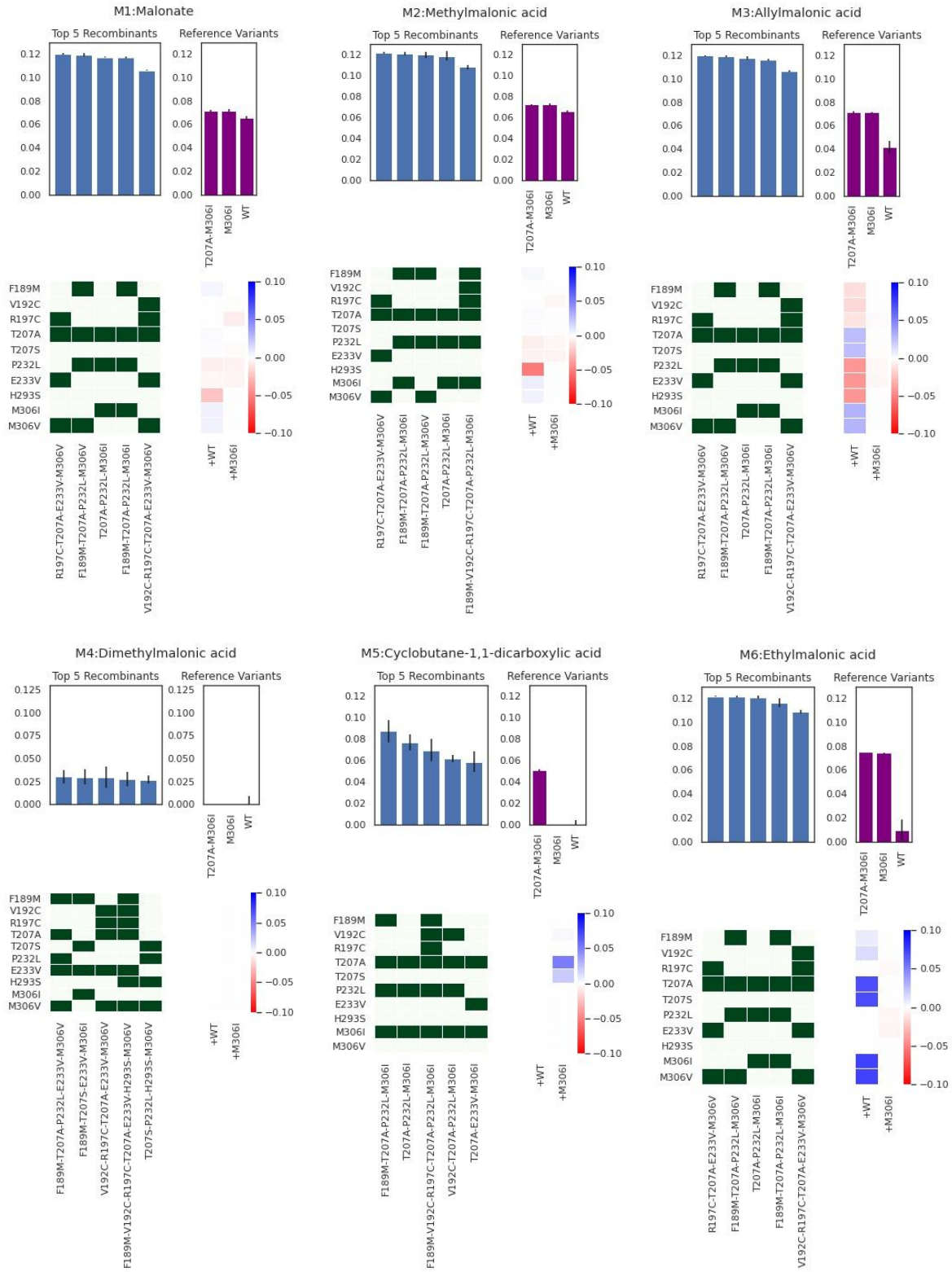
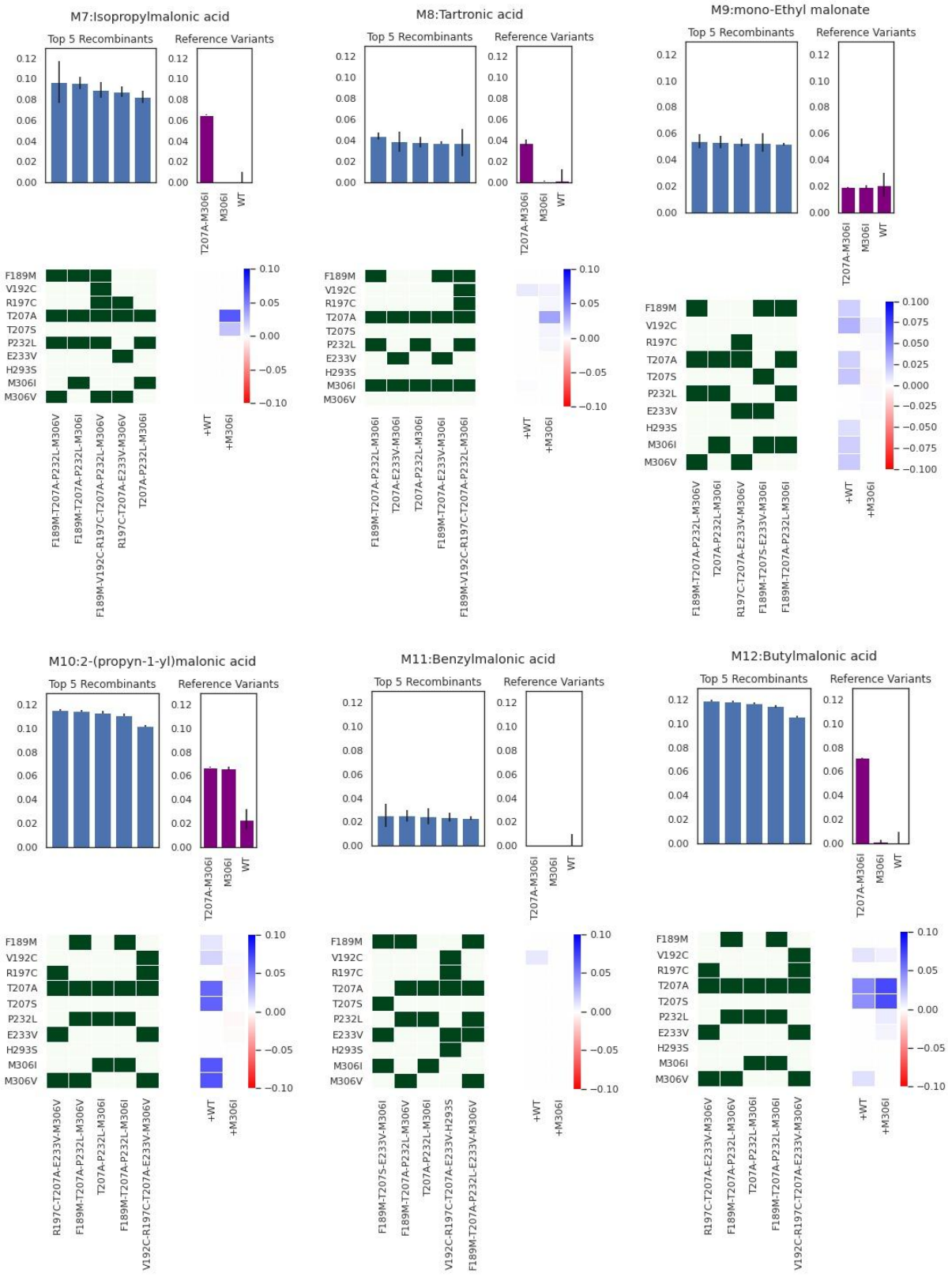


Figure S5: Reaction of Mesoxalic acid with NADH in the presence of coupling enzymes. The formation of AMP in the synthetase (matB) reaction is coupled to NADH oxidation by purified myokinase, pyruvate kinase, and lactate dehydrogenase, which can be monitored at 340 nm. Abbr.: pyk (pyruvate kinase), ldh (lactate dehydrogenase), myk (myokinase).


M1:Malonate
Top 5 Recombinants
Reference Variants
T207A-M306I
M306I
WT
F189M
V192C
R197C
T207A
T207S
P232L
E233V
H293S
M306I
M306V
R197C-T207A-E233V-M306V
F189M-T207A-P232L-M306V
T207A-P232L-M306I
F189M-T207A-P232L-M306I
V192C-R197C-T207A-E233V-M306V
+WT
+M306I
M2:Methylmalonic acid
F189M-V192C-R197C-T207A-P232L-M306I
M3:Allylmalonic acid
M4:Dimethylmalonic acid
F189M-T207A-P232L-E233V-M306V
F189M-T207S-E233V-M306I
V192C-R197C-T207A-E233V-M306V
F189M-V192C-R197C-T207A-E233V-H293S-M306V
T207S-P232L-H293S-M306V
M5:Cyclobutane-1,1-dicarboxylic acid
F189M-T207A-P232L-M306I
T207A-P232L-M306I
V192C-T207A-P232L-M306I
T207A-E233V-M306I
M6:Ethylmalonic acid


M7:Isopropylmalonic acid
Top 5 Recombinants
Reference Variants
T207A-M306I
M306I
WT
F189M
V192C
R197C
T207A
T207S
P232L
E233V
H293S
M306I
M306V
F189M-T207A-P232L-M306V
F189M-T207A-P232L-M306I
F189M-V192C-R197C-T207A-P232L-M306V
R197C-T207A-E233V-M306V
T207A-P232L-M306I
+WT
+M306I
M8:Tartronic acid
F189M-T207A-P232L-M306I
T207A-E233V-M306I
T207A-P232L-M306I
F189M-T207A-E233V-M306I
F189M-V192C-R197C-T207A-P232L-M306I
M9:mono-Ethyl malonate
F189M-T207A-P232L-M306V
T207A-P232L-M306I
R197C-T207A-E233V-M306V
F189M-T207S-E233V-M306I
F189M-T207A-P232L-M306I
M10:2-(propyn-1-yl)malonic acid
R197C-T207A-E233V-M306V
F189M-T207A-P232L-M306V
T207A-P232L-M306I
F189M-T207A-P232L-M306I
V192C-R197C-T207A-E233V-M306V
M11:Benzylmalonic acid
F189M-T207S-E233V-M306I
F189M-T207A-P232L-M306V
T207A-P232L-M306I
V192C-R197C-T207A-E233V-H293S
F189M-T207A-P232L-E233V-M306V
M12:Butylmalonic acid
R197C-T207A-E233V-M306V
F189M-T207A-P232L-M306V
T207A-P232L-M306I
F189M-T207A-P232L-M306I
V192C-R197C-T207A-E233V-M306V

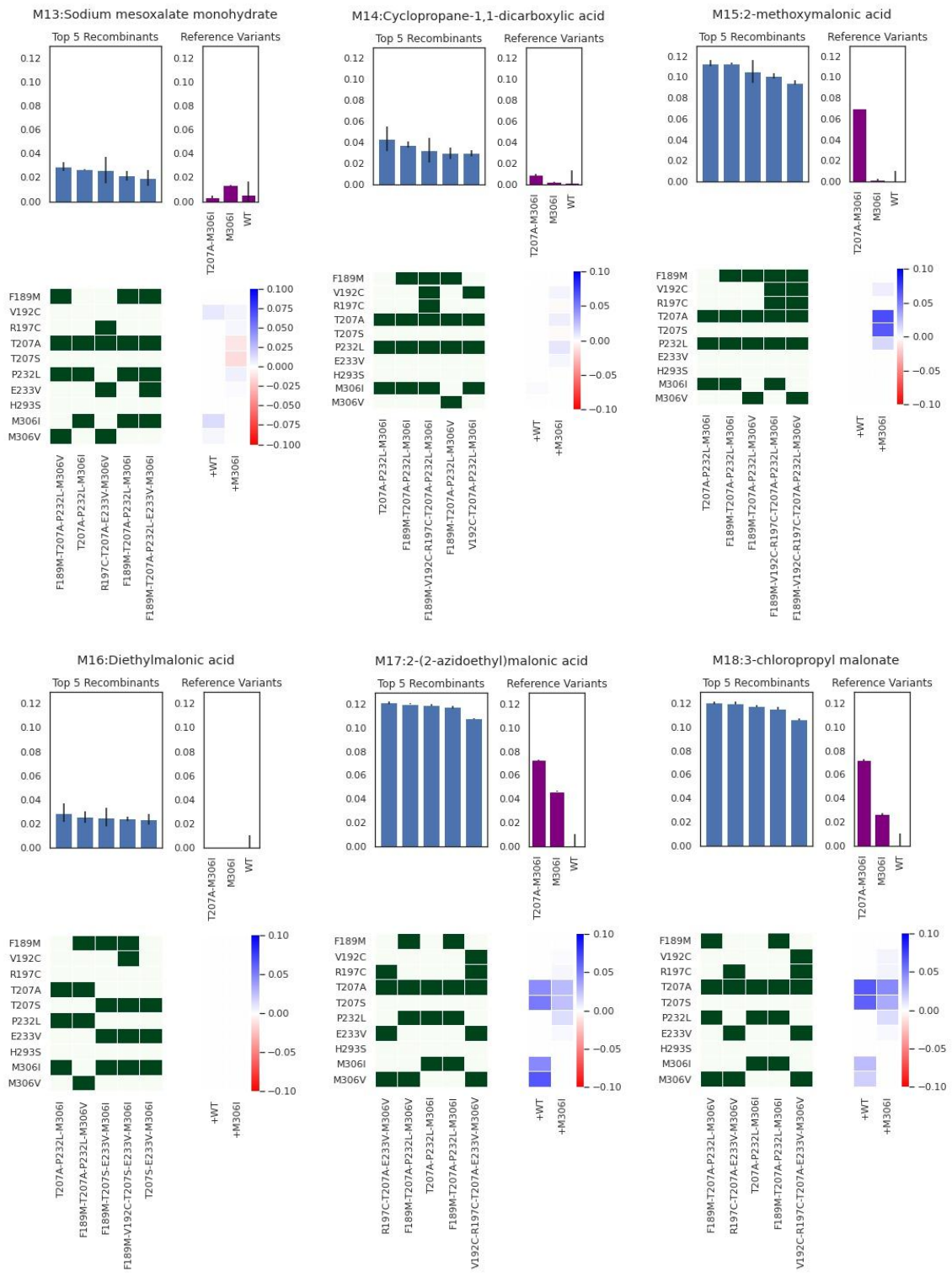
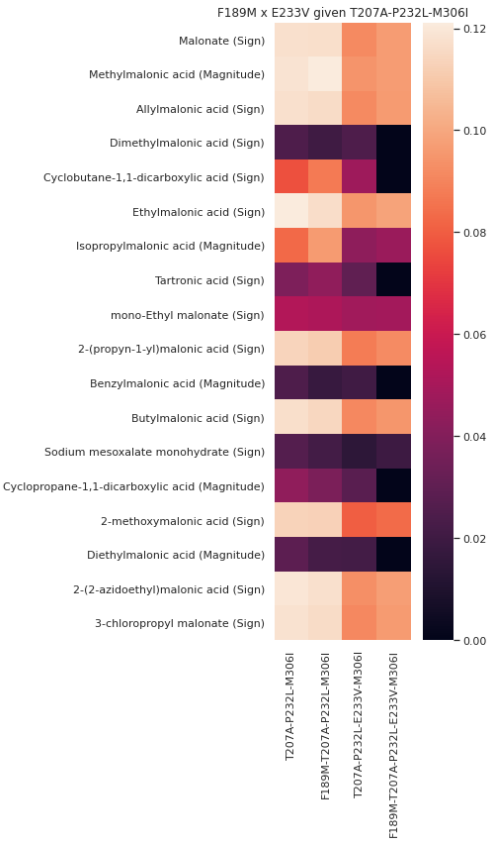
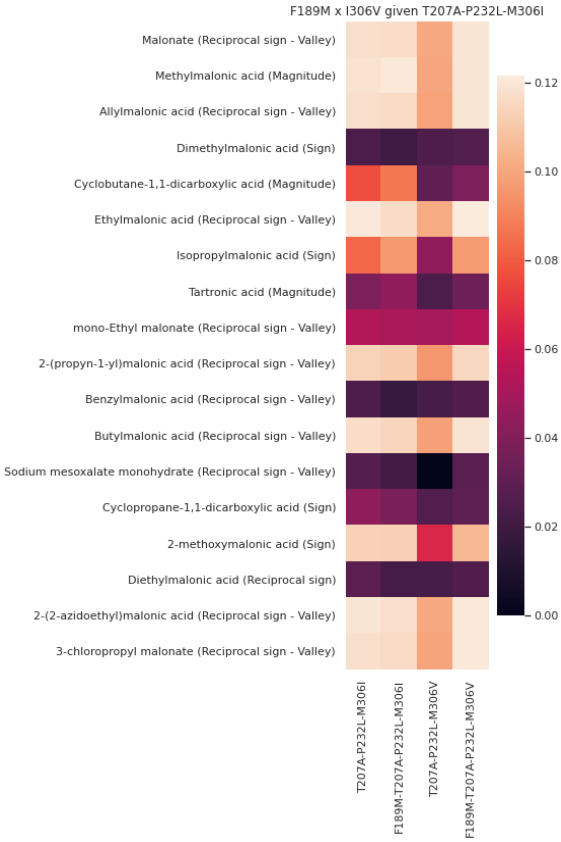
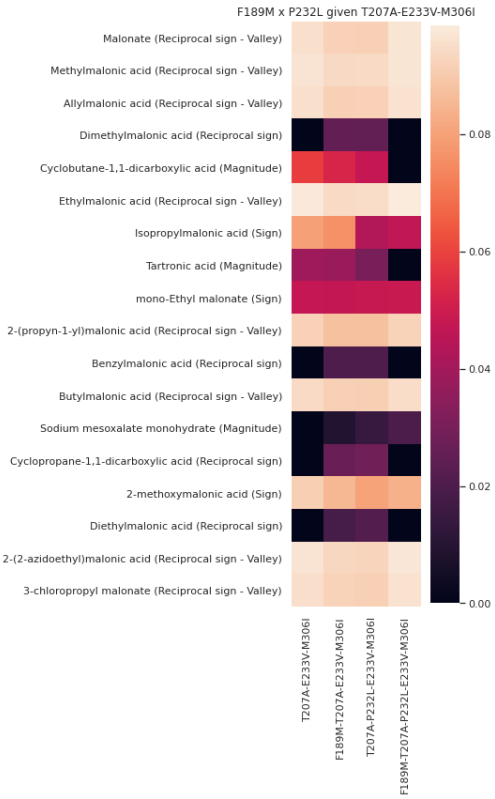
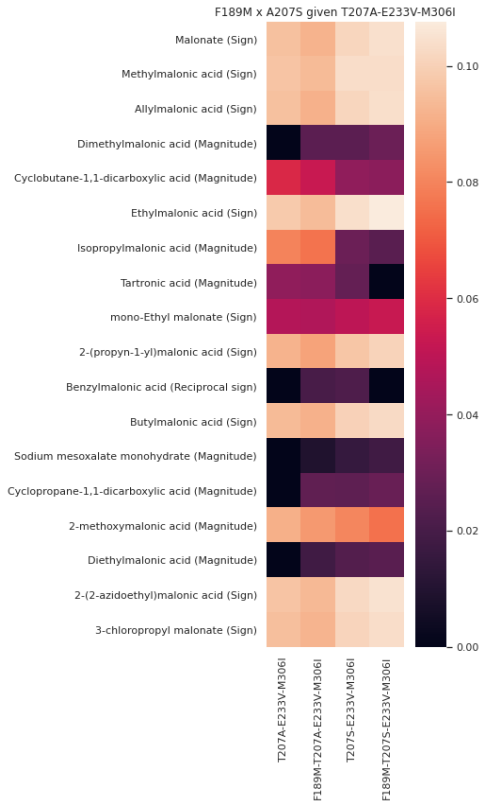


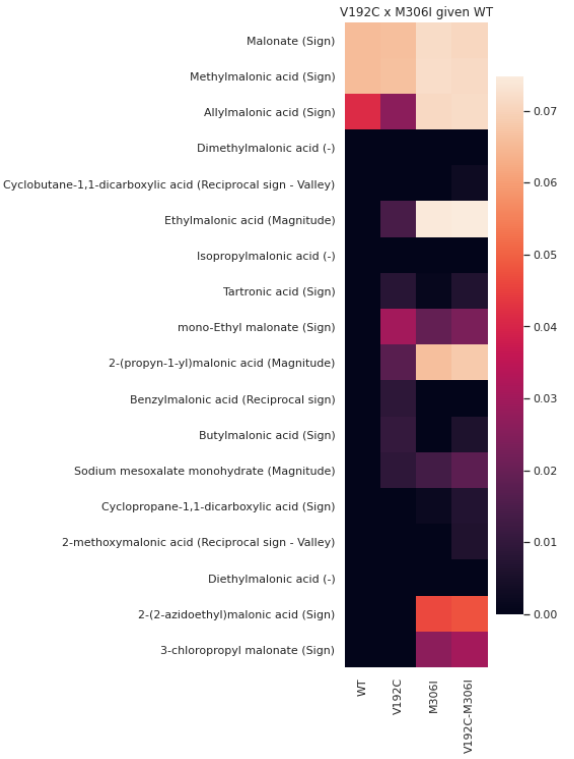
Figure S6. The top 5 active recombinants for all substrates contain mutations that are not directly beneficial given the WT or the M306I sequence.

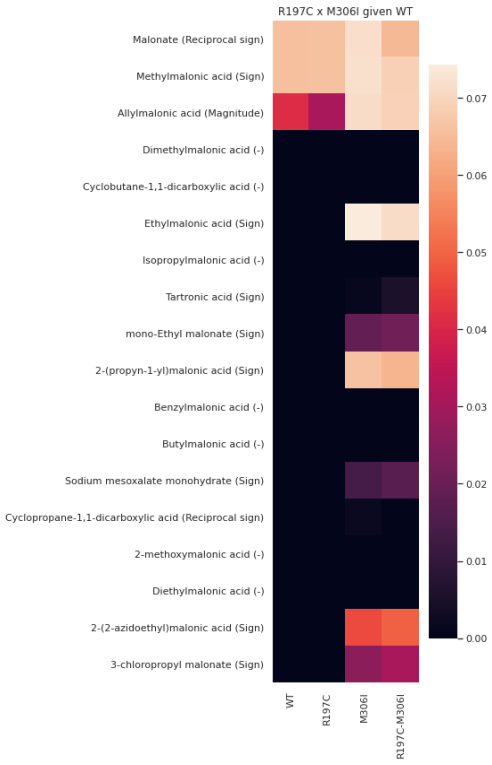

F189M x E233V given T207A-P232L-M306I
Malonate (Sign)
Methylmalonic acid (Magnitude)
Allylmalonic acid (Sign)
Dimethylmalonic acid (Sign)
Cyclobutane-1,1-dicarboxylic acid (Sign)
Ethylmalonic acid (Sign)
Isopropylmalonic acid (Magnitude)
Tartronic acid (Sign)
mono-Ethyl malonate (Sign)
2-(propyn-1-yl)malonic acid (Sign)
Benzylmalonic acid (Magnitude)
Butylmalonic acid (Sign)
Sodium mesoxalate monohydrate (Sign)
Cyclopropane-1,1-dicarboxylic acid (Magnitude)
2-methoxymalonic acid (Sign)
Diethylmalonic acid (Magnitude)
2-(2-azidoethyl)malonic acid (Sign)
3-chloropropyl malonate (Sign)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
T207A-P232L-M306I
F189M-T207A-P232L-M306I
T207A-P232L-E233V-M306I
F189M-T207A-P232L-E233V-M306I

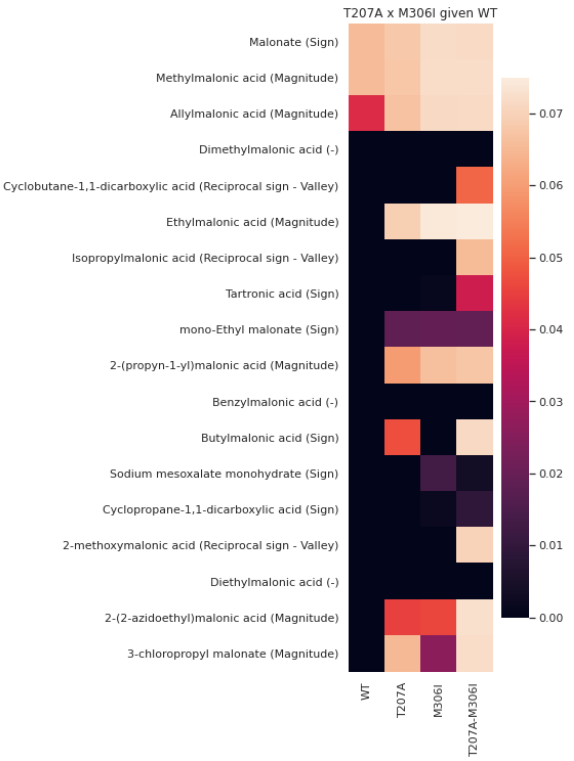

F189M x I306V given T207A-P232L-M306I
Malonate (Reciprocal sign - Valley)
Methylmalonic acid (Magnitude)
Allylmalonic acid (Reciprocal sign - Valley)
Dimethylmalonic acid (Sign)
Cyclobutane-1,1-dicarboxylic acid (Magnitude)
Ethylmalonic acid (Reciprocal sign - Valley)
Isopropylmalonic acid (Sign)
Tartronic acid (Magnitude)
mono-Ethyl malonate (Reciprocal sign - Valley)
2-(propyn-1-yl)malonic acid (Reciprocal sign - Valley)
Benzylmalonic acid (Reciprocal sign - Valley)
Butylmalonic acid (Reciprocal sign - Valley)
Sodium mesoxalate monohydrate (Reciprocal sign - Valley)
Cyclopropane-1,1-dicarboxylic acid (Sign)
2-methoxymalonic acid (Sign)
Diethylmalonic acid (Reciprocal sign)
2-(2-azidoethyl)malonic acid (Reciprocal sign - Valley)
3-chloropropyl malonate (Reciprocal sign - Valley)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
T207A-P232L-M306I
F189M-T207A-P232L-M306I
T207A-P232L-M306V
F189M-T207A-P232L-M306V

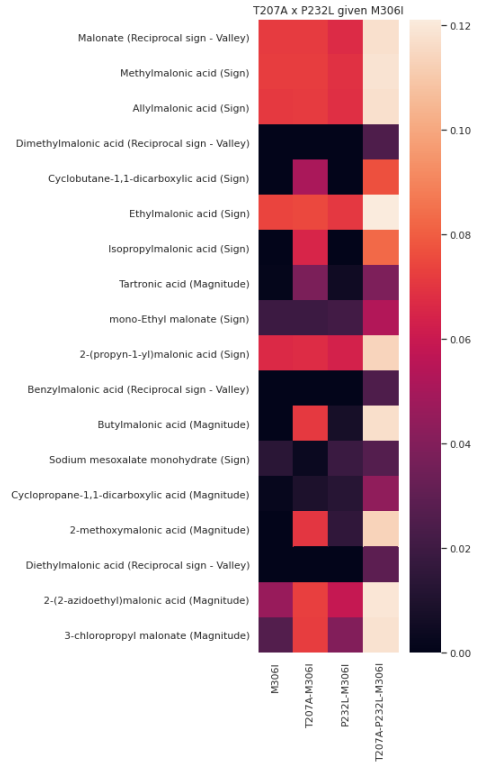

F189M x P232L given T207A-E233V-M306I
Malonate (Reciprocal sign - Valley)
Methylmalonic acid (Reciprocal sign - Valley)
Allylmalonic acid (Reciprocal sign - Valley)
Dimethylmalonic acid (Reciprocal sign)
Cyclobutane-1,1-dicarboxylic acid (Magnitude)
Ethylmalonic acid (Reciprocal sign - Valley)
Isopropylmalonic acid (Sign)
Tartronic acid (Magnitude)
mono-Ethyl malonate (Sign)
2-(propyn-1-yl)malonic acid (Reciprocal sign - Valley)
Benzylmalonic acid (Reciprocal sign)
Butylmalonic acid (Reciprocal sign - Valley)
Sodium mesoxalate monohydrate (Magnitude)
Cyclopropane-1,1-dicarboxylic acid (Reciprocal sign)
2-methoxymalonic acid (Sign)
Diethylmalonic acid (Reciprocal sign)
2-(2-azidoethyl)malonic acid (Reciprocal sign - Valley)
3-chloropropyl malonate (Reciprocal sign - Valley)
0.08
0.06
0.04
0.02
0.00
T207A-E233V-M306I
F189M-T207A-E233V-M306I
T207A-P232L-E233V-M306I
F189M-T207A-P232L-E233V-M306I

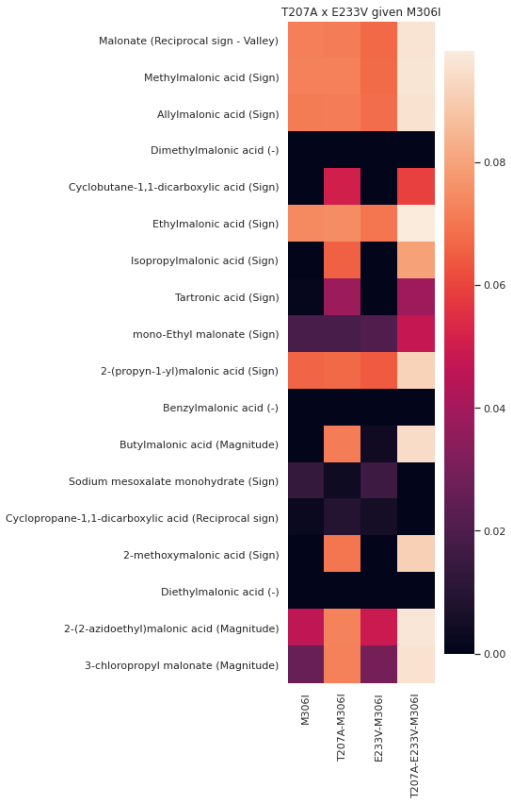

F189M x A207S given T207A-E233V-M306I
Malonate (Sign)
Methylmalonic acid (Sign)
Allylmalonic acid (Sign)
Dimethylmalonic acid (Magnitude)
Cyclobutane-1,1-dicarboxylic acid (Magnitude)
Ethylmalonic acid (Sign)
Isopropylmalonic acid (Magnitude)
Tartronic acid (Magnitude)
mono-Ethyl malonate (Sign)
2-(propyn-1-yl)malonic acid (Sign)
Benzylmalonic acid (Reciprocal sign)
Butylmalonic acid (Sign)
Sodium mesoxalate monohydrate (Magnitude)
Cyclopropane-1,1-dicarboxylic acid (Magnitude)
2-methoxymalonic acid (Magnitude)
Diethylmalonic acid (Magnitude)
2-(2-azidoethyl)malonic acid (Sign)
3-chloropropyl malonate (Sign)
0.10
0.08
0.06
0.04
0.02
0.00
T207A-E233V-M306I
F189M-T207A-E233V-M306I
T207S-E233V-M306I
F189M-T207S-E233V-M306I

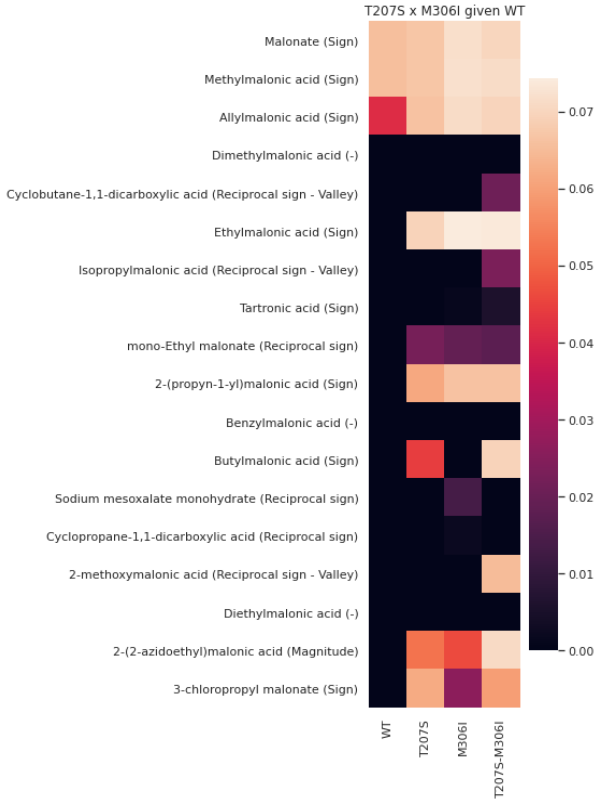

V192C x M306I given WT
Malonate (Sign)
Methylmalonic acid (Sign)
Allylmalonic acid (Sign)
Dimethylmalonic acid (-)
Cyclobutane-1,1-dicarboxylic acid (Reciprocal sign - Valley)
Ethylmalonic acid (Magnitude)
Isopropylmalonic acid (-)
Tartronic acid (Sign)
mono-Ethyl malonate (Sign)
2-(propyn-1-yl)malonic acid (Magnitude)
Benzylmalonic acid (Reciprocal sign)
Butylmalonic acid (Sign)
Sodium mesoxalate monohydrate (Magnitude)
Cyclopropane-1,1-dicarboxylic acid (Sign)
2-methoxymalonic acid (Reciprocal sign - Valley)
Diethylmalonic acid (-)
2-(2-azidoethyl)malonic acid (Sign)
3-chloropropyl malonate (Sign)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
WT
V192C
M306I
V192C-M306I

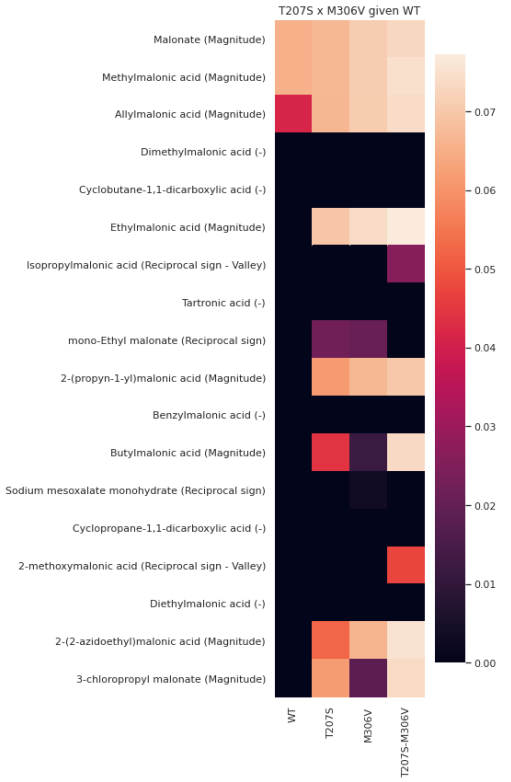

R197C x M306I given WT
Malonate (Reciprocal sign)
Methylmalonic acid (Sign)
Allylmalonic acid (Magnitude)
Dimethylmalonic acid (-)
Cyclobutane-1,1-dicarboxylic acid (-)
Ethylmalonic acid (Sign)
Isopropylmalonic acid (-)
Tartronic acid (Sign)
mono-Ethyl malonate (Sign)
2-(propyn-1-yl)malonic acid (Sign)
Benzylmalonic acid (-)
Butylmalonic acid (-)
Sodium mesoxalate monohydrate (Sign)
Cyclopropane-1,1-dicarboxylic acid (Reciprocal sign)
2-methoxymalonic acid (-)
Diethylmalonic acid (-)
2-(2-azidoethyl)malonic acid (Sign)
3-chloropropyl malonate (Sign)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
WT
R197C
M306I
R197C-M306I

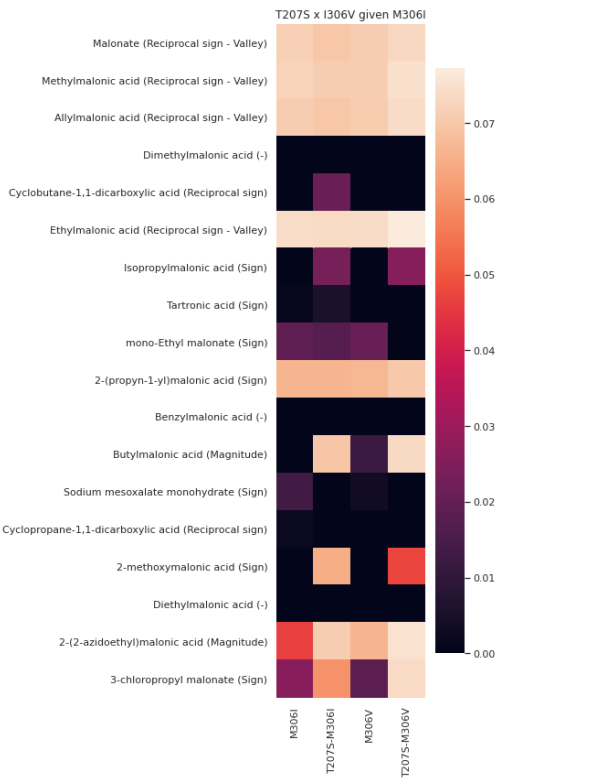

T207A x M306I given WT
Malonate (Sign)
Methylmalonic acid (Magnitude)
Allylmalonic acid (Magnitude)
Dimethylmalonic acid (-)
Cyclobutane-1,1-dicarboxylic acid (Reciprocal sign - Valley)
Ethylmalonic acid (Magnitude)
Isopropylmalonic acid (Reciprocal sign - Valley)
Tartronic acid (Sign)
mono-Ethyl malonate (Sign)
2-(propyn-1-yl)malonic acid (Magnitude)
Benzylmalonic acid (-)
Butylmalonic acid (Sign)
Sodium mesoxalate monohydrate (Sign)
Cyclopropane-1,1-dicarboxylic acid (Sign)
2-methoxymalonic acid (Reciprocal sign - Valley)
Diethylmalonic acid (-)
2-(2-azidoethyl)malonic acid (Magnitude)
3-chloropropyl malonate (Magnitude)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
WT
T207A
M306I
T207A-M306I

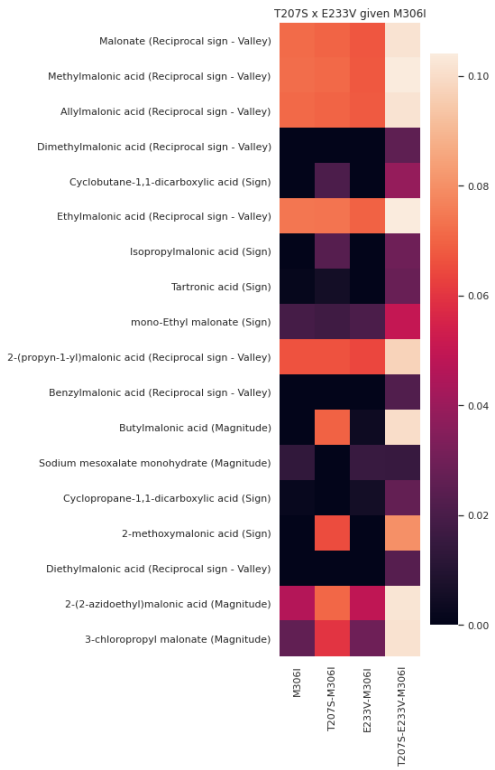

T207A x P232L given M306I
Malonate (Reciprocal sign - Valley)
Methylmalonic acid (Sign)
Allylmalonic acid (Sign)
Dimethylmalonic acid (Reciprocal sign - Valley)
Cyclobutane-1,1-dicarboxylic acid (Sign)
Ethylmalonic acid (Sign)
Isopropylmalonic acid (Sign)
Tartronic acid (Magnitude)
mono-Ethyl malonate (Sign)
2-(propyn-1-yl)malonic acid (Sign)
Benzylmalonic acid (Reciprocal sign - Valley)
Butylmalonic acid (Magnitude)
Sodium mesoxalate monohydrate (Sign)
Cyclopropane-1,1-dicarboxylic acid (Magnitude)
2-methoxymalonic acid (Magnitude)
Diethylmalonic acid (Reciprocal sign - Valley)
2-(2-azidoethyl)malonic acid (Magnitude)
3-chloropropyl malonate (Magnitude)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
M306I
T207A-M306I
P232L-M306I
T207A-P232L-M306I

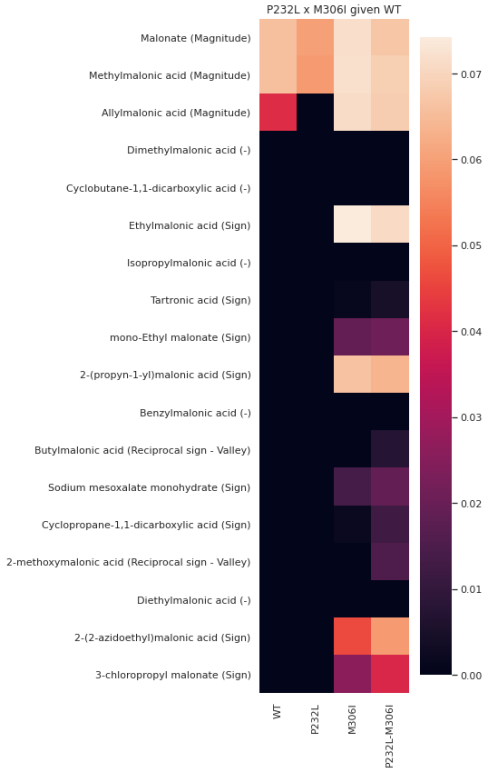

T207A x E233V given M306I
Malonate (Reciprocal sign - Valley)
Methylmalonic acid (Sign)
Allylmalonic acid (Sign)
Dimethylmalonic acid (-)
Cyclobutane-1,1-dicarboxylic acid (Sign)
Ethylmalonic acid (Sign)
Isopropylmalonic acid (Sign)
Tartronic acid (Sign)
mono-Ethyl malonate (Sign)
2-(propyn-1-yl)malonic acid (Sign)
Benzylmalonic acid (-)
Butylmalonic acid (Magnitude)
Sodium mesoxalate monohydrate (Sign)
Cyclopropane-1,1-dicarboxylic acid (Reciprocal sign)
2-methoxymalonic acid (Sign)
Diethylmalonic acid (-)
2-(2-azidoethyl)malonic acid (Magnitude)
3-chloropropyl malonate (Magnitude)
M306I
T207A-M306I
E233V-M306I
T207A-E233V-M306I
0.08
0.06
0.04
0.02
0.00

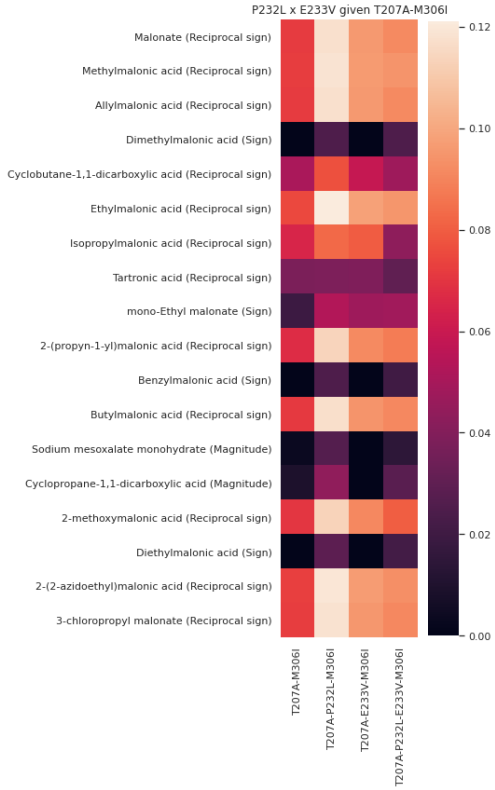

T207S x M306I given WT
Malonate (Sign)
Methylmalonic acid (Sign)
Allylmalonic acid (Sign)
Dimethylmalonic acid (-)
Cyclobutane-1,1-dicarboxylic acid (Reciprocal sign - Valley)
Ethylmalonic acid (Sign)
Isopropylmalonic acid (Reciprocal sign - Valley)
Tartronic acid (Sign)
mono-Ethyl malonate (Reciprocal sign)
2-(propyn-1-yl)malonic acid (Sign)
Benzylmalonic acid (-)
Butylmalonic acid (Sign)
Sodium mesoxalate monohydrate (Reciprocal sign)
Cyclopropane-1,1-dicarboxylic acid (Reciprocal sign)
2-methoxymalonic acid (Reciprocal sign - Valley)
Diethylmalonic acid (-)
2-(2-azidoethyl)malonic acid (Magnitude)
3-chloropropyl malonate (Sign)
WT
T207S
M306I
T207S-M306I
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00

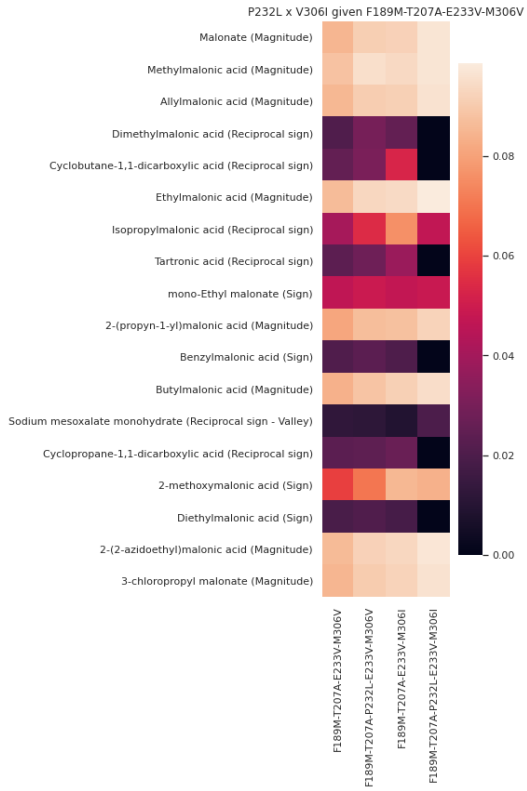

T207S x M306V given WT
Malonate (Magnitude)
Methylmalonic acid (Magnitude)
Allylmalonic acid (Magnitude)
Dimethylmalonic acid (-)
Cyclobutane-1,1-dicarboxylic acid (-)
Ethylmalonic acid (Magnitude)
Isopropylmalonic acid (Reciprocal sign - Valley)
Tartronic acid (-)
mono-Ethyl malonate (Reciprocal sign)
2-(propyn-1-yl)malonic acid (Magnitude)
Benzylmalonic acid (-)
Butylmalonic acid (Magnitude)
Sodium mesoxalate monohydrate (Reciprocal sign)
Cyclopropane-1,1-dicarboxylic acid (-)
2-methoxymalonic acid (Reciprocal sign - Valley)
Diethylmalonic acid (-)
2-(2-azidoethyl)malonic acid (Magnitude)
3-chloropropyl malonate (Magnitude)
WT
T207S
M306V
T207S-M306V
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00

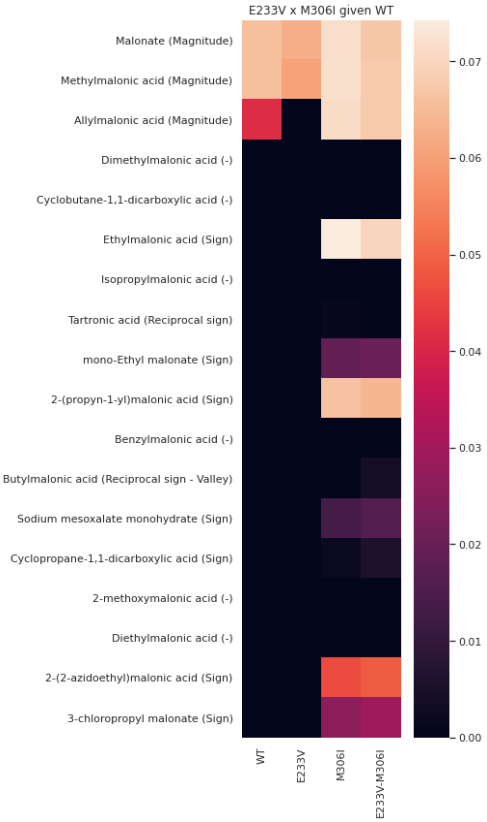

T207S x I306V given M306I
Malonate (Reciprocal sign - Valley)
Methylmalonic acid (Reciprocal sign - Valley)
Allylmalonic acid (Reciprocal sign - Valley)
Dimethylmalonic acid (-)
Cyclobutane-1,1-dicarboxylic acid (Reciprocal sign)
Ethylmalonic acid (Reciprocal sign - Valley)
Isopropylmalonic acid (Sign)
Tartronic acid (Sign)
mono-Ethyl malonate (Sign)
2-(propyn-1-yl)malonic acid (Sign)
Benzylmalonic acid (-)
Butylmalonic acid (Magnitude)
Sodium mesoxalate monohydrate (Sign)
Cyclopropane-1,1-dicarboxylic acid (Reciprocal sign)
2-methoxymalonic acid (Sign)
Diethylmalonic acid (-)
2-(2-azidoethyl)malonic acid (Magnitude)
3-chloropropyl malonate (Sign)
M306I
T207S-M306I
M306V
T207S-M306V
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00

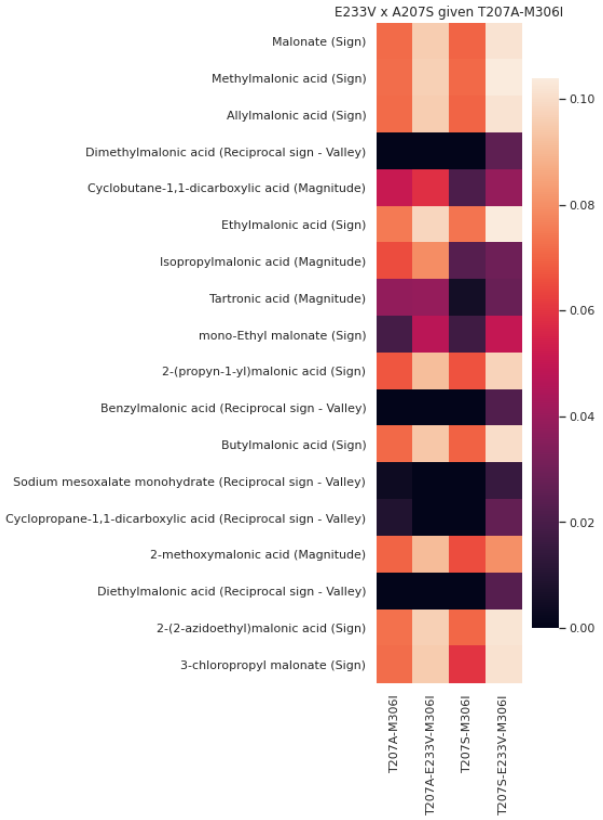

T207S x E233V given M306I
Malonate (Reciprocal sign - Valley)
Methylmalonic acid (Reciprocal sign - Valley)
Allylmalonic acid (Reciprocal sign - Valley)
Dimethylmalonic acid (Reciprocal sign - Valley)
Cyclobutane-1,1-dicarboxylic acid (Sign)
Ethylmalonic acid (Reciprocal sign - Valley)
Isopropylmalonic acid (Sign)
Tartronic acid (Sign)
mono-Ethyl malonate (Sign)
2-(propyn-1-yl)malonic acid (Reciprocal sign - Valley)
Benzylmalonic acid (Reciprocal sign - Valley)
Butylmalonic acid (Magnitude)
Sodium mesoxalate monohydrate (Magnitude)
Cyclopropane-1,1-dicarboxylic acid (Sign)
2-methoxymalonic acid (Sign)
Diethylmalonic acid (Reciprocal sign - Valley)
2-(2-azidoethyl)malonic acid (Magnitude)
3-chloropropyl malonate (Magnitude)
0.10
0.08
0.06
0.04
0.02
0.00
M306I
T207S-M306I
E233V-M306I
T207S-E233V-M306I

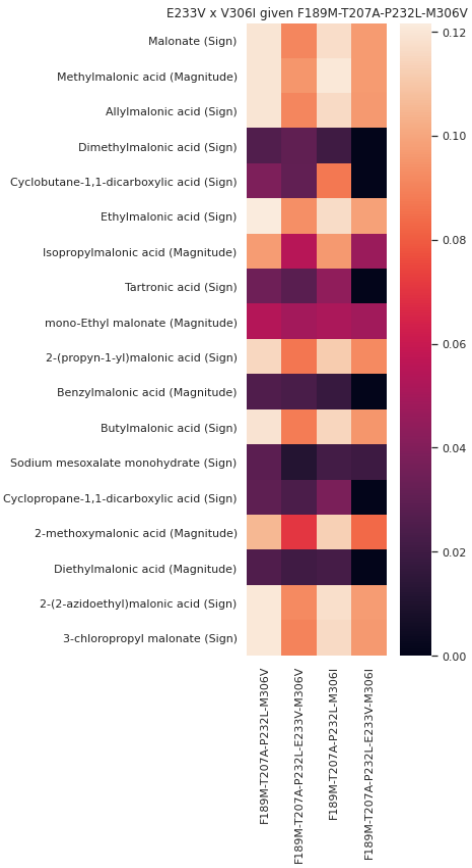

P232L x M306I given WT
Malonate (Magnitude)
Methylmalonic acid (Magnitude)
Allylmalonic acid (Magnitude)
Dimethylmalonic acid (-)
Cyclobutane-1,1-dicarboxylic acid (-)
Ethylmalonic acid (Sign)
Isopropylmalonic acid (-)
Tartronic acid (Sign)
mono-Ethyl malonate (Sign)
2-(propyn-1-yl)malonic acid (Sign)
Benzylmalonic acid (-)
Butylmalonic acid (Reciprocal sign - Valley)
Sodium mesoxalate monohydrate (Sign)
Cyclopropane-1,1-dicarboxylic acid (Sign)
2-methoxymalonic acid (Reciprocal sign - Valley)
Diethylmalonic acid (-)
2-(2-azidoethyl)malonic acid (Sign)
3-chloropropyl malonate (Sign)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
WT
P232L
M306I
P232L-M306I


P232L x E233V given T207A-M306I
Malonate (Reciprocal sign)
Methylmalonic acid (Reciprocal sign)
Allylmalonic acid (Reciprocal sign)
Dimethylmalonic acid (Sign)
Cyclobutane-1,1-dicarboxylic acid (Reciprocal sign)
Ethylmalonic acid (Reciprocal sign)
Isopropylmalonic acid (Reciprocal sign)
Tartronic acid (Reciprocal sign)
mono-Ethyl malonate (Sign)
2-(propyn-1-yl)malonic acid (Reciprocal sign)
Benzylmalonic acid (Sign)
Butylmalonic acid (Reciprocal sign)
Sodium mesoxalate monohydrate (Magnitude)
Cyclopropane-1,1-dicarboxylic acid (Magnitude)
2-methoxymalonic acid (Reciprocal sign)
Diethylmalonic acid (Sign)
2-(2-azidoethyl)malonic acid (Reciprocal sign)
3-chloropropyl malonate (Reciprocal sign)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
T207A-M306I
T207A-P232L-M306I
T207A-E233V-M306I
T207A-P232L-E233V-M306I


P232L x V306I given F189M-T207A-E233V-M306V
Malonate (Magnitude)
Methylmalonic acid (Magnitude)
Allylmalonic acid (Magnitude)
Dimethylmalonic acid (Reciprocal sign)
Cyclobutane-1,1-dicarboxylic acid (Reciprocal sign)
Ethylmalonic acid (Magnitude)
Isopropylmalonic acid (Reciprocal sign)
Tartronic acid (Reciprocal sign)
mono-Ethyl malonate (Sign)
2-(propyn-1-yl)malonic acid (Magnitude)
Benzylmalonic acid (Sign)
Butylmalonic acid (Magnitude)
Sodium mesoxalate monohydrate (Reciprocal sign - Valley)
Cyclopropane-1,1-dicarboxylic acid (Reciprocal sign)
2-methoxymalonic acid (Sign)
Diethylmalonic acid (Sign)
2-(2-azidoethyl)malonic acid (Magnitude)
3-chloropropyl malonate (Magnitude)
0.08
0.06
0.04
0.02
0.00
F189M-T207A-E233V-M306V
F189M-T207A-P232L-E233V-M306V
F189M-T207A-E233V-M306I
F189M-T207A-P232L-E233V-M306I


E233V x M306I given WT
Malonate (Magnitude)
Methylmalonic acid (Magnitude)
Allylmalonic acid (Magnitude)
Dimethylmalonic acid (-)
Cyclobutane-1,1-dicarboxylic acid (-)
Ethylmalonic acid (Sign)
Isopropylmalonic acid (-)
Tartronic acid (Reciprocal sign)
mono-Ethyl malonate (Sign)
2-(propyn-1-yl)malonic acid (Sign)
Benzylmalonic acid (-)
Butylmalonic acid (Reciprocal sign - Valley)
Sodium mesoxalate monohydrate (Sign)
Cyclopropane-1,1-dicarboxylic acid (Sign)
2-methoxymalonic acid (-)
Diethylmalonic acid (-)
2-(2-azidoethyl)malonic acid (Sign)
3-chloropropyl malonate (Sign)
WT
E233V
M306I
E233V-M306I
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00


E233V x A207S given T207A-M306I
Malonate (Sign)
Methylmalonic acid (Sign)
Allylmalonic acid (Sign)
Dimethylmalonic acid (Reciprocal sign - Valley)
Cyclobutane-1,1-dicarboxylic acid (Magnitude)
Ethylmalonic acid (Sign)
Isopropylmalonic acid (Magnitude)
Tartronic acid (Magnitude)
mono-Ethyl malonate (Sign)
2-(propyn-1-yl)malonic acid (Sign)
Benzylmalonic acid (Reciprocal sign - Valley)
Butylmalonic acid (Sign)
Sodium mesoxalate monohydrate (Reciprocal sign - Valley)
Cyclopropane-1,1-dicarboxylic acid (Reciprocal sign - Valley)
2-methoxymalonic acid (Magnitude)
Diethylmalonic acid (Reciprocal sign - Valley)
2-(2-azidoethyl)malonic acid (Sign)
3-chloropropyl malonate (Sign)
T207A-M306I
T207A-E233V-M306I
T207S-M306I
T207S-E233V-M306I
0.10
0.08
0.06
0.04
0.02
0.00


E233V x V306I given F189M-T207A-P232L-M306V
Malonate (Sign)
Methylmalonic acid (Magnitude)
Allylmalonic acid (Sign)
Dimethylmalonic acid (Sign)
Cyclobutane-1,1-dicarboxylic acid (Sign)
Ethylmalonic acid (Sign)
Isopropylmalonic acid (Magnitude)
Tartronic acid (Sign)
mono-Ethyl malonate (Magnitude)
2-(propyn-1-yl)malonic acid (Sign)
Benzylmalonic acid (Magnitude)
Butylmalonic acid (Sign)
Sodium mesoxalate monohydrate (Sign)
Cyclopropane-1,1-dicarboxylic acid (Sign)
2-methoxymalonic acid (Magnitude)
Diethylmalonic acid (Magnitude)
2-(2-azidoethyl)malonic acid (Sign)
3-chloropropyl malonate (Sign)
F189M-T207A-P232L-M306V
F189M-T207A-P232L-E233V-M306V
F189M-T207A-P232L-M306I
F189M-T207A-P232L-E233V-M306I
0.12
0.10
0.08
0.06
0.04
0.02
0.00

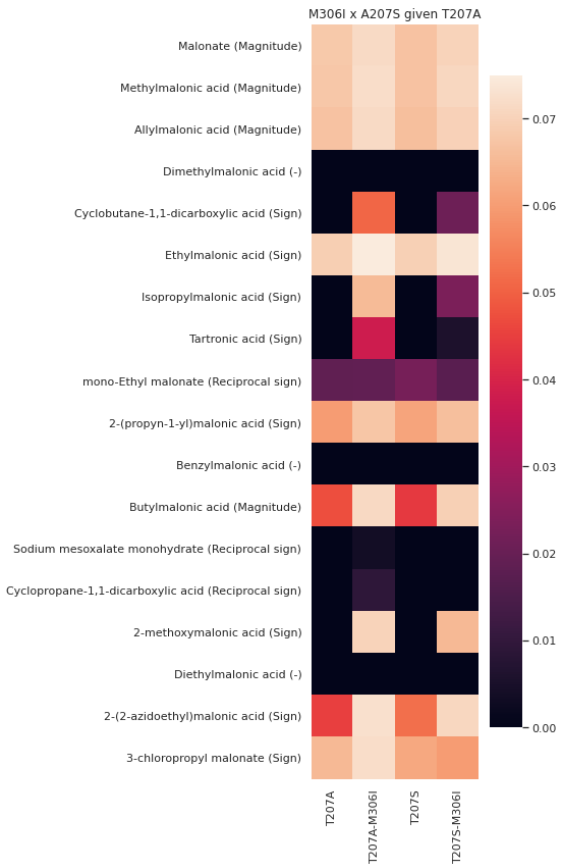

M306I x A207S given T207A
Malonate (Magnitude)
Methylmalonic acid (Magnitude)
Allylmalonic acid (Magnitude)
Dimethylmalonic acid (-)
Cyclobutane-1,1-dicarboxylic acid (Sign)
Ethylmalonic acid (Sign)
Isopropylmalonic acid (Sign)
Tartronic acid (Sign)
mono-Ethyl malonate (Reciprocal sign)
2-(propyn-1-yl)malonic acid (Sign)
Benzylmalonic acid (-)
Butylmalonic acid (Magnitude)
Sodium mesoxalate monohydrate (Reciprocal sign)
Cyclopropane-1,1-dicarboxylic acid (Reciprocal sign)
2-methoxymalonic acid (Sign)
Diethylmalonic acid (-)
2-(2-azidoethyl)malonic acid (Sign)
3-chloropropyl malonate (Sign)
T207A
T207A-M306I
T207S
T207S-M306I
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00

Figure S7. All possible pairwise epistasis in the sequence space of our assayed variants. Type of epistasis is widely substrate-dependent.

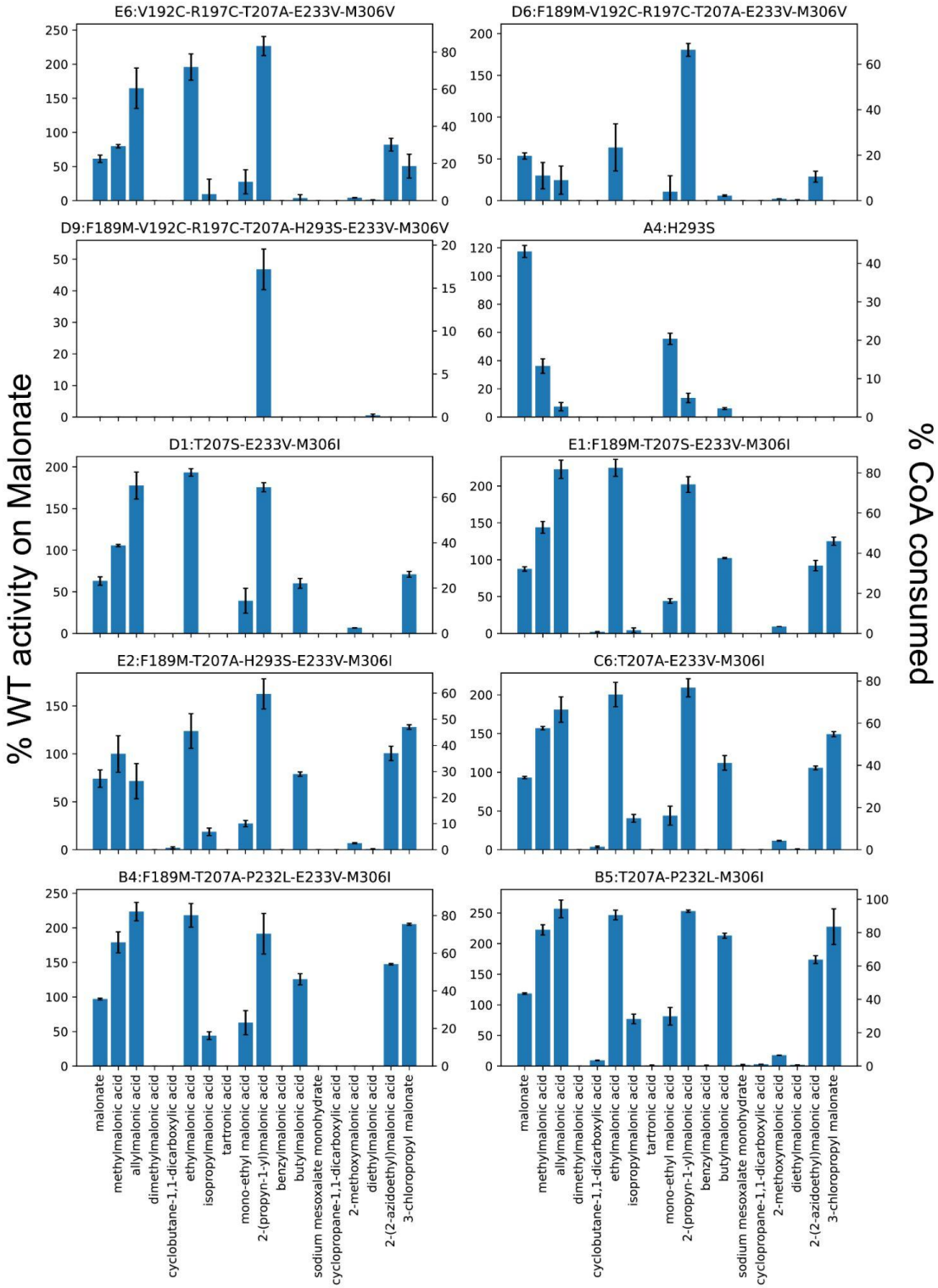


Figure S8. Complete specificity profile of 10 assayed purified recombinants.