



Supporting Information

Hydroxamate Assays for High-Throughput Screening of Transketolase Libraries Against Arylated Substrates

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Supporting Information

High-Throughput Assay for Screening of Transketolase Libraries Against Arylated Substrates

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Effect of pH and buffer component in the iron (III) assay with **2b**

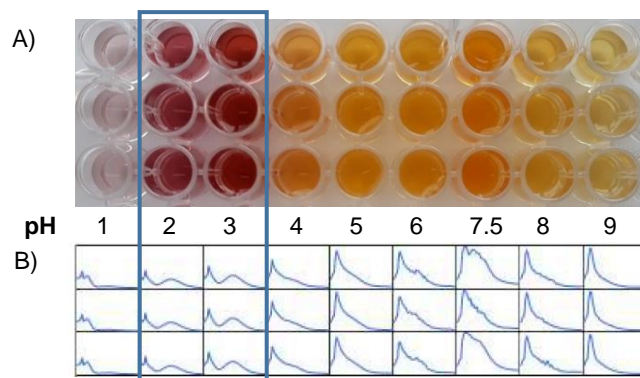


Figure S1. Effect of TEA buffer (2 mM, pH 7.45) at different pHs in iron(III)-HA-complex formation. Conditions: FeCl_3 (2 mM) and **2b** (2 mM). Total volume: 200 μL . Experiments performed in triplicates. (A) Color comparison, (B) spectrum of absorbance comparison.

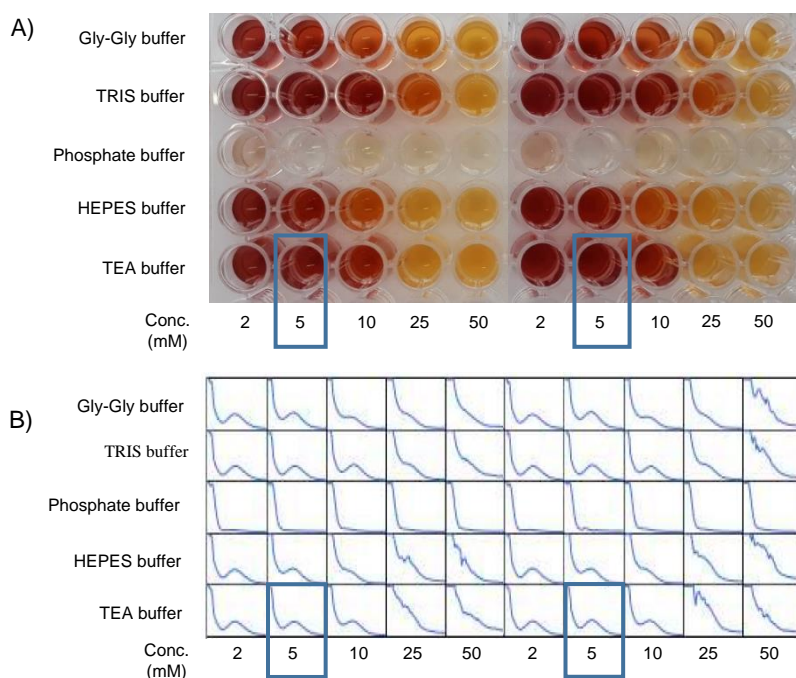


Figure S2. Effect of various buffers (initial pH 7.45) at different concentrations in iron(III)-HA complex formation: Gly-Gly buffer, TRIS buffer, phosphate buffer, HEPES buffer, and TEA buffer. Fixed conditions: FeCl_3 (2 mM), and **2b** (2 mM). Total volume: 200 μL . Experiment performed in duplicates. (A) Color comparison, (B) spectrum of absorbance comparison.

Standard curve calibration of the iron (III) assay with **2b**

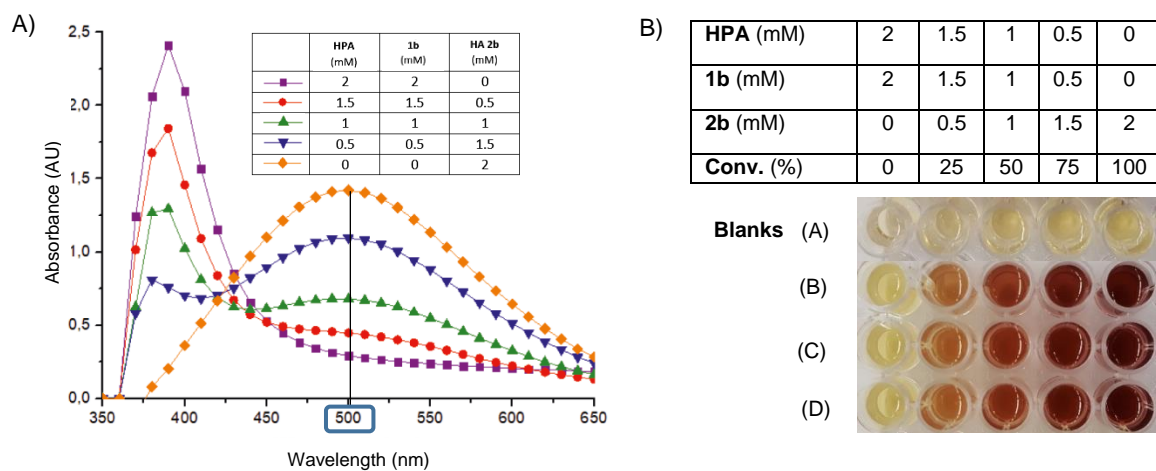


Figure S3. (A) Standard curve spectrum for HA **2b**, (B) standard curve microplate for **2b**. Conditions: nitroso **1b** (2-0 mM), HPA (2-0 mM), Br-HA **2b** (0-2 mM), TEA buffer (5 mM, pH 7.45), FeCl₃ (6 mM). A) Blanks, B, C, D) Experiments performed in triplicates.

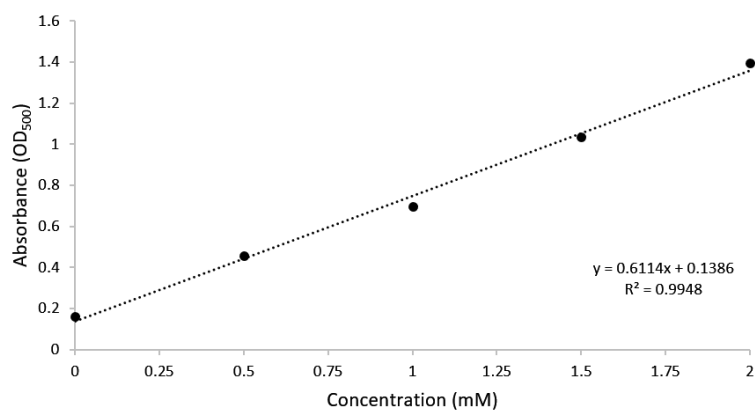


Figure S4. Calibration curve for **2b**. The linear range of the colorimetric assay was found to be up to 2 mM of **2b** per 200 μ L well.

SCREENING WITH THE LIQUID-PHASE ASSAY

Color and absorbance results with the TK_{gst} L382X/D470X library

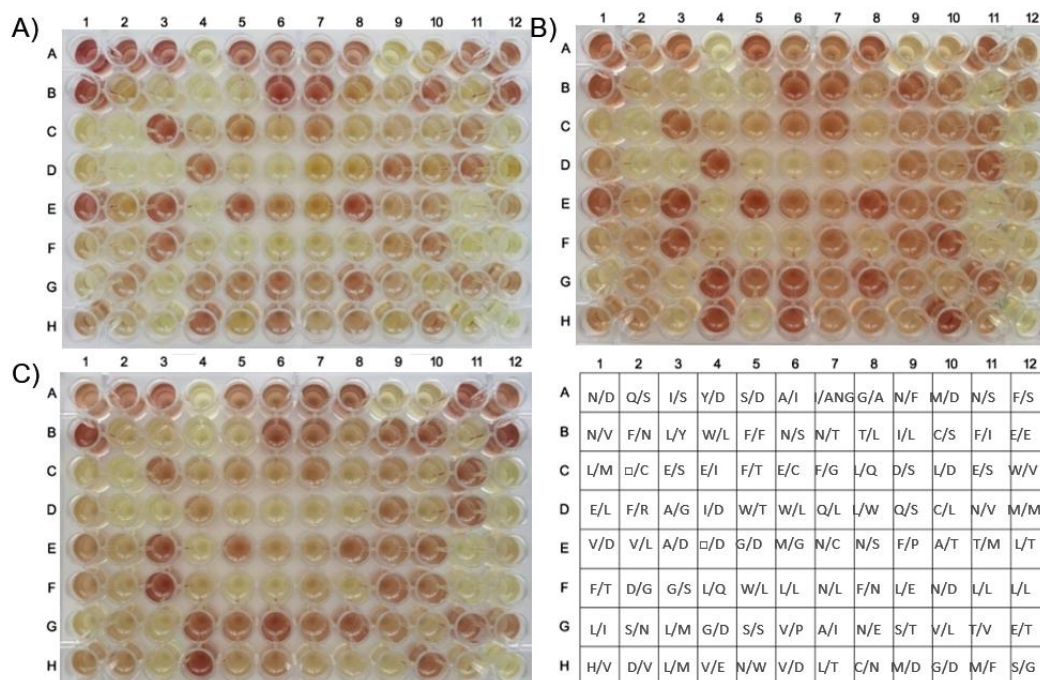


Figure S5. Color screening results with the three nitrosoarenes. A) **2b**; B) **2c**; C) **2d**. For A and C, the lysates were diluted 1:1 prior to use. Plate A was incubated for 15 minutes at RT, and plate B and C for 30 minutes. The experiments were performed in duplicates, yet only one plate for each set of experiments is shown. Corresponding mutations from the L382X/D470X library are shown in template in a one letter code fashion. □: Stop codon; ANG: A+overlapped peaks (ATGC)+G.

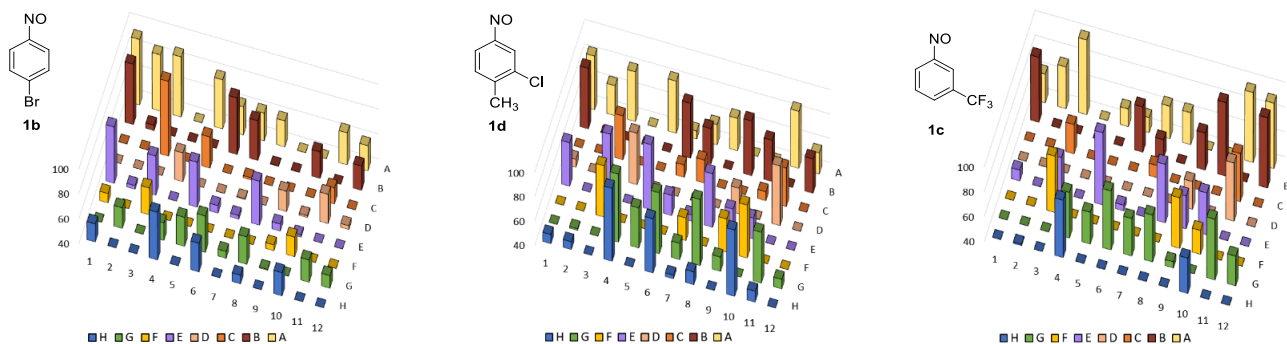


Figure S6. Absorbance screening results with the three nitrosoarenes: A) **1b**; B) **1c**; C) **1d**. The experiments were performed in duplicates, being the average of absorbance values represented in the graphic.

Raw and treated results from liquid-phase assay

- *N*-(4-Bromophenyl)-*N*,2-dihydroxyacetamide (*2b*)

Plate 1:

0.505	0.576	0.600	0.289	0.573	0.446	0.461	0.461	0.294	0.372	0.503	0.453
0.507	0.412	0.290	0.254	0.299	0.539	0.503	0.372	0.345	0.414	0.273	0.409
0.403	0.300	0.697	0.382	0.526	0.362	0.374	0.337	0.406	0.413	0.465	0.340
0.405	0.246	0.258	0.497	0.270	0.236	0.410	0.383	0.476	0.416	0.484	0.491
0.556	0.390	0.500	0.215	0.564	0.396	0.399	0.532	0.507	0.420	0.247	0.421
0.502	0.336	0.478	0.270	0.241	0.285	0.308	0.285	0.454	0.463	0.311	0.326
0.425	0.514	0.281	0.503	0.583	0.583	0.471	0.486	0.447	0.321	0.509	0.453
0.483	0.388	0.320	0.605	0.410	0.520	0.397	0.441	0.372	0.512	0.347	0.195

Plate 2:

0.767	0.625	0.631	0.288	0.574	0.539	0.521	0.508	0.290	0.385	0.512	0.510
0.726	0.399	0.255	0.202	0.228	0.664	0.571	0.402	0.306	0.560	0.277	0.548
0.299	0.221	0.671	0.319	0.483	0.317	0.413	0.296	0.301	0.275	0.428	0.244
0.346	0.211	0.218	0.494	0.256	0.223	0.367	0.338	0.450	0.369	0.511	0.308
0.646	0.408	0.571	0.187	0.551	0.433	0.400	0.575	0.318	0.367	0.171	0.340
0.339	0.274	0.498	0.245	0.184	0.222	0.246	0.232	0.361	0.457	0.232	0.185
0.273	0.414	0.233	0.402	0.410	0.467	0.352	0.495	0.331	0.295	0.433	0.414
0.426	0.346	0.248	0.531	0.351	0.470	0.320	0.397	0.338	0.436	0.283	0.164

Average plate minus the smallest value (not normalized):

0.457	0.421	0.436	0.109	0.394	0.313	0.312	0.305	0.113	0.199	0.328	0.302
0.437	0.226	0.093	0.049	0.084	0.422	0.358	0.208	0.146	0.307	0.096	0.299
0.171	0.081	0.505	0.171	0.325	0.160	0.214	0.137	0.174	0.164	0.267	0.113
0.196	0.049	0.059	0.316	0.083	0.050	0.209	0.181	0.284	0.213	0.318	0.220
0.421	0.219	0.356	0.022	0.378	0.235	0.220	0.374	0.233	0.214	0.030	0.201
0.241	0.126	0.309	0.078	0.033	0.074	0.097	0.079	0.228	0.281	0.092	0.076
0.169	0.285	0.078	0.273	0.317	0.346	0.232	0.311	0.210	0.128	0.291	0.254
0.275	0.187	0.104	0.389	0.201	0.316	0.179	0.239	0.175	0.295	0.135	0.000

Final average plate normalized and used for the graphical results:

90.483	83.362	86.294	21.575	78.014	62.050	61.763	60.426	22.298	39.361	64.952	59.792
86.541	44.699	18.445	9.611	16.583	83.640	70.815	41.124	28.864	60.872	18.950	59.218
33.924	16.098	100.000	33.795	64.348	31.627	42.402	27.071	34.439	32.548	52.939	22.367
38.807	9.730	11.602	62.565	16.524	9.879	41.391	35.806	56.187	42.114	62.931	43.620
83.461	43.471	70.498	4.343	74.924	46.492	43.531	74.122	46.175	42.362	5.868	39.797
47.789	24.902	61.139	15.415	6.601	14.692	19.267	15.722	45.165	55.583	18.178	15.068
33.558	56.375	15.375	54.068	62.822	68.448	45.967	61.594	41.510	25.417	57.702	50.295
54.504	37.123	20.683	76.974	39.827	62.496	35.469	47.393	34.756	58.376	26.804	0.000

To further exemplify the applicability of the ferric chloride as semi-quantitative method, the standard curve can be used to extrapolate the concentration of the final product in the different reaction mixtures. The approximate concentration values of the **2b** assay were calculated by plotting the values in the standard curve with equation $y = 0.6114x + 0.1386$. The maximum concentration of product **2b** achieved by TK_{gst} (0.893 mM) was obtained by variant E/S in well C3. The very active variants offer HA yields between 0.5 and 1, which is in accordance with the visual color results from the standard curve.

Average plate (not normalized):

0.636	0.600	0.615	0.288	0.573	0.493	0.491	0.485	0.292	0.378	0.507	0.481
0.616	0.405	0.273	0.228	0.263	0.602	0.537	0.387	0.325	0.487	0.275	0.478
0.351	0.261	0.684	0.350	0.504	0.339	0.394	0.316	0.353	0.344	0.447	0.292
0.375	0.229	0.238	0.495	0.263	0.229	0.388	0.360	0.463	0.392	0.497	0.400
0.601	0.399	0.535	0.201	0.558	0.414	0.399	0.554	0.413	0.393	0.209	0.380
0.421	0.305	0.488	0.257	0.213	0.254	0.277	0.259	0.407	0.460	0.271	0.256
0.349	0.464	0.257	0.452	0.497	0.525	0.412	0.490	0.389	0.308	0.471	0.433
0.455	0.367	0.284	0.568	0.381	0.495	0.359	0.419	0.355	0.474	0.315	0.179

Final concentration values:

0.814	0.755	0.779	0.245	0.711	0.579	0.577	0.566	0.251	0.392	0.603	0.561
0.781	0.436	0.219	0.146	0.204	0.757	0.652	0.406	0.305	0.569	0.223	0.556
0.347	0.200	0.893	0.346	0.598	0.328	0.417	0.290	0.351	0.336	0.504	0.251
0.387	0.147	0.163	0.583	0.203	0.148	0.409	0.362	0.531	0.415	0.586	0.427
0.756	0.426	0.649	0.103	0.685	0.451	0.426	0.679	0.448	0.417	0.115	0.395
0.461	0.272	0.572	0.194	0.121	0.188	0.226	0.197	0.440	0.526	0.217	0.191
0.344	0.532	0.194	0.513	0.586	0.632	0.446	0.575	0.410	0.277	0.543	0.482
0.517	0.373	0.238	0.702	0.396	0.583	0.360	0.458	0.354	0.549	0.288	0.067

• *N*-(3-(Trifluoromethyl)phenyl)-*N*,2-dihydroxyacetamide (*1c*)

Plate 1:

0.685	0.590	0.674	0.278	0.666	0.460	0.502	0.528	0.402	0.411	0.709	0.565
0.709	0.374	0.308	0.251	0.304	0.694	0.609	0.459	0.747	0.666	0.271	0.657
0.358	0.242	0.624	0.383	0.421	0.515	0.493	0.370	0.427	0.502	0.586	0.256
0.476	0.244	0.240	0.667	0.254	0.247	0.409	0.346	0.578	0.457	0.728	0.452
0.634	0.434	0.718	0.251	0.721	0.497	0.463	0.648	0.543	0.503	0.290	0.405
0.426	0.390	0.659	0.362	0.225	0.332	0.562	0.355	0.597	0.698	0.335	0.337
0.315	0.442	0.345	0.735	0.631	0.730	0.519	0.770	0.491	0.454	0.676	0.521
0.479	0.462	0.349	0.765	0.314	0.685	0.456	0.519	0.414	0.709	0.495	0.234

Plate 2:

0.697	0.575	0.672	0.277	0.701	0.504	0.574	0.660	0.396	0.441	0.703	0.520
0.736	0.372	0.307	0.256	0.287	0.698	0.606	0.407	0.645	0.603	0.273	0.547
0.377	0.232	0.668	0.370	0.427	0.489	0.608	0.362	0.430	0.471	0.658	0.231
0.488	0.220	0.223	0.695	0.232	0.232	0.385	0.337	0.548	0.438	0.710	0.418
0.656	0.424	0.758	0.218	0.765	0.579	0.487	0.733	0.568	0.579	0.245	0.417
0.400	0.381	0.702	0.350	0.223	0.337	0.535	0.365	0.599	0.678	0.330	0.331
0.314	0.464	0.355	0.779	0.627	0.721	0.522	0.727	0.530	0.431	0.682	0.458
0.496	0.500	0.364	0.794	0.302	0.702	0.469	0.489	0.439	0.789	0.496	0.224

Average plate minus the smallest value (not normalized):

0.467	0.359	0.449	0.054	0.460	0.258	0.314	0.370	0.175	0.202	0.482	0.319
0.499	0.149	0.084	0.030	0.072	0.473	0.384	0.209	0.472	0.411	0.048	0.378
0.144	0.013	0.423	0.153	0.200	0.278	0.327	0.142	0.205	0.263	0.399	0.020
0.258	0.008	0.008	0.458	0.019	0.016	0.174	0.118	0.340	0.224	0.495	0.211
0.421	0.206	0.514	0.011	0.520	0.314	0.251	0.467	0.332	0.318	0.044	0.187
0.189	0.162	0.457	0.132	0.000	0.111	0.325	0.137	0.374	0.465	0.109	0.110
0.091	0.229	0.127	0.533	0.406	0.502	0.297	0.525	0.287	0.219	0.455	0.266
0.264	0.258	0.133	0.556	0.085	0.470	0.239	0.281	0.203	0.526	0.272	0.006

Final average plate normalized and used for the graphical results:

84.048	64.527	80.739	9.684	82.717	46.417	56.524	66.541	31.544	36.364	86.737	57.279
89.749	26.877	15.125	5.395	12.886	84.983	69.005	37.667	84.911	73.905	8.704	68.060
25.942	2.410	76.000	27.426	35.995	50.040	58.844	25.555	36.912	47.253	71.666	3.588
46.426	1.457	1.385	82.304	3.489	2.913	31.265	21.266	61.056	40.284	89.048	37.982
75.776	36.966	92.429	1.888	93.427	56.542	45.158	83.958	59.671	57.099	7.877	33.702
34.071	29.098	82.178	23.793	0.000	19.926	58.475	24.602	67.296	83.554	19.630	19.836
16.392	41.255	22.786	95.909	72.961	90.226	53.394	94.371	51.605	39.376	81.827	47.765
47.406	46.309	23.955	100.000	15.196	84.498	42.955	50.445	36.436	94.506	48.934	1.016

• *N-(3-Chloro-4-methylphenyl)-N,2-dihydroxyacetamide (1d)*

Plate 1:

0.443	0.489	0.672	0.239	0.430	0.424	0.486	0.476	0.264	0.289	0.645	0.615
0.524	0.292	0.297	0.235	0.254	0.551	0.436	0.339	0.593	0.763	0.248	0.620
0.266	0.240	0.465	0.314	0.341	0.350	0.388	0.275	0.345	0.264	0.546	0.253
0.292	0.215	0.214	0.365	0.234	0.214	0.264	0.261	0.539	0.343	0.678	0.239
0.422	0.332	0.457	0.206	0.784	0.429	0.385	0.679	0.571	0.605	0.223	0.332
0.340	0.306	0.522	0.311	0.197	0.231	0.368	0.282	0.672	0.492	0.235	0.249
0.266	0.330	0.305	0.550	0.507	0.593	0.576	0.571	0.415	0.373	0.657	0.509
0.306	0.342	0.244	0.497	0.336	0.337	0.296	0.290	0.242	0.499	0.272	0.195

Plate 2:

0.525	0.575	0.653	0.248	0.456	0.443	0.509	0.515	0.294	0.278	0.627	0.623
0.711	0.279	0.263	0.217	0.222	0.538	0.455	0.321	0.437	0.535	0.219	0.654
0.275	0.206	0.506	0.336	0.365	0.351	0.458	0.288	0.385	0.300	0.564	0.258
0.349	0.193	0.208	0.353	0.270	0.246	0.263	0.253	0.422	0.290	0.508	0.242
0.414	0.295	0.559	0.192	0.504	0.369	0.333	0.508	0.427	0.460	0.217	0.305
0.380	0.298	0.649	0.301	0.278	0.203	0.320	0.288	0.457	0.440	0.218	0.227
0.262	0.350	0.264	0.543	0.483	0.598	0.457	0.531	0.386	0.320	0.546	0.467
0.438	0.425	0.250	0.683	0.325	0.419	0.293	0.246	0.255	0.517	0.295	0.174

Average plate minus the smallest value (not normalized):

0.299	0.348	0.478	0.059	0.259	0.249	0.313	0.311	0.094	0.099	0.451	0.435
0.433	0.101	0.095	0.042	0.053	0.360	0.261	0.145	0.330	0.464	0.049	0.452
0.086	0.038	0.301	0.140	0.168	0.166	0.239	0.097	0.180	0.098	0.371	0.071
0.136	0.019	0.026	0.174	0.067	0.046	0.079	0.073	0.296	0.132	0.408	0.056
0.233	0.129	0.324	0.014	0.460	0.214	0.175	0.409	0.315	0.348	0.036	0.134
0.175	0.118	0.401	0.122	0.053	0.032	0.160	0.101	0.380	0.282	0.042	0.054
0.080	0.156	0.101	0.362	0.311	0.411	0.332	0.366	0.216	0.162	0.417	0.304
0.188	0.199	0.062	0.405	0.146	0.194	0.110	0.084	0.064	0.324	0.099	0.000

Final average plate normalized and used for the graphical results:

62.599	72.665	100.000	12.350	54.048	51.999	65.400	65.034	19.730	20.734	94.355	90.874
90.509	21.131	19.929	8.734	11.148	75.320	54.665	30.372	69.090	97.084	10.218	94.596
17.932	8.033	62.996	29.337	35.211	34.710	49.877	20.305	37.699	20.462	77.536	14.765
28.386	4.030	5.514	36.445	14.096	9.570	16.563	15.225	61.856	27.602	85.366	11.660
48.811	27.016	67.731	2.995	96.101	44.839	36.518	85.575	65.808	72.759	7.427	28.020
36.664	24.622	83.902	25.500	11.075	6.768	33.393	21.016	79.470	58.909	8.796	11.190
16.688	32.567	21.016	75.728	64.982	86.014	69.362	76.564	45.194	33.967	87.184	63.456
39.215	41.556	13.061	84.759	30.591	40.469	23.054	17.472	13.427	67.689	20.755	0.000

BCA assay and SDS-PAGE gel results from the liquid-phase assay

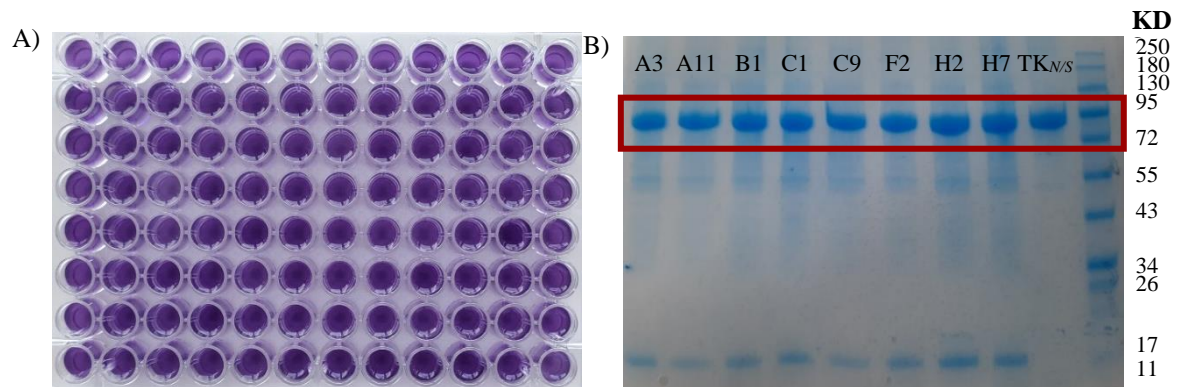


Figure S7. A) 96-well microtiter plate with BCA method. B) SDS gels of 8 random active and inactive variants from the liquid-phase assay: A3: I/S; A11: N/S; B1: N/V; C1: L/M; C9: D/S; F2: D/G; H2: D/V; H7: L/T; TK_{N/S}: TK_{gst} variant (N/S) (1 mg/mL) purified as positive control for quantification and comparison. The last column shows the ladder, and with the red square the corresponding TK_{gst} bands are highlighted. The last bands that can be seen in the gel at the bottom correspond to the lysozyme (14.3 KDa) from cell lysis. 10 μ L of samples were mixed with 10 μ L of protein loading buffer and 7.5 μ L were added to the gel.

SDS-PAGE gel ImageJ results from the liquid-phase assay

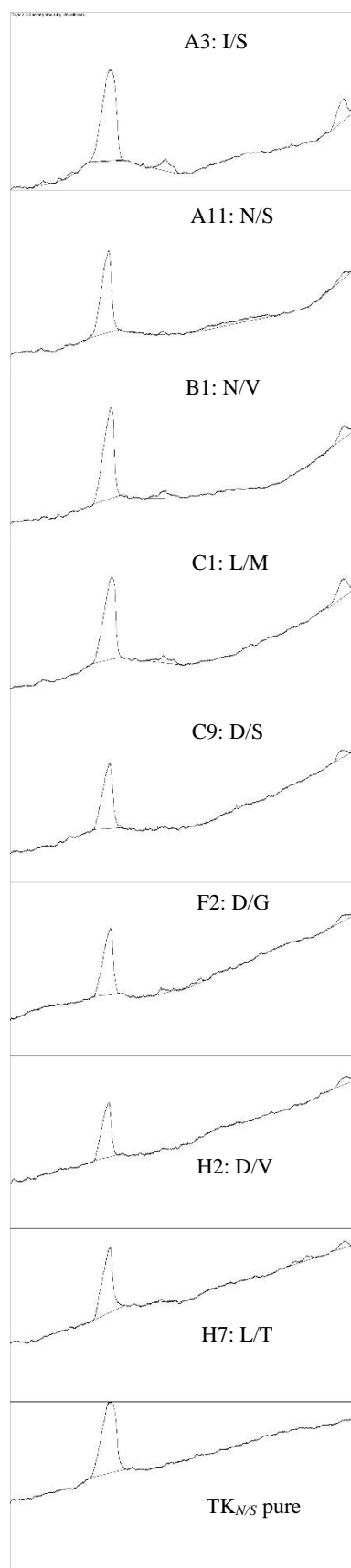


Figure S8. SDS-PAGE gel results of lysates treated with the program ImageJ.

SCREENING WITH THE SOLID-SUPPORTED ASSAY

Cell permeabilization experiment

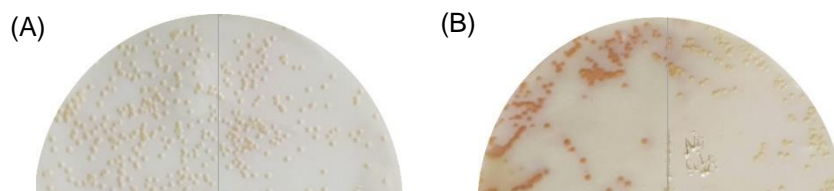


Figure S9. Membranes with *E. coli* colonies containing TK_{gst} N/S mutations after spraying with FeCl₃ (252 mM) solution in the presence of the reagents (left half of circumference) or their absence (right half). Conditions: commercial **1a** (10 mM), DMSO (20%), HPA (7.5 mM), TEA buffer (5 mM, pH 7.45), and FeCl₃ (252 mM). A) No cell permeabilization, B) cell permeabilization by immersing the membranes in liquid nitrogen.

Experiments in the presence and absence of cofactors

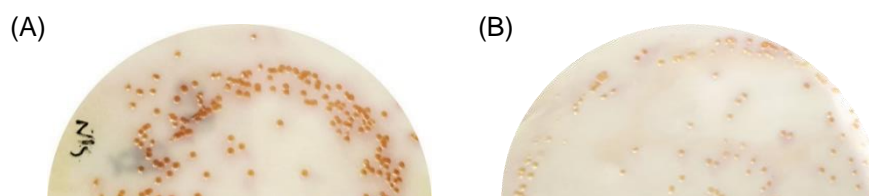


Figure S10. Membranes with *E. coli* colonies containing TK_{gst} N/S mutations after spraying with FeCl₃ (252 mM) solution. Conditions: A) **1a** (5 mM), DMSO (20%), HPA (5 mM), ThDP (0.2 mM), MgCl₂ (1 mM), TEA buffer (5 mM, pH 7.45); B) equal treatment of membrane yet in the absence of cofactors.

Tests with L382N/D470S variant and BL21(DE3) untransformed

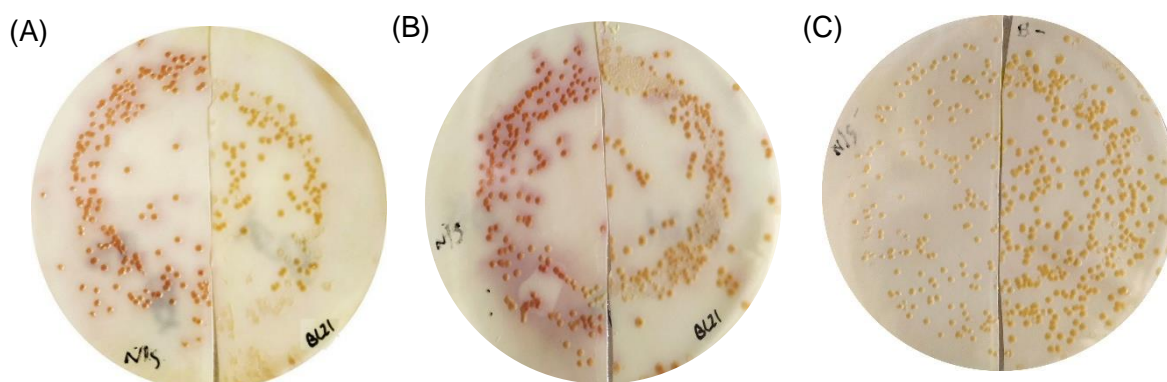


Figure S11. Membranes with *E. coli* colonies containing TK_{gst} N/S mutations (left half) or untransformed BL21(DE3) (right half) after spraying with FeCl₃ (252 mM) solution. Common conditions for A and B: ThDP (0.2 mM), MgCl₂ (1 mM), DMSO (20%), TEA buffer (5 mM, pH 7.45). A) **1a** (5 mM), HPA (5 mM); B) **1a** (10 mM), HPA (10 mM); C) Absence of substrates.

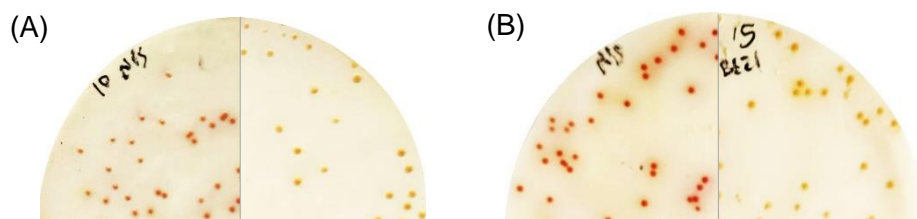


Figure S12. Membranes with *E. coli* colonies containing TK_{gst} N/S mutations (left half) or untransformed BL21(DE3) (right half) with A) 10 mM or B) 15 mM of both substrates, after dipping the membrane in aqueous FeCl₃ (252 mM) solution. Common conditions for A and B: ThDP (0.6 mM), MgCl₂ (2.5 mM), DMSO (20%), TEA buffer (5 mM, pH 7.45).

Tests with N/S variant and TK_{gst} empty-plasmid containing cells

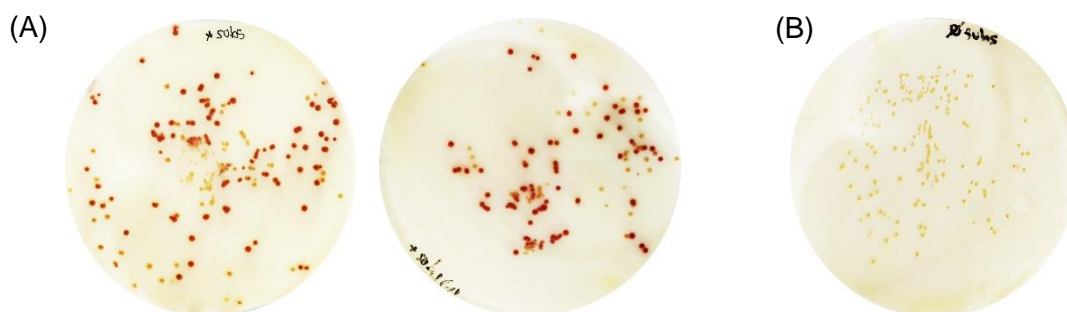


Figure S13. Membranes with *E. coli* colonies containing TK_{gst} N/S mutations and the empty vector in A) the presence or B) absence of substrates after dipping the membrane in aqueous FeCl₃ (252 mM) solution. Conditions for the reactions with substrates: **1a** (15 mM), DMSO (20%), HPA (15 mM), ThDP (0.6 mM), MgCl₂ (2.5 mM), TEA buffer (5 mM, pH 7.45). The experiments were performed in duplicates with similar result.

Screening results of TK_{gst} L382X/D470X library with solid assay



Figure S14. Membranes with *E. coli* colonies containing positive hits of the TK_{gst} L382X/D470X library in A) the presence or B) absence of substrates after dipping the membrane in aqueous FeCl₃ (252 mM) solution. Conditions for the reactions with substrates: **1a** (15 mM), DMSO (20%), HPA (15 mM), ThDP (0.6 mM), MgCl₂ (2.5 mM), TEA buffer (5 mM, pH 7.45). The experiments were performed in duplicates with similar result.

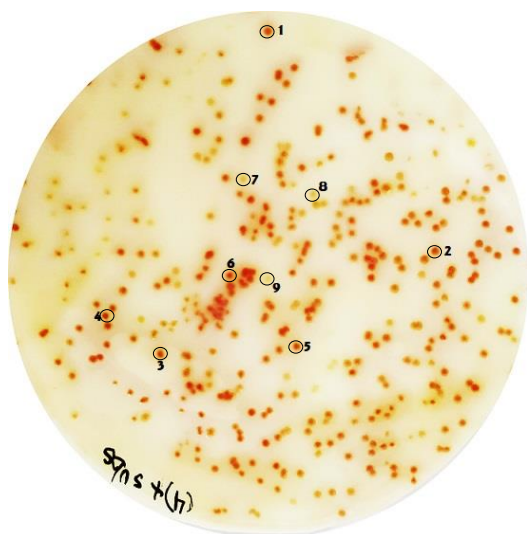


Figure S15. Membranes with *E. coli* colonies containing positive hits of the TK_{gst} L382X/D470X library after dipping the membrane in aqueous FeCl₃ (252 mM) solution. Picked colonies are numbered from 1 to 9 and circled in black.

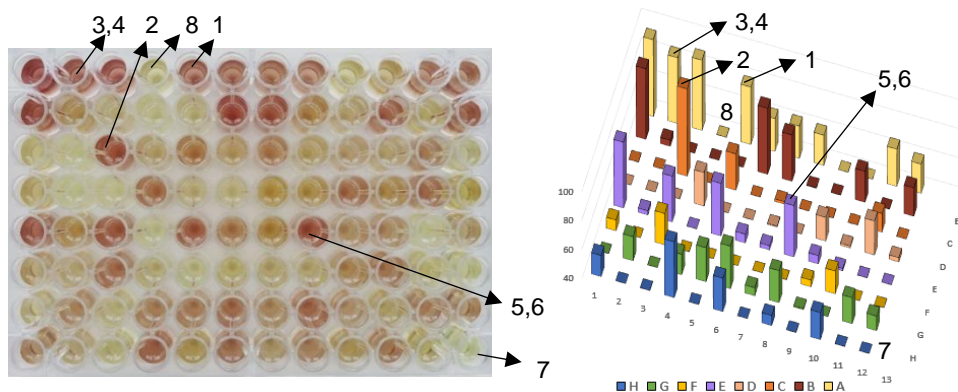


Figure S16. Results of screening of positive hits of the TK_{gst} L382X/D470X library towards **2b** with liquid-phase assay. Variants with equivalent sequence to the picked colonies are pointed with an arrow and numbered. 1: S/D; 2: E/S; 3,4: Q/S; 5,6: N/S; 7: S/G; 8: Y/D.

Sequences of colonies picked from the assay membrane

WILD-TYPE: L382/D470, TTG (Leu)/GAC (Asp)

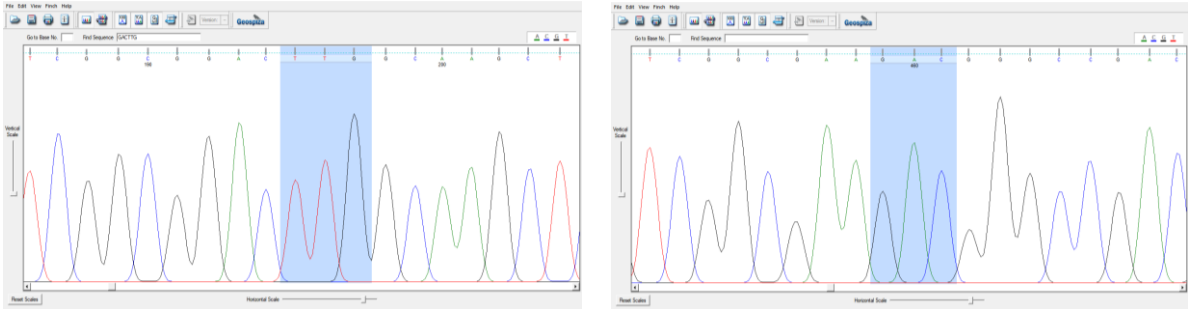


Figure S17. Wild-type sequence L382/D470.

1: L382S/D470D, TCC (Ser)/GAC (Asp)

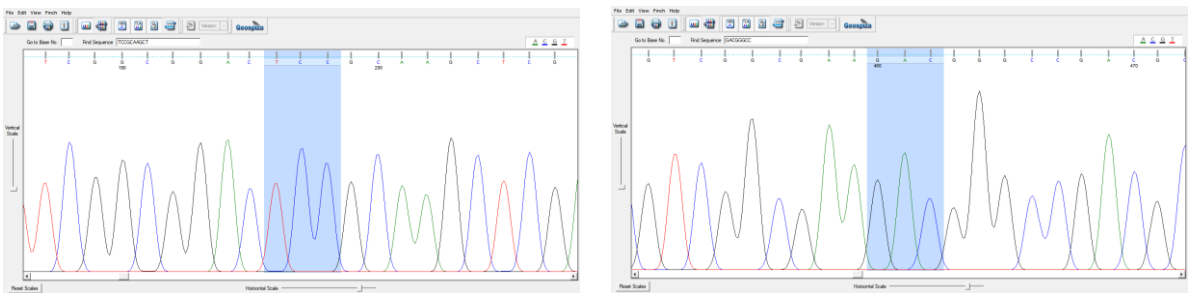


Figure S18. Sequence L382S/D470D.

2: L382E/D470S, GAG (Glu)/TCC (Ser)

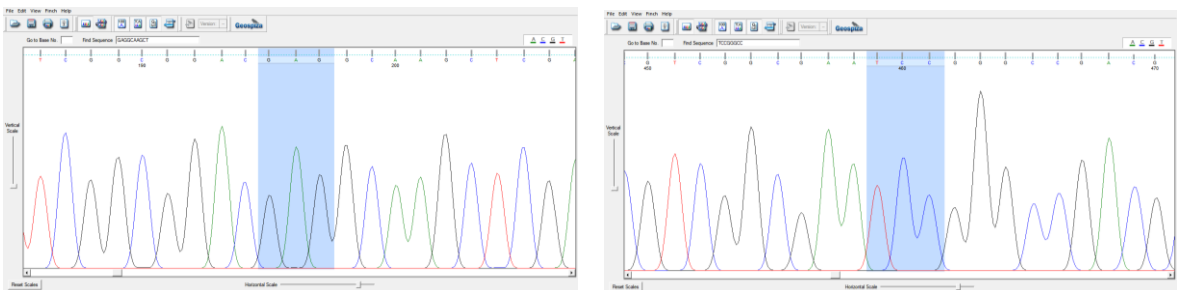


Figure S19. Sequence L382E/D470S.

3: L382Q/D470S, CAG (Gln)/AGC (Ser)

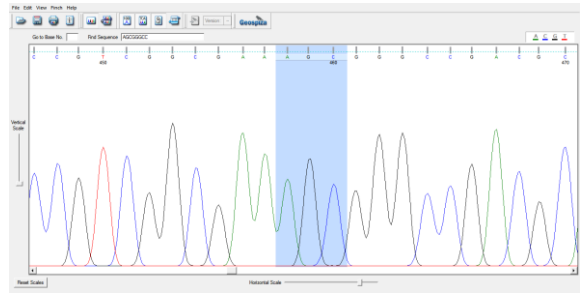
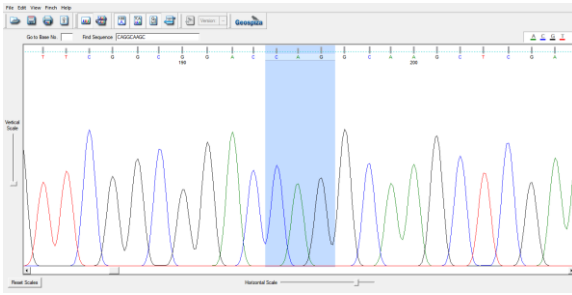


Figure S20. Sequence L382Q/D470S.

4: L382Q/D470S, CAG (Gln)/AGC (Ser)

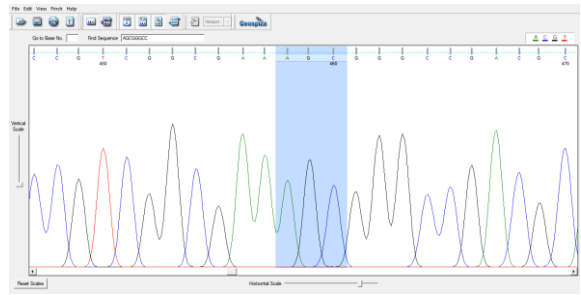
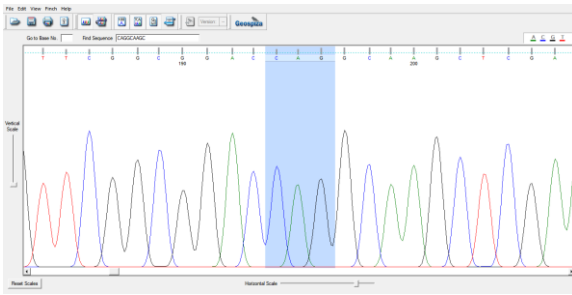


Figure S21. Sequence L382Q/D470S.

5: L382N/D470S, AAC (Asn)/AGC (Ser)

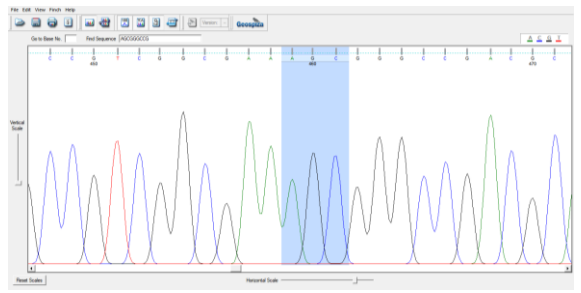
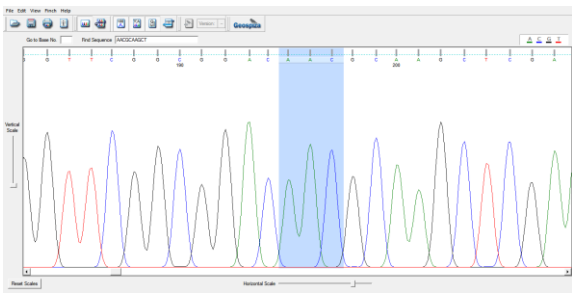


Figure S22. Sequence L382N/D470S.

6: L382N/D470S, AAC (Asn)/TCC (Ser)

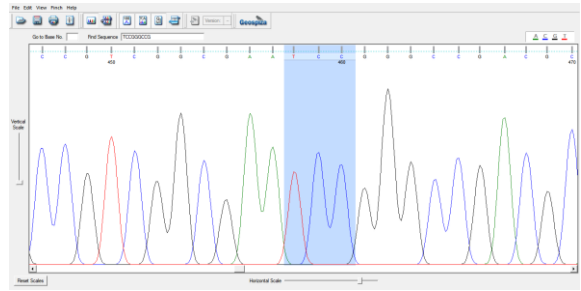
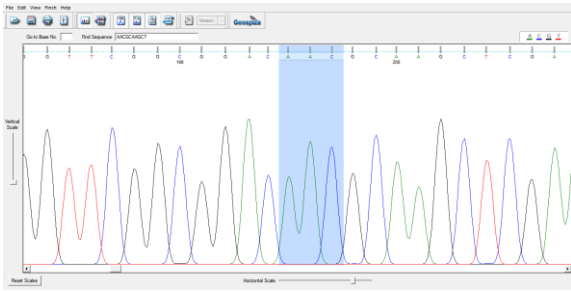


Figure S23. Sequence L382N/D470S.

7: L382S/D470G, AGC (Ser)/GGG (Gly)

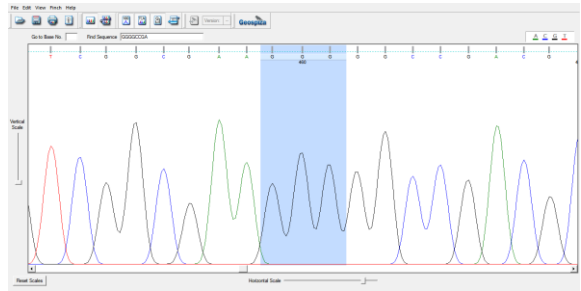
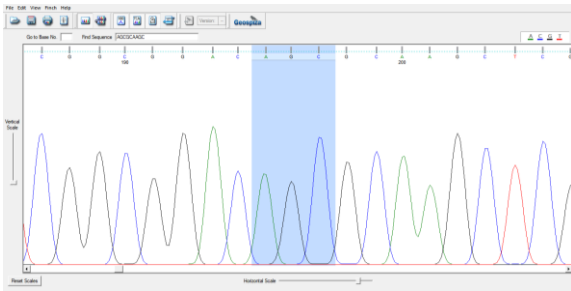


Figure S24. Sequence L382S/D470G.

8: L382Y/D470D, TAC (Tyr)/GAC (Asp)

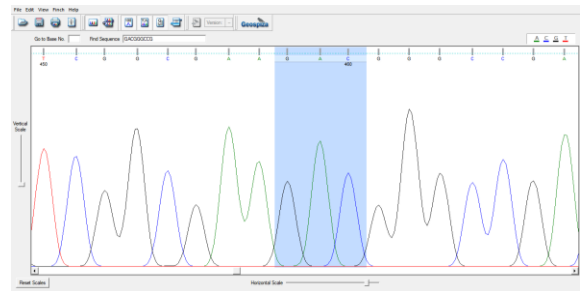
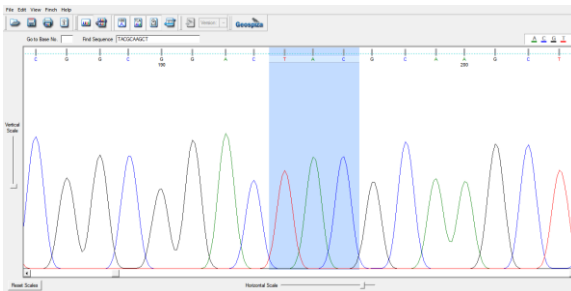


Figure S25. Sequence L382Y/D470D.

9: L382□/D470C, TAG (STOP)/TGC (Cys)

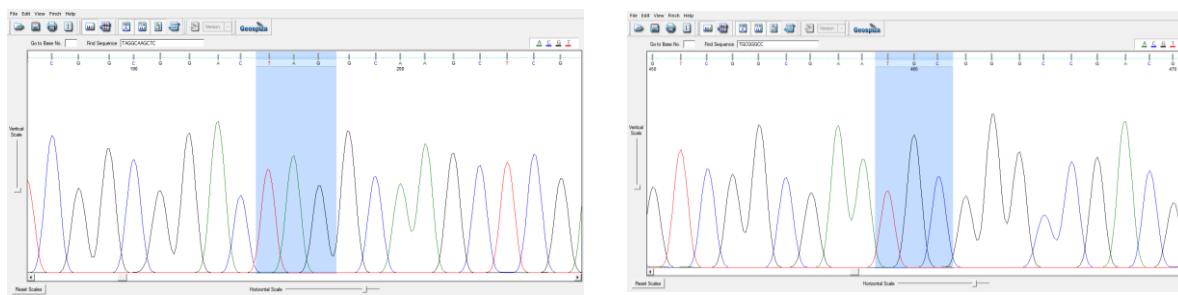


Figure S26. Sequence L382□/D470C.

Solid-supported assay membrane over time

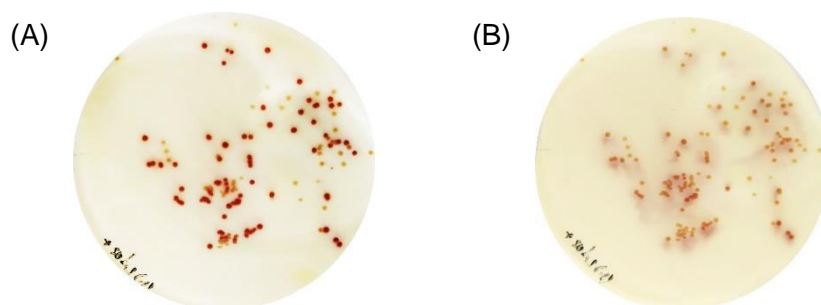


Figure S27. Membranes with *E. coli* colonies containing TK_{gst} N/S mutations and the empty vector A) immediately after dipping the membrane in aqueous $FeCl_3$ solution and B) after 20 minutes.