Supporting Information

An Enzyme Cascade with Horseradish Peroxidase Readout for High-throughput Screening and Engineering of Human Arginase-1

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Detailed chemicals, materials, protein synthesis and nanoDSF experiments

Chemicals and materials

Unless otherwise stated, all the chemicals and the HRP (#77332) were purchased from Sigma-Aldrich. The primary anti-6x-His mouse monoclonal antibodies (#MA1-21315), the goat anti-Mouse IgG (H+L) secondary antibodies conjugated with Alexa Fluor™ 594 (#A-11005) and the Amplex™ UltraRed Reagent (#A36006) were purchased from Thermo Fisher Scientific. DNA oligos to synthesize the arginase library were provided by CodexDNA and DNA sanger sequencing was performed by Mycrosynth.

Protein expression and purification

Sequences for arginase (EC 3.5.3.1), engineered ornithine decarboxylase[33] and putrescine oxidase were cloned into pET28a vectors (GenBank accession numbers: OP832346 - OP832358) with C-terminal 6x His-Tags. Arginase variants were synthesized and cloned on a BioXP 3250 synthetic biology workstation (Codex DNA). ODC, PO, hArg1 and its variants were expressed in *E. coli* BL21 (DE3) from overnight pre-cultures in LB broth, induced with 0.5 mM IPTG at OD_{600} 0.6 - 0.7, and expanded overnight at 25 ºC with 50 µg/mL kanamycin. These proteins were then purified with an Ni-NTA sepharose resin using 500 mM imidazole (HisTrap FF 5 mL, Cytiva) followed by size exclusion chromatography column (Superdex 30 mL, Cytiva) into HEPES buffer (pH 7.4). Protein size and purity was then verified by SDS-PAGE and all samples were stored at - 20 ºC in 20% (v/v) glycerol.

Differential Scanning Fluorimetry (NanoDSF)

A Prometheus NT.48 instrument (NanoTemper Technologies) was used to characterize the thermal denaturation temperatures of human arginase variants following standard protocols. Standard capillaries were filled with 10 µL of each sample at 1 mg/mL and placed on the sample holder unsealed. After a discovery scan that yielded a 70% intensity optimal setting, a one-step temperature gradient of 1 °C$\cdot$min^{-1}, from 20 to 95 °C was applied. The intrinsic protein fluorescence at 330 and 350 nm was then recorded. Data were visualized as the first derivative of the 300/350 nm ratio. The melting temperature corresponds to the maximum of the first derivative from the average of three technical replicates.

Kinetic parameters of hArg1 compared to other publications

Table S1: Kinetic parameters of hArg1 compared to other publications all measured at pH 7.4.

K_M (mM)	k_{cat} (s^{-1})	Assay	Reference
1.16 ± 0.02	16 ± 0.5	Peroxidase cascade	This study
1.65 ± 0.14	26 ± 1.3	Arginase assay kit	This study
1.26	Not reported	Urease coupling	27
1.5 ± 0.2	190 ± 10	Urea derivatization	37
1.9 ± 0.7	Not reported	Urease coupling	19
2.33 ± 0.3	300 ± 12	Urea derivatization	10
2.3	57	Urea derivatization	4
3.3 ± 0.2	(v_{max}) 34 ± 1 nmol $min^{-1}mg^{-1}$	UPLC-MS analysis	38
5.01	93	SAMDI	29

Kinetic and thermal parameters of hArg1 and library members.

Table S2: Kinetic and thermal parameters of hArg1 and library members.

Mutant	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (mM^{-1} s^{-1})	k_{cat} (%)	T_m (℃)
R21E	18 ± 0.2	1.39 ± 0.07	12.95	113	77.5
WT	16 ± 0.5	1.16 ± 0.02	13.79	100	80.5
Q19E	9 ± 0.3	1.77 ± 0.09	5.08	58	78.5
T246E	12 ± 0.3	4.47 ± 0.17	2.68	75	79.5
R21D	8 ± 0.1	2.18 ± 0.16	3.67	52	77.8
V145K	4 ± 0.3	27.25 ± 8.88	0.37	25	70.5
Q19D	3 ± 0.1	9.99 ± 1.19	0.16	19	79.6
T246D	2 ± 0.3	4.70 ± 2.06	0.26	13	81.5
E277D	~0	n.d.	n.d.	n.d.	72.3
V145R	~0	n.d.	n.d.	n.d.	67.1
E186K	~0	n.d.	n.d.	n.d.	66.6
E186R	~0	n.d.	n.d.	n.d.	65.9
G18D	~0	n.d.	n.d.	n.d.	73.1
G18E	~0	n.d.	n.d.	n.d.	72.9

Primary amino acid sequences

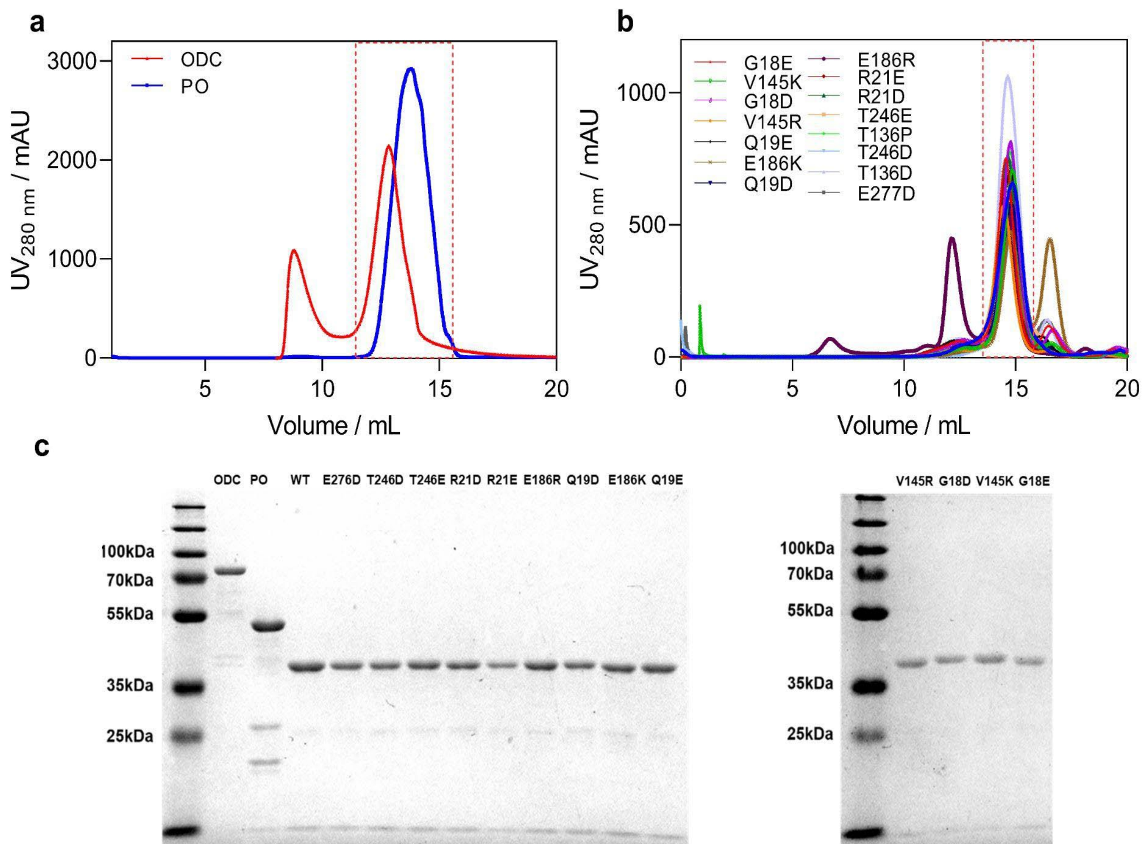
Ornithine decarboxylase (EC 4.1.1.17) with 6x-HisTag
MKSMNIAASSELVSRLSSHRRVVALGDTDFTDVAAVVITAADSRSGILALLKRTGFHLPVFLYSEHAVELPAGVTAVI
NGNEQQWLELESAACQYEENLLPPFYDTLTQYVEMGNSTFACPGHQHGAFFKKHPAGRHFYDFFGENVFRADM
CNADVKLGDLLTHTGSAKDAQKFAAKVFHADKTYFVLNGTSAANKVVTNALLTRGDLVLFDRNNHKSNHHGALIQ
AGATPVYLEASRNPFGFIGGIDAHCFNEEYLRQQIRDVAPEKADLPRPYRLAIIQLGTYDGTVYNARQVIDTVGHLCD
YILFDSAWVGYEQFIPMMADSSPLLLELNENDPGIFVTQSVHKQQAGFSQTSQIHKKDNHIRGQARFCPHKRLNN
AFMLHASTSPFYPLFAALDVNAKIHEGESGRRLWAECVEIGIEARKAILARCKLFRPFIPPVVDGKLWQDYPTSVLAS
DRRFFSFEPGAKWHGFEGYAADQYFVDPCKLLLTTPGIDAETGEYSDFGVPATILAHYLRENGIVPEKCDLNSILFLLT
PAESHEKLAQLVAMLAQFEQHIEDDSPLVEVLPSVYNKYPVRYRDYTLRQLCQEMHDLYVSFDVKDLQKAMFRQQ
SFPSVVMNPQDAHSAYIRGDVELVRIRDAEGRIAAEGALPYPPGVLCVVPGEVWGGAVQRYFLALEEGVNLLPGFS
PELQGVYSETDADGVKRLYGYVLKTRTGHHHHHH*

Putrescine oxidase (EC 1.4.3.10) with 6x-HisTag
MQNLDRDVVIVGAGPSGLTAARELKKAGLSVAVLEARDRVGGRTWTDTIDGAMLEIGGQWVSPDQTVLMELLD
ELGLKMYSRYRDGESVYIGADGKRTQYTGDTFPVNETTKAEMDKLVAILDELAAEIGPTEPWAHPKARELDTISFHH
WLRQNSNDEEACNNIGLFIAGGMLTKPAHAFSALQAVLMAASAGSFSHLTDEDFILDKRVIGGMQQVSLLQAAEL
GDDVVLNSPVRTIKWDENGVSVVSERATVNARFVIMAVPPNLYSRVSFDPPLPRRQHQMHQHQSLGLVIKVHAV
YDTPFWREEGLSGTGFSAGALVQEVYDNTNHGDSRGTLVGFVSDEKADAVFELSAEDRKKAILESIAGFLGDKALTP
EVYYESDWGSEEWTRGAYASSYDLGGLHRYGKDQHANVGPIYWSSSDLAAEGYQHVDGAVRMGQATAARIVEA
NKLASVPVATRTGHHHHHH*

Human Arginase 1 (WT) (EC 1.4.3.10) with 6x-HisTag
MSAKSRTIGIIGAPFSKGQPRGGVEEGPTVLRKAGLLEKLKEQECDVKDYGDLPFADIPNDSPFQIVKNPRSVGKASE
QLAGKVAEVKKNGRISLVLGGDHSLAIGSISGHARVHPDLGVIWVDAHTDINTPLTTTSGNLHGQPVSFLLKELKGKI
PDVPGFSWVTPCISAKDIVYIGLRDVDPGEHYILKTLGIKYFSMTEVDRLGIGKVMEETLSYLLGRKKRPIHLSFDVDG
LDPSFTPATGTPVVGGLTYREGLYITEEIYKTGLLSGLDIMEVNPSLGKTPEEVTRTVNTAVAITLACFGLAREGNHKPI
DYLNPPKHHHHHH*

Primary nucleotide sequences for the mutants can be found under the following GenBank accession numbers: OP832346 - OP832358

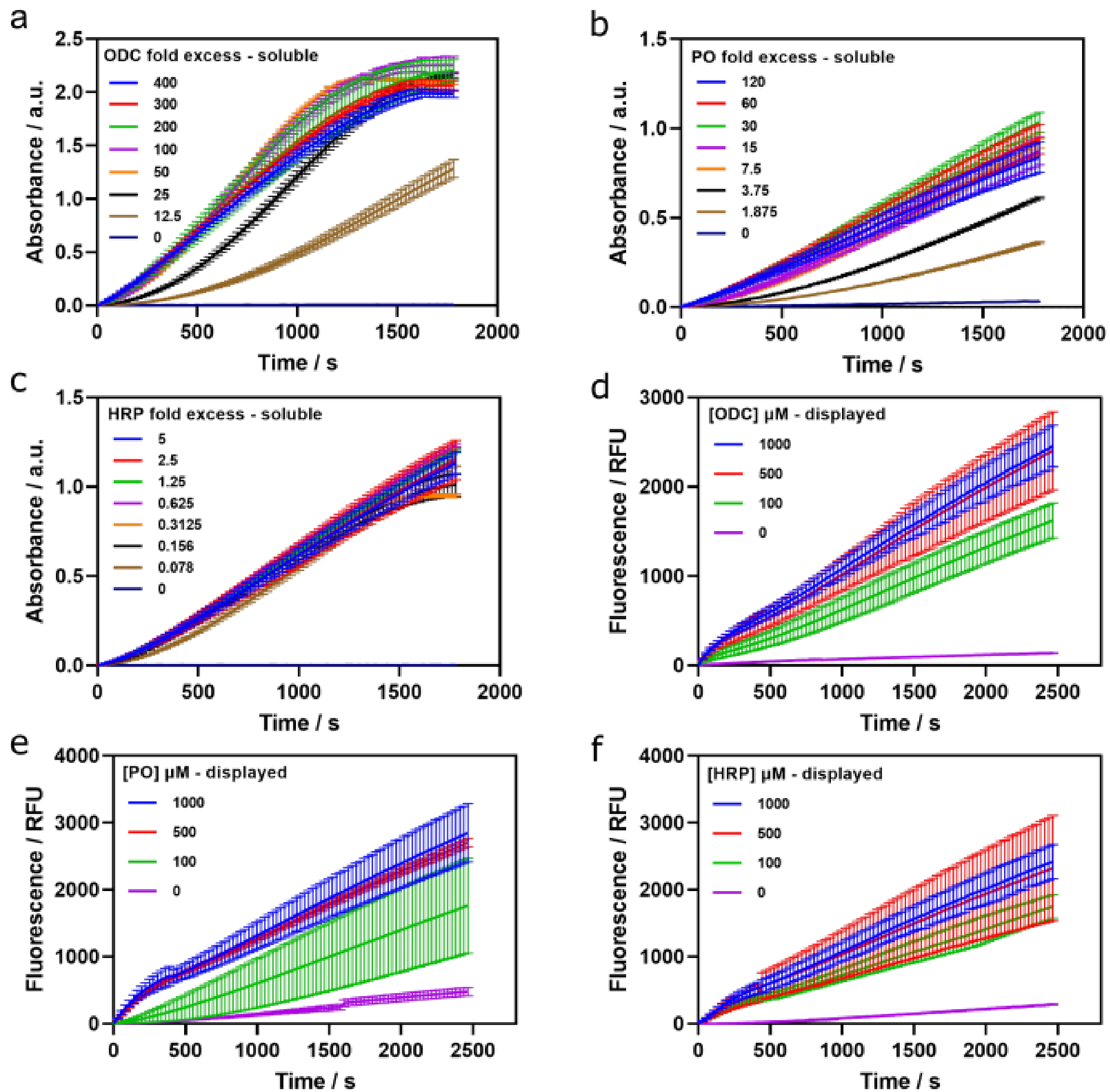
Size exclusion chromatograms and SDS-PAGE analysis for purified proteins



Supplementary Figure 1: Protein purification of auxiliary enzymes, WT hArg1 and hArg1 variants.

(a) Size exclusion chromatograms of auxiliary enzymes (ODC and PO) and, **(b)** hArg1 WT and its mutants after processing cell cultures with Ni-NTA affinity chromatography column. Further size and purity verification was carried out in a 12% SDS-PAGE **(c)**. Theoretical molecular weights of ODC, PO, and hArg1 are 80.6, 51.1 and 37 kDa respectively.

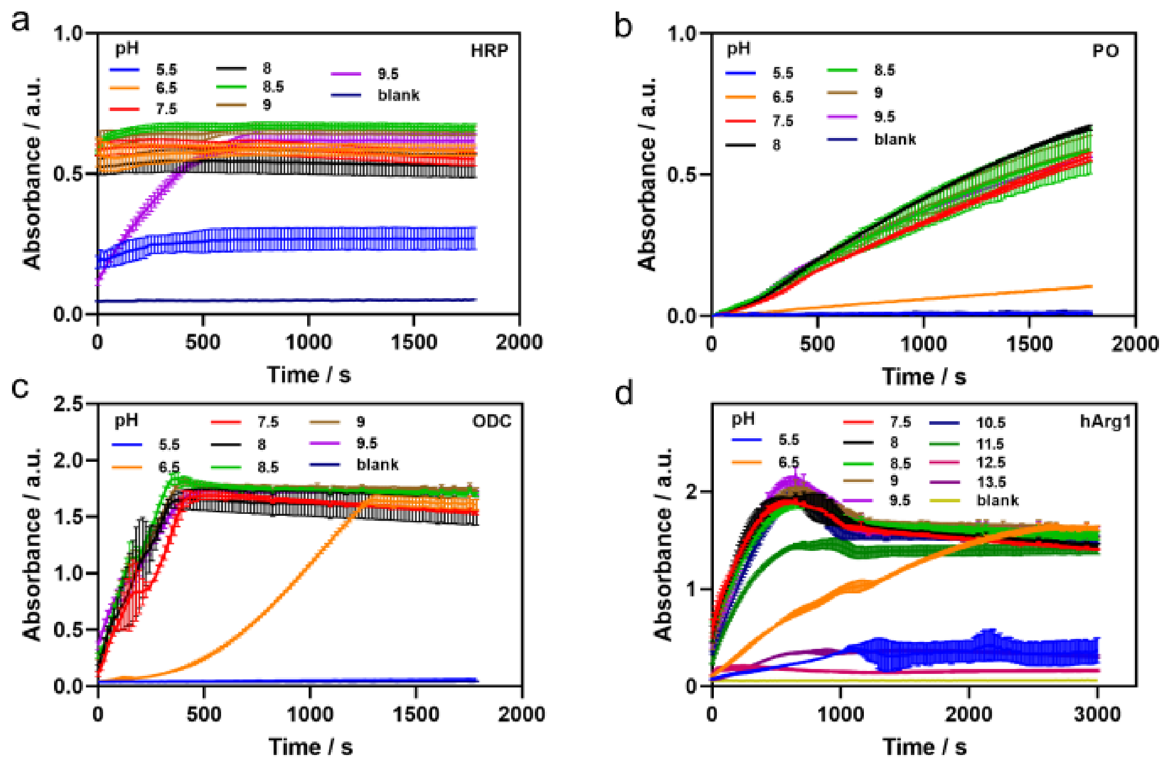
Determination of the concentration of auxiliary enzymes



Supplementary Figure 2: Saturation of auxiliary enzymes.

(a)-(c), Time course experiments of 5 nM soluble hArg1 with excess ODC, PO and HRP to ensure that hArg1 is the rate-determining enzyme in the cascade. When not specified, L-arginine, hArg1, ODC, PO and HRP were at 20 mM, 5 nM, 1.5 µM, 1.5 µM and 250 nM, respectively. The fold excess concentration refers to the 5 nM of hArg1. **(d)-(f),** Time course experiments with hArg1-displaying cells (2.5 million cells/mL) with excess ODC, PO and HRP to ensure that hArg1 is the rate-determining enzyme in the cascade. In all panels, data points represent the mean of triplicates and the SD is indicated by error bars.

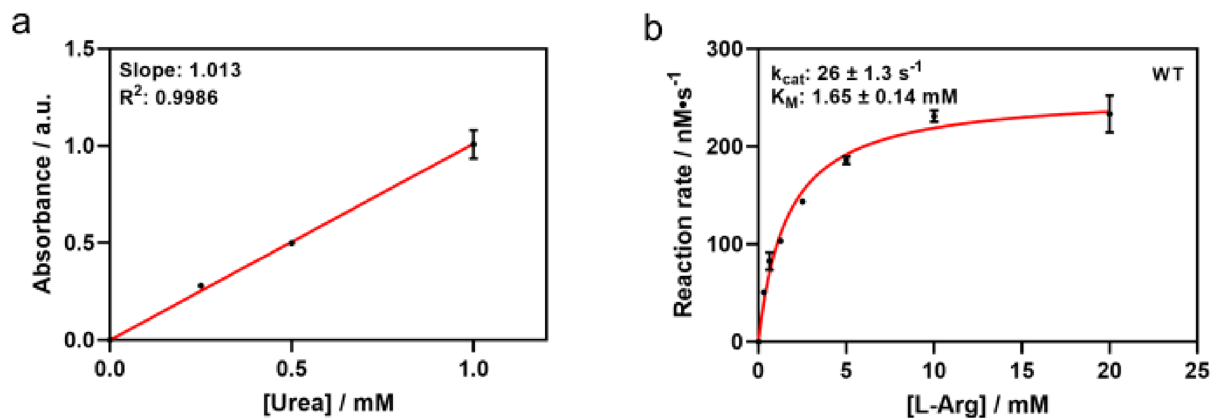
Characterization of the pH range of the assay



Supplementary Figure 3: Characterization of the pH range of hArg1 in our system.

Time course experiments with 50 nM of HRP **(a)** 10 nM of PO **(b)** 12.5 nM of ODC **(c)** or 1 nM of hArg1 **(d)** as the starting point for the cascade with 100 µM H_2O_2, 25 mM putrescine, 25 mM L-ornithine and 20 mM L-arginine, respectively. The downstream enzyme concentrations were 5 µM ODC, 2 µM PO and 500 nM HRP. Data points represent the mean of triplicates and error bars are the SD. Reaction rates were estimated from a linear regression performed in the linear range of the reaction.

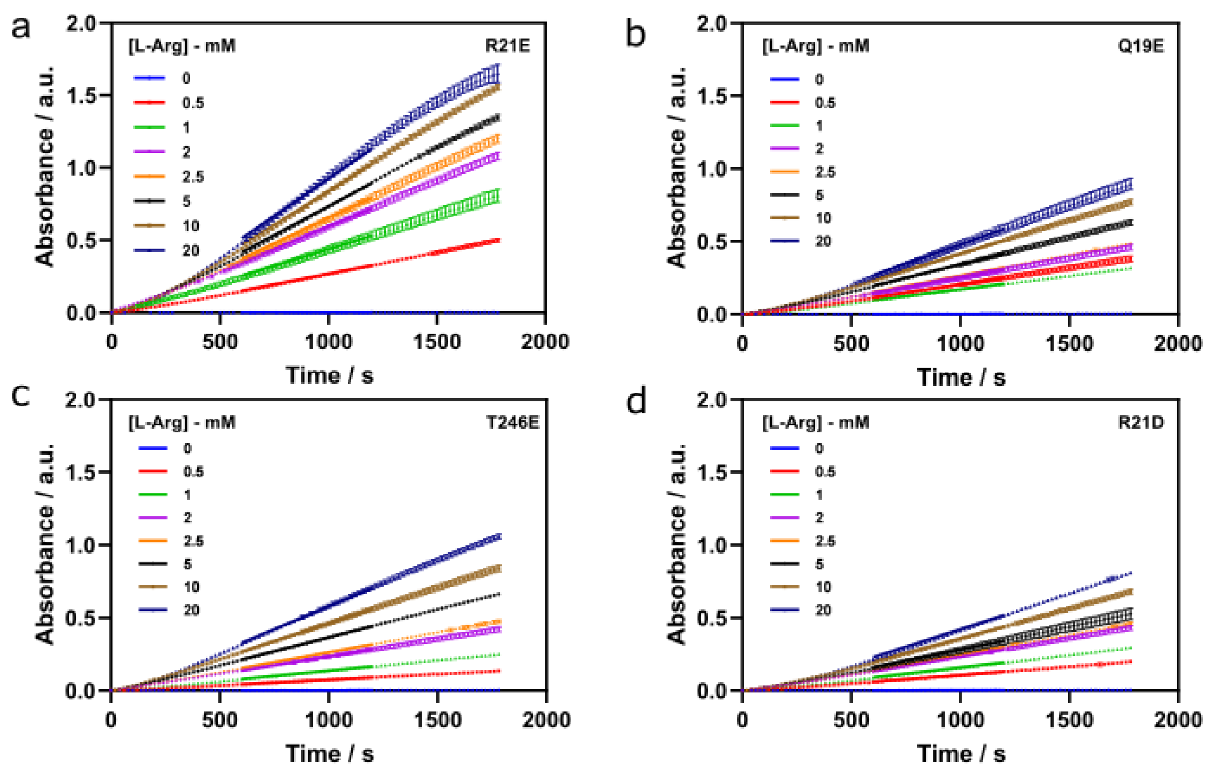
Characterization of hArg1 with the commercial Arginase Activity Assay Kit



Supplementary Figure 4: Arginase assay kit.

(a) Standard curve for urea conversion of the commercial *Arginase Activity Assay Kit* from 0 - 1 mM. Data represents the mean of duplicates, and the error bars are SD. **(b)** Kinetic parameter estimation of 10 nM WT hArg1 after 1 hour incubation with L-arginine (0 - 20 mM) at pH 7.4 and 37 ºC using a Michaelis-Menten fit with *Arginase Activity Assay Kit*(Sigma-Aldrich), following manufacturer instructions with minor adaptations. The reaction was quenched with 200 µL of the provided urea reagent and incubated for another hour at room temperature. Absorbance was recorded at 430 nm and data points represent the mean and error bars are the SD (n = 3).

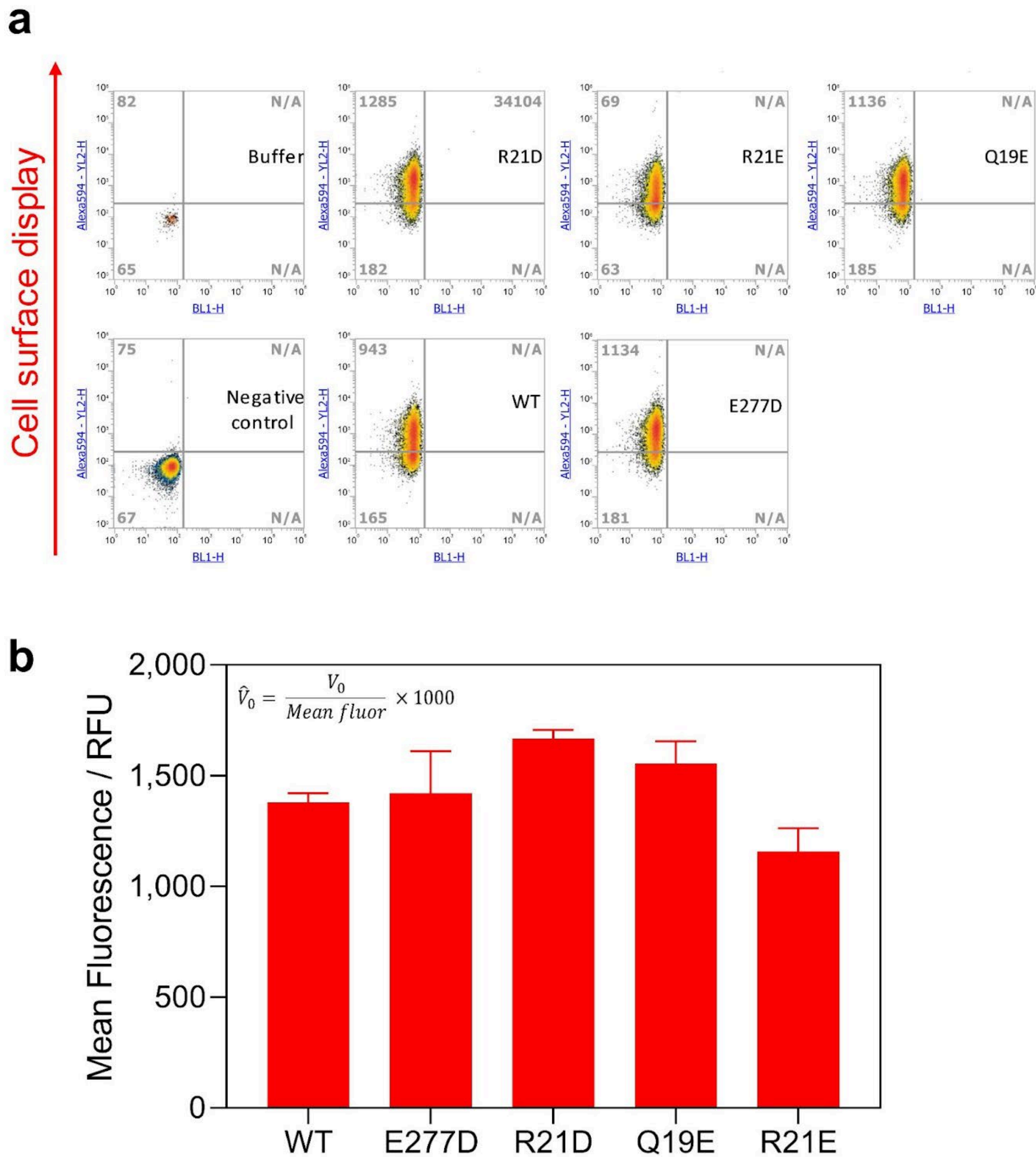
Screening of active members of the library



Supplementary Figure 5: Screening of active members of the library.

(a)-(e), Time course experiments with 5 nM of hArg1 or its active mutants with 0 - 20 mM of L-arginine. All mutants were activated with Mn^{2+} at pH 7.4 and measurements were taken in triplicates at 37 ºC. Data points represent the mean and error bars are the SD (n = 3). Reaction rates were estimated from a linear regression performed from 600-1200 s, here shown in a solid line.

Expression level by mutant and normalization



Supplementary Figure 6: Expression level by mutant and normalization equation inset.

(a) Immunolabeling of $2 \cdot 10^7$ hArg1- and mutant-hArg1-displaying NiCo21 cells/mL analyzed with Attune NxT (life technologies) Flow Analyzer. Cells were labeled with anti-6xHisTag mouse antibodies (dilution 1:500) for 30 mins at room temperature and with anti-mouse goat antibodies conjugated with AlexaFluor594 (dilution 1:500) for 20 mins at 4 ºC. Fluorophore was detected by the YL2 laser here represented in the y-axis. The negative control consisted of the WT cells labeled only with the secondary antibody. **(b)** The expression level of each mutant was considered the median of the expressing cells for each mutant and the reaction velocity normalization was performed following the inset equation. The error bars represent the SEM (n = 10,000).