Supporting Information

Evolving a fluorescent light-up aptamer for rotationally quenched asymmetric rhodamines

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Experimental methods

General

Reagents were purchased from THERMO FISHER SCIENTIFIC, NEW ENGLAND BIOLABS or SIGMA-ALDRICH and used without further purification. DNA oligonucleotides and the 15% RhoBAST doped DNA library were purchased from INTEGRATED DNA TECHNOLOGIES. RNA folding structures were predicted by mfold[1] and depicted using the software VARNA.[2] All experiments were conducted using deionized water purified by Milli-Q purification systems A10 or EQ7000 from MERCK. For absorbance measurements, a Cary50 UV/Vis spectrometer (VARIAN) was used. Fluorescence measurements were conducted at either a FP-6500 or a FP-8500 spectrofluorometer (JASCO) at 25°C using a 20 µL or 100 µL quartz glass cuvette (pathlength 1 cm). Stopped-flow experiments were recorded on a SX-18M stopped-flow spectrometer (APPLIED PHOTOPHYSICS).

NMR-spectra were recorded using a Mercury Plus 300 or 500 (VARIAN). The chemical shifts δ are given in ppm (parts per million) with respect to tetramethylsilane. The following spectroscopic experiments were conducted: ^{1}H, ^{13}C, COSY, HSQC, HMBC. High resolution mass measurements were obtained utilizing a BRUKER micrOTOF QII-ESI system.

For column chromatography, high-quality silica gel (pore size 60 Å) purchased from SIGMA-ALDRICH was used. Preparative reversed-phase high performance liquid chromatography (HPLC) was performed on an AGILENT 1100 Series HPLC system with a diode array detector. A PHENOMENEX Luna 5 µm C-18(2) 100 Å (250 x 21.2 mm) column was used applying a constant flow rate of 5 mL/min. As solvent systems either buffer A (100 mM triethylammonium acetate pH = 7.0 in water) and buffer B (100 mM triethylammonium acetate pH = 7.0 in 80% acetonitrile) or water and acetonitrile, each containing 0.1% trifluoroacetic acid (TFA), were used.

In vitro synthesis of RNA

RNA aptamers were synthesized by *in vitro* transcription using T7 RNA polymerase (lab stock), 1 µM dsDNA template, 2 mM NTP, 10 mM DTT in transcription buffer (40 mM Tris-HCl (pH 8.1), 1 mM spermidine, 22 mM $MgCl_2$, 0.01% Triton X-100, 5% DMSO, 10 mM DTT). The mixture was incubated for 4 h at 37°C. Thereafter, DNaseI (10 U/µL) digestion was performed by incubation for 30 min at 37°C. The RNA was purified by denaturing polyacrylamide gel electrophoresis (PAGE, 10%). The RNA product was visualized by UV-shadowing, excised and eluted with NaOAc buffer (0.3 M, pH 5.5). The product was obtained by ethanol or isopropanol precipitation.

SELEX

HPLC-purified **PhTMR** was immobilized on NHS-activated Sepharose 4 Fast Flow resin (GE Healthcare) according to the manufacturer´s protocol yielding a final concentration of 4.4 mM loaded dye. Following the same procedure, a mock resin was prepared using 2,2'-(ethane-1,2-diylbis(oxy))bis(ethan-1-amine).

The initial DNA library (15% doped RhoBAST) was PCR amplified using Taq polymerase and *in vitro* transcribed as stated above. After DNaseI digestion, the RNA library was phenol-chloroform-isoamylalcohol extracted and isopropanol precipitated. Starting with a diversity of $4 \cdot 10^{14}$ molecules, an RNA concentration of 390 µM was applied in the first SELEX round. Thereafter, the selection was conducted with 3.2 µM in the second round and 2.5 µM for all following rounds.

Prior to every selection round, the RNA was freshly folded by incubating for 2 min at 75°C and cooling to 25°C with a rate of 5°C/min. Then, aptamer selection buffer supplemented with $MgCl_2$ (ASBMg: 20 mM HEPES (pH 7.4), 5 mM $MgCl_2$,125 mM KCl) was added.

The resins were washed with ASBMg. The freshly folded RNA was incubated with the mock resin (250 µL) for 1 h at 25°C while shaking. Thereafter, the unbound RNA was incubated with the PhTMR-loaded resin for 30 min at 25°C while shaking. After washing the resins six times with ASBMg, the bound RNA was eluted three times by incubation for 5 min at 65°C using formamide elution buffer (formamide 98%, 10 mM EDTA). The eluted RNA was ethanol precipitated, reverse transcribed by SuperScript IV reverse transcriptase (THERMO FISHER SCIENTIFIC) using primer B (5 µM, SI Table S5), dNTP (0.5 mM), DTT

(5 mM), 5X SuperScript IV RT reaction buffer according to the manufacturer´s protocol and PCR-amplified using Taq polymerase. The obtained DNA pool was *in vitro* transcribed by T7 polymerase and purified by denaturing PAGE as described above. Following this protocol, six rounds of SELEX were performed. The RNA elution yields (Fig. 1D) are given as the ratio of eluted RNA to initially added RNA amount determined by NanoDrop ND-1000 spectrophotometer.

For analysis of the enriched sequences from the SELEX, the reverse-transcribed and amplified DNA of selection round six was converted into 5´-phosphorylated blunt-end DNA using the Fast DNA End Repair Kit (THERMO FISHER SCIENTIFIC). After purification with a QIAquick PCR purification kit (QIAGEN), the DNA pool was cloned into EcoRV-digested pDisplay-AP-CFP-TM vector (Addgene, plasmid #20861)[3] by blunt-end ligation using T4 DNA-ligase. The obtained vector was transformed into competent DH5 α *E. coli* cells by heat-shock procedure, bacteria were stroked on LB-agar plates with ampicillin (100 µg/mL) and grown over night at 37°C. For Sanger sequencing by MICROSYNTH SEQLAB 96 colonies were selected. The sequences were truncated by excluding the constant primer regions and aligned using the CrystalW algorithm by the software MEGA X.[4] Folding predictions of RNA aptamers were performed using m-fold.[1] The obtained sequences are listed in Table S6.

***In vitro* characterization of dyes and aptamer:dye complexes**

Prior to all *in vitro* experiments, RNA aptamers were freshly folded as stated above. As buffers either ASB (aptamer selection buffer) containing 20 mM HEPES (pH 7.4), 1 mM $MgCl_2$ and 125 mM KCl or ASBT (aptamer selection buffer supplemented with 0.05% (v/v) Tween20) were used.

Fluorophores and aptamer:dye complexes were excited with the maximum excitation wavelength in fluorescence measurements. Fluorescence intensities were detected at the maximum emission wavelength. If required, minimal and maximal absorbance or fluorescence intensities were normalized to 0 and 1, respectively.

The magnesium ion (Mg^{2+}) dependence on the folding of the aptamer was determined by measuring the fluorescence intensities of equimolar concentrations of aptamer and fluorogen (20 µM) within ASB containing different $MgCl_2$ concentrations.

The D_{50} values as the dielectric constant at half-maximum absorbance were obtained by measuring the absorbance of the fluorophore (5 µM) at different water-dioxane mixtures (10–90% (v/v)) at 25°C.

Molar extinction coefficients (ε) at the respective maximum absorbance wavelength were determined by absorbance measurements of dye (0.1–1 µM) in the presence and absence of aptamer (5 µM).

The fluorescence turn-ons were determined as ratio of the fluorescence intensities of fluorophore in the presence and absence of aptamer.

Dissociation constants (K_D) were determined as previously described.[5] In detail, K_D values were determined by measuring the fluorescence changes of dye upon binding to aptamer in ASBT (aptamer selection buffer supplemented with 0.05% (v/v) Tween20). A fixed concentration of fluorophore (50 nM) was titrated with different aptamer concentrations. The measured fluorescence intensities versus increasing RNA concentrations were fitted using equation (1) with F as fluorescence at any aptamer concentration, F_0 as fluorescence of free fluorophore with a starting concentration of P_0, F_∞ as the maximum fluorescence of the complex, [*Apt*] as aptamer concentration and K_D as dissociation constant.[5]

$$F = F_0 + \frac{(F_\infty - F_0) \times \left\{ (K_D + P_0 + [Apt]) - \sqrt{([Apt] - P_0)^2 + K_D \times (K_D + 2[Apt] + 2P_0)} \right\}}{2P_0} \quad (1)$$

Association (k_a) and dissociation rate coefficients (k_d) were obtained as previously described.[5] Concisely, fluorophore (10 nM) was titrated with different aptamer concentrations and the fluorescence increase over time was recorded using a stopped-flow spectrofluorometer and measured in five technical replicates. Linear fitting of the observed kinetic rates versus the aptamer concentration yielded the kinetic rate coefficients.

Monitoring *in vitro* transcription

In vitro transcription was monitored in real-time using a Tecan Spark multimode microplate reader. DNA-templates (5 µmol, RhoBAST^{G36C}, RhoBAST or SiRA, see SI Table S5) with T7 promoter were transcribed using T7 RNA polymerase (lab stock), 2 mM NTP, 10 mM DTT, pyrophosphatase (0.1 U/µL, THERMO FISHER SCIENTIFIC) in transcription buffer (40 mM Tris-HCl (pH 8.1), 1 mM Spermidine, 22 mM $MgCl_2$, 0.01% Triton X-100, 5% DMSO, 10 mM DTT) in the presence of 500 nM fluorogen at 37°C. The fluorescence intensity was recorded every 30 s over 2 hours.

DNA cloning and plasmids

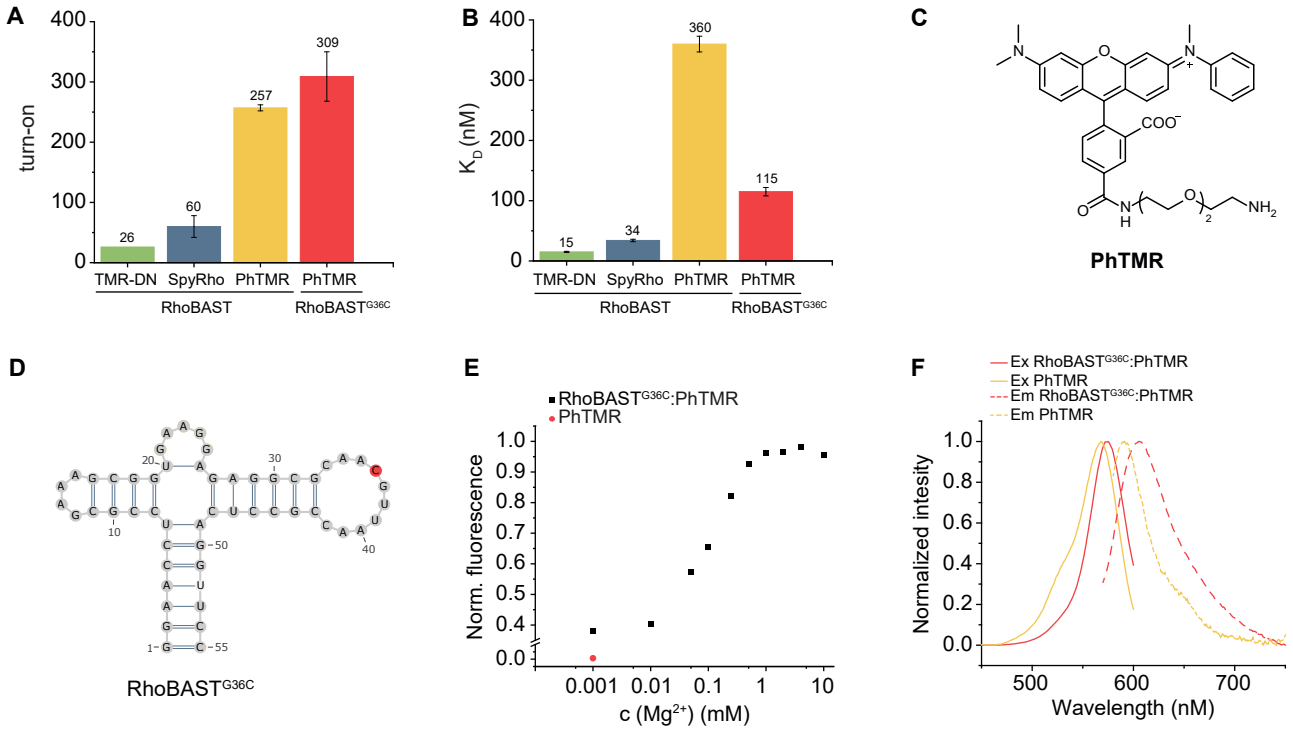
DNA sequences are listed in Table S5. To prepare the vector pET-1GFP-(RhoBAST^{G36C})$_{16}$ the synonymous repeats (RhoBAST^{G36C})$_2$ and (RhoBAST$^{G36C\ \prime}$)$_2$ were synthesized by PCR and digested with XhoI and SalI. The pET-His6-GFP-TEV-LIC (1GFP) cloning vector (Addgene, plasmid #29663)[6] was double digested using XhoI and SalI, dephosphorylated, and ligated with double-digested (RhoBAST^{G36C})$_2$. After insertion of (RhoBAST^{G36C})$_2$ the plasmid was only single digested with XhoI and deposphorylated for all further repeats. All inserts having more than four repeats were amplified by PCR with the plasmid as template. Finally, the vector pET-1GFP-(RhoBAST^{G36C})$_{16}$ was obtained.

Confocal microscopy

A point scanning Nikon A1R confocal microscope based on a fully inverted Nikon Ti2 was used for confocal laser scanning microscopy. It was equipped with a hybrid scanner (galvo/resonant) and four-channel detection. As objective, a Nikon N Apo 60× NA 1.4 λs OI (WD 0.14 mm, FOV 0.21 × 0.21 mm) was used. GFP was excited at 488 nm and the emission was detected via a 525±25 nm filter set. SpyRho/TMR was excited at 561 nm and the emission was detected via a 595±50 nm filter set. Images were recorded using the Nikon Elements software and analyzed with Fiji.[7]

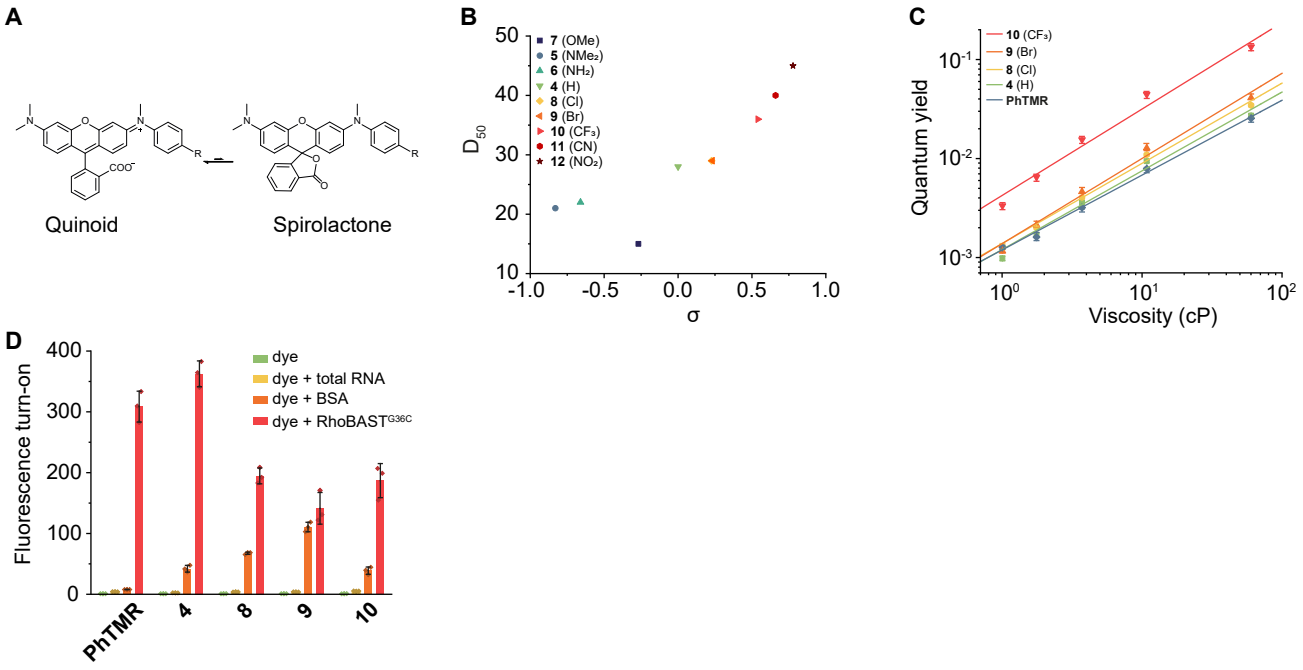
Live *E. coli* imaging

Competent BL21(DE3) *E. coli* were transformed using pET-1GFP (pET-His6-GFP-TEV-LIC (1GFP), ADDGENE #29663), pET-1GFP-(RhoBAST^{G36C})$_{16}$, pET-28a(+)-tRNA or pET-28a(+)-tRNA-RhoBAST^{G36C}. Bacteria were stroked on LB-agar plates with kanamycin (30 µg/mL) and grown over night at 37°C. Next, single colonies were picked and grown in LB medium with kanamycin (30 µg/mL) and glucose (1% w/v) overnight shaking at 37°C. The overnight cultures were diluted with fresh LB medium containing kanamycin (30 µg/mL) to an OD_{600} of 0.05 and were grown until an OD_{600} of 0.3. Expression was induced with isopropyl-β-D-thiogalacto-pyranosid (IPTG, 100 µM) and bacteria were further grown at 37°C for 2–4 h. Bacteria were seeded on poly-D-lysine-coated glass slides (8-well glass bottom µ-slides IBIDI). Prior to imaging bacteria were incubated with PhTMR in premwarmed life cell imaging solution (1x LCIS, INVITROGEN) for 30 min.



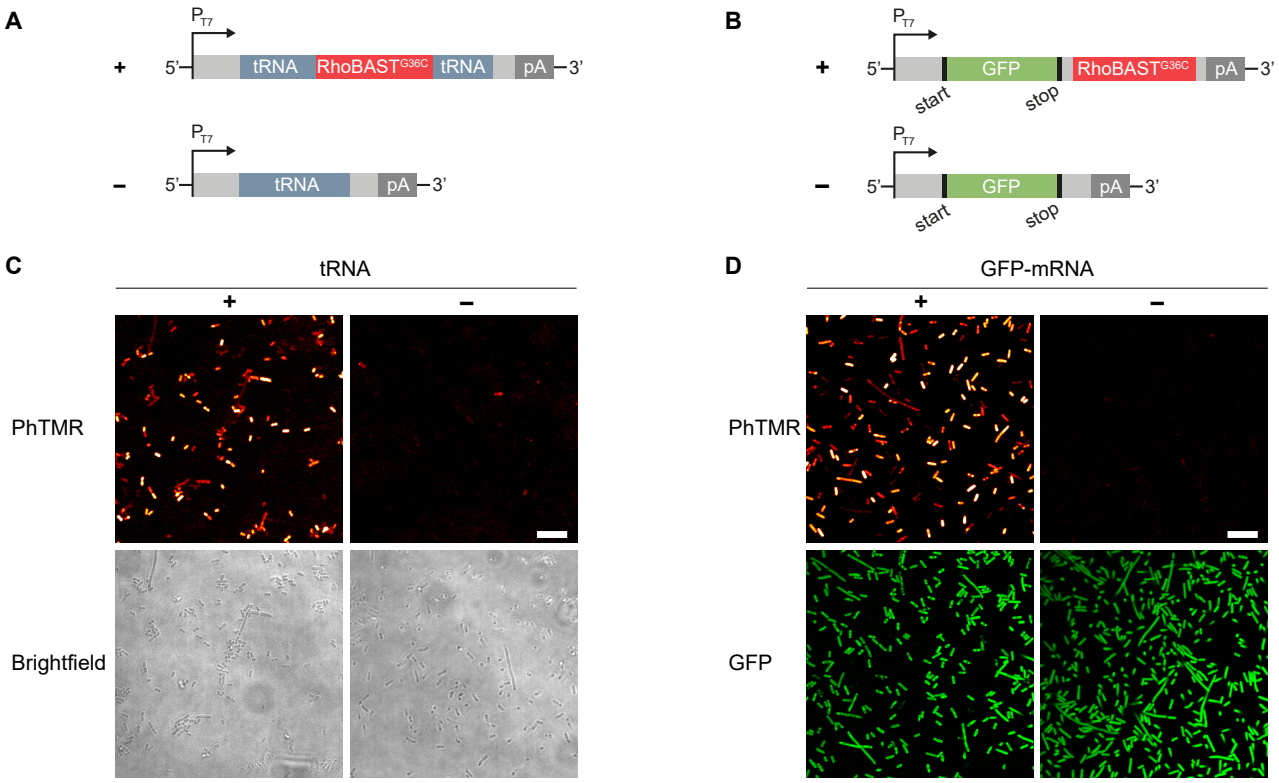
Supplementary Figure S1: *In vitro* properties of RhoBAST^{G36C}:PhTMR in comparison to predecessor RhoBAST.

A) Comparison of fluorescence turn-on values of RhoBAST with TMR-DN,[5] SpyRho,[8] PhTMR and of RhoBAST^{G36C} with PhTMR. **B)** Comparison of dissociation constants K_D of RhoBAST with TMR-DN,[5] SpyRho,[8] PhTMR and of RhoBAST^{G36C} with PhTMR. **C)** Chemical structure of PhTMR. **D)** Predicted secondary folding of RhoBAST^{G36C}.[1] **E)** Dependence of RhoBAST^{G36C}:PhTMR fluorescence intensity on Mg^{2+} concentration. **F)** Normalized excitation (solid lines) and emission (dashed lines) spectrum of PhTMR in the presence and absence of RhoBAST^{G36C}. The maximum excitation and emission wavelength were determined ($\lambda_{Ex,\,(PhTMR)}$ = 568 nm, $\lambda_{Em,\,(PhTMR)}$ = 591 nm, $\lambda_{Ex,\,(RhoBASTG36C:PhTMR)}$ = 574 nm, $\lambda_{Em,\,(RhoBASTG36C\,PhTMR)}$ = 606 nm).



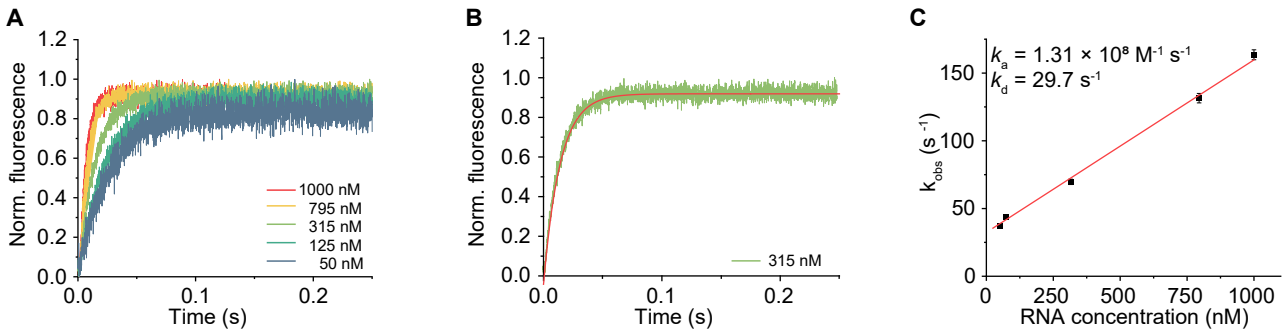
Supplementary Figure S2: *In vitro* characterization of aryl-substituted rhodamines.

A) Equilibrium between quinoid and spirolactone form of asymmetric, aryl-substituted rhodamines. **B)** Obtained D_{50} values (data given in Fig. 2B) versus the *Hammett* parameter (σ)[9] of the para-substituents (R) at the phenyl moiety. **C)** Quantum yields of aryl-substituted rhodamines depending on viscosity measured in different ratios of water/glycerol mixtures. Values are given as mean ± s.d. (*N* = 3 independent measurements). **D)** Fluorescence turn-ons of aryl-substituted rhodamines in the presence and absence of *E. coli* total RNA (500 ng/µL), BSA (500 ng/µL) and RhoBAST^{G36C} (5 µM, 100 ng/µL) in ASB. Values are given as mean ± s.d. (*N* = 3 independent measurements).



Supplementary Figure S3: Application of RhoBAST^{G36C}:PhTMR in confocal imaging of RNAs in living *E. coli*.

A, B) Scheme of used genes for imaging in *E. coli* of A) tRNA and B) GFP-mRNA. **C)** Confocal images of living *E. coli.* tRNA was visualized using 1xRhoBAST^{G36C} (+) with PhTMR (1 µM). As negative control, 0xRhoBAST^{G36C}(–) was used. Scale bar, 10 µm. **D)** Confocal images of living *E. coli*. *GFP*-mRNA was visualized using 16xRhoBAST^{G36C} (+) and PhTMR (2 µM). As negative control, 0xRhoBAST^{G36C} (–) was used. Scale bar, 10 µm.



Supplementary Figure S4: Binding kinetics of RhoBAST^{G36C} with PhTMR measured by stopped-flow spectrometry.

A) Normalized fluorescence increase over time upon mixing different concentrations of RhoBAST^{G36C} with PhTMR (50 nM) in ASBT at 25°C using a stopped-flow spectrometer. Shown data represent averaged technical replicates (N = 5). **B)** Exponential fitting of recorded data shown in (A) gives observed kinetic rate coefficients k_{obs}. **C)** Linear fitting of observed kinetic rate coefficients k_{obs} versus the RNA concentration (RhoBAST^{G36C}) yields the association (k_a) and dissociation (k_d) rate coefficients. Data point represent mean ± s.d. (N = 2 independent measurements).

Supplementary Table S1: *In vitro* properties of aryl-substituted rhodamines and TMR-DN with RhoBAST and RhoBAST mutants.

Aptamer	Dye	λ_{ex} [nm]	λ_{em} [nm]	K_D [nM]
RhoBAST[5]	TMR-DN[5]	564	590	15 ± 1
RhoBAST	SpyRho	551	573	34 ± 2
RhoBAST	Ph-5C-TMR	578	607	1897 ± 107
RhoBAST	**4**	572	602	2330 ± 20
RhoBAST	PhTMR	578	607	360 ± 13
RhoBAST^{G36C}	PhTMR	574	606	115 ± 7
Mutant G36A	PhTMR	579	607	384 ± 22
Mutant G36U	PhTMR	572	604	247 ± 23
Mutant G36C, U38C	PhTMR	576	608	897 ± 110

Supplementary Table S2: *In vitro* properties of aryl-substituted rhodamines in the presence and absence of RhoBAST^{G36C}. Stokes shifts were calculated as ($\Delta_{Stokes} = \lambda_{em} - \lambda_{ex}$). Bathochromic shifts induced by aptamer-binding were calculated as ($\Delta_{batho.\ ex} = \lambda_{ex,\ dye} - \lambda_{ex}$) and ($\Delta_{batho.\ em} = \lambda_{em,\ dye} - \lambda_{em}$). Fluorescence turn-ons are given as mean ± s.d. (N = 3 independent experiments).

		Unbound dye					RhoBAST^{G36C}:dye complex						
Dye	**R**	D_{50}	ε_{dye} [M^{-1} cm^{-1}]	$\lambda_{ex,\ dye}$ [nm]	$\lambda_{em,\ dye}$ [nm]	$\Delta_{Stokes,\ dye}$ [nm]	λ_{ex} [nm]	λ_{em} [nm]	Δ_{Stokes} [nm]	$\Delta_{batho.\ ex}$ [nm]	$\Delta_{batho.\ em}$ [nm]	K_D [nM]	**Turn-on** [n-fold]
4	H	28	65000	554	580	26	564	598	34	10	18	500 ± 30	363 ± 40
5	NMe_2	21	63000	548	568	20	559	577	18	11	9	n.d.	2.5 ± 0.2
6	NH_2	22	58000	547	565	18	569	594	25	22	29	n.d.	3.8 ± 0.1
7	OMe	15	49000	555	575	20	568	601	33	13	26	n.d.	8.3 ± 0.2
8	Cl	29	56000	554	581	27	561	597	36	7	16	570 ± 20	195 ± 37
9	Br	29	45000	553	582	29	561	597	36	8	15	1130 ± 50	141 ± 28
10	CF_3	36	51000	553	583	30	559	589	30	6	6	4100 ± 300	187 ± 62
11	CN	40	43000	554	584	30	570	593	23	16	9	768 ± 60	83 ± 17
12	NO_2	45	49000	541	562	21	577	592	15	36	30	1271 ± 89	24 ± 2
PhTMR	H	28	64000	568	591	23	574	606	32	6	15	115 ± 7	309 ± 45

Supplementary Table S3: Photophysical properties such as molar extinction coefficient ε, quantum yield QY and brightness ($\varepsilon \cdot QY$) of PhTMR and aryl-substituted rhodamines in the presence and absence of RhoBAST^{G36C}.

		Unbound dye			RhoBAST^{G36C}:dye complex		
Dye	**Residue**	**ε_{dye}** [M^{-1} cm^{-1}]	**QY_{dye}**	**$Brightness_{dye}$** [M^{-1} cm^{-1}]	**ε** [M^{-1} cm^{-1}]	**QY**	**Brightness** [M^{-1} cm^{-1}]
PhTMR	H	64000	0.0013 ± 0.0001	83	54000	0.12 ± 0.01	7100
4	H	65000	0.0010 ± 0.0001	65	59000	0.15 ± 0.01	8100
8	Cl	56000	0.0013 ± 0.0001	73	57000	0.14 ± 0.02	8000
9	Br	45000	0.0012 ± 0.0001	54	55000	0.12 ± 0.02	6600
10	CF_3	51000	0.0034 ± 0.0001	170	59000	0.23 ± 0.02	13500

Supplementary Table S4: Kinetic rate coefficients of the association (k_a) and the dissociation (k_d) of aptamer:dye systems. Measurements were conducted using a stopped-flow spectrometer and recording five technical replicates. Values are given as mean ± s.d. (N = 2 independent experiments). The times T were calculated as $T_{fluorescent} = (k_d)^{-1}$, $T_{dark} = (k_a \cdot c)^{-1}$ with c as concentration (100 nM) and $T_{blinking\ interval} = T_{fluorescent} + T_{dark}$.

Aptamer:Dye	**k_a** [M^{-1} s^{-1}]	**k_d** [s^{-1}]	**$T_{fluorescent}$** [ms]	**$T_{dark,\ c=100\ nM}$** [ms]	**$T_{blinking\ interval}$** [ms]
RhoBAST^{G36C}:PhTMR	(1.31 ± 0.05) · 10^8	29.7 ± 1.1	34	76	110
RhoBAST:TMR-DN[5]	(1.59 ± 0.01) · 10^8	2.94 ± 0.10	340	63	403
RhoBAST:SpyRho[8]	(2.1 ± 0.1) · 10^7	1.8 ± 0.1	556	476	1032

Supplementary Table S5: RNA and DNA (italic) sequences. Sequences highlighted in blue represent the 15%-doped DNA library (with a probability of 85% the stated nucleotide and with a probability of 5% each other nucleotide is present). The T7 promoter is underlined.

Name	**Sequence (5' → 3')**
RhoBAST	GGAACCUCCGCGAAAGCGGUGAAGGAGAGGCGCAAGGUUAACCGCCUCAGGUUCC
RhoBAST^{G36C}	GGAACCUCCGCGAAAGCGGUGAAGGAGAGGCGCAACGUUAACCGCCUCAGGUUCC
RhoBAST 36G-36A	GGAACCUCCGCGAAAGCGGUGAAGGAGAGGCGCAAAGUUAACCGCCUCAGGUUCC
RhoBAST 36G-36U	GGAACCUCCGCGAAAGCGGUGAAGGAGAGGCGCAAUGUUAACCGCCUCAGGUUCC
SiRA	GGCCACCGGGUUUGAAAACCUGGCUGCUUCGGCAGUUGUAUCCUUUGGCC
RhoBAST DNA-template	*TAATACGACTCACTATAGGAACCTCCGCGAAAGCGGTGAAGGAGAGGCGCAAGGTTA ACCGCCTCAGGTTCC*
RhoBAST^{G36C} DNA-template	*TAATACGACTCACTATAGGAACCTCCGCGAAAGCGGTGAAGGAGAGGCGCAACGTTAA CCGCCTCAGGTTCC*
SiRA DNA-template	*TAATACGACTCACTATAGGCCACCGGGTTTGAAAACCTGGCTGCTTCGGCAGTTGTAT CCTTTGGCC*
(RhoBAST^{G36C})$_4$ (synonymous repeats)	*ATAGAGGAACCTCCGCGAAAGCGGTGAAGGAGAGGCGCAACGTTAACCGCCTCAGGT TCCTCATAACAAGGCCTCCGCGAAAGCGGTGAAGGAGCGGCACAACGTTAACTGCCG CAGGCCTTGTATAATAGGAAGACCTCCGCGAAAGCGGTGAAGGAGCGGTGCAACGTT AACCACCGCAGGTCTTCCATAAGCAGACCTCCGCGAAAGCGGTGAAGGAGTGGCGCA ACGTTAACCGCCACAGGTCTGCTATA*
tRNA-RhoBAST^{G36C}	*GCGGATTTAGCTCAGTTGGGAGAGCGCCAGGGAACCTCCGCGAAAGCGGTGAAGGA GAGGCGCAACGTTAACCGCCTCAGGTTCCCTGGAGGTCCTGTGTTCGATCCACAGAAT TCGCACCA*
Primer A (forward)	*TTCTAATACGACTCACTATAGGAACACTATCCGACTGGCACC*
Primer B (revers)	*CCTTGGTCATTAGGATCCG*
Initial DNA library (15%-doped)	*GGAACACTATCCGACTGGCACCGGAACCTCCGCGAAAGCGGTGAAGGAGAGGCGCA AGGTTAACCGCCTCAGGTTCCCGGATCCTAATGACCAAGG*

Supplementary Table S6: Truncated RNA sequences obtained after Sanger sequencing from round six of SELEX.

Name	Sequence
A01-1	GGAUCCUACGUGAAAGCGGUUGAAGGAGAGGGGCAAGGUUAACCUCCUCAGGAUCC
A01-2	GGAGCGUCCUAUAGAGAGGUGAAGGAGAGACGCAAUGUUAACCGCCUCACGCUCC
A02-1	GGACCCUCCGUAAAAUCGGUGAAGGAGACGCGCAAUGCUAACCGCAUCAGGGUCC
A02-2	GGAAACUCCGCGAAAGCGGUGAAGGAGAGGAGCGAGGUUUAACCUCCUCAGUCUCC
A02-3	GGAACGUCCGCGAGAGCGGUGAAGGAGAUGCCCAAGGUUAACGGUUUCACGUUCC
A02-4	UCCGCGUAACCGGUGCAGGGAGGGGCGCAUUGUUAACCGCCUCACGGUCC
A04-1	GGAAACUCCACGAAAGUGGUGACGGAGAGAUGCAAGGGUAACCAUCUCAGGUUCC
A04-2	GGAAACUACGUGAAAACGUUGAAGGAGAGGAGCAAGGUUAACCUCCUCAGCUUCC
A05-1	GGAAGGUUCUCAAAAGCUUUGUAUGAUAGUAGCAGUCUUAACCGCCUCAGGUCC
A06-1	GGAACGUCGGCGAAAGCCGUGAAGGAGAGGAGCAAGGUUAACCCCCUCACGUUCC
A10-1	GGAACGUCCUCGAAAGAGGUGAAGGAGAUGCCCAAGGAUAACCGCCUCACGUUCC
A10-2	GGAAGCUACGCGAAAGCGGUGAAGGAAAGGUACAAGGGUAACUGCCUUAGCUUCC
A11-1	GGACCUGCGCGUAAGCGUUGAAUGAGAGUCGGGUGGUUAAUCCCCUCUGGUUCC
B02-1	GGAUCCUGUGUGAAAUCUGUCACGGGGAGGUGCAGUGUUCACCGCCCCAAGUUCC
B02-2	GGAACUUCCGCGGAUGCGGUGAUGGAGAGGCGCACUGUUAACCGUCUCAUGUUCC
B02-3	GGAUCCUACGUAAUAACGUUGAAGGAGAGGAGCAAAGUUAACCUCCACAGGGUCC
B02-4	GGAAACACCGCAAAAUCGCUGAAGGAGAGGCGCAAGGUUAACUGCCUCGGUGUCC
B02-5	GGAAACACCGCAAAAUCGCUGAAGGAGAGGCGCAAGGUUAACUGCCUCGGUGUCC
B06-1	GGAACGUCCGAGAAAGUGCUGAAGGACAGUCGCUAGGAGUUUCGCCUCAGGUUCC
B06-2	GGAAGUUACUUGAAACUGGUGCCGGAGUAGUGUAAGUUUAACCCCCUCAGCCUCC
B06-3	GGAAGCUGCCCGAAAGCGCUGAAGGAGAUGCGCAAGGUUAACCGCAUCAGCUUCC
B07-1	GGAAGCUACGCCAAAGCGGUGAAAUGAAGCGCAAGCUUCACCUCCUCAGGUUCC
B09-1	GGAACAUCUGCGAUAACAGUGAAGGAGAGGAGCAAGGCUAACCUCCUCAUGUUCC
B09-2	GGAACGUCCGUGAAAGCGGCGACGGAAAGGCACAAGGUUAACUGCCUCACGUUCC
B10-1	GGAACAUCCGCGUAAGCGGUGAUGGAGAGGUGCAAGGUUAACCUCCUCAUGUUCC
B10-2	GGAACGUCUGCGAGAGCGGUGACGGAGAGAUGCAAGGAUAACCAUCUCACGUUCC
C01-1	GGAACAUGCGCGAAAGCGCUGAAGGAGCGGCACAACGUUAACUGCCCCAUGUUCC
C02-1	GGAACCUCCGUGAAAACGGUGAAGGAGAAGCGCAACGUUAACUGCUUCAGGUUCC
C04-1	GGAACCACCUCGAUAGCGGUGAUGGAGAGGAGCAAAGUUAACCUCCUCUGGUUCC
C06-1	GGAACGACCGCGCAAACGGUGAAGGACAGGCACAAGGGUAACUGCCUGACGUUCC
C07-1	GGAAUGUCCCCGAAAGGGGUGAAGGAGAGGCGCAAUGUUAACCGCUUCAUGUUCC
C09-1	GGAAUCUCCUCAAAUGCGGUGCAAGAGAGACGCGAUGUGAACCGCCUCAGGCUCC
D01-1	GGAACAUGCGCGAAAGCGCUGAAGGAGCGGCACAACGUUAACUGCCCCAUGUUCC
D03-1	GGAACCUGCGUGAAAGCGCUGAAGGAGAUGAGCAAUGUUAACCUCAUCUGGCUCC
D03-2	GGAACCUGCGCGCAAGCGCUGAAGGAGAGAUGCAAGGUUAACCACCUCAGGUUCC
D05-1	GGAGUCUCCGCGAAAGCGGUGAUGGAGUGGCGCAACGUUAACCGCCUCAGGUUCC
D06-1	GGAACCUCCGCGUAAGCGGUGAAGAAGAGGUAUAAGGUUAACUACCUCAGGCUCC
D06-2	GGAAUCUUAGCGUAAGCGUGAAGGAGAGCCACWAGGUUAACUGGCUCACGUUCCC
D07-1	GGAAACUCCUCGGAAGAGGUGACGGAGAGGCGCAAUGUUAACCGCCUCAGAUUCC
D08-1	GGAACAUCCGCGAAAGCGGUGACGGAGAGGCGCAAGGCUAACCGCCUCAUGUUCC
D08-2	GGACCCUCCGCGAGGCCGGUGAAGGAGGUAAGUUUAACCGCCCCAAGUUCC
D09-1	GGAACCUNCNCCAAAGNNGUGANGGAGANGCACAAGGNUAACUGCAUCAGGUUCC
D10-1	GGAACUUCMGCGAAAGCKGUGAAGGAGAAGCGCAAUGUUAACCGCUUCRGGUUCC
D10-2	GGAAGCUCCGUGUAAUCGGUGAUGGAGAGGGGCAAGGGUAACCUCCUCAGCUUCC
D12-1	GGAACCUCCUCUAAAUAGGUGAAGGAUAGGCGCAAGGGUAACCGCCUAAGGCUCC
D12-2	GGAAACUCCACGAAAGUGGUGAAGGAGAGUCACAACGUUAACUGCCUCAGCUUC
D12-3	GGAAGCUCCACGAUAGUGGUGAGGGAGAGGCACAACGUUAACUGCCUUAGCUUCC
E05-1	GGAACUUUCGCGAAAGCUAUGAUGGAGUGGCACACGGUUAACUGCUACAAGUUCC
E05-2	GGAACGUUCGCGAAAGCGAUGAAGGAGAGGAGCACGGUUAACCUCCUCACGUUCC
E05-3	GGAACCGGCGCGAAAGCGUUGAUGGAUAGGAGCAAGGUUAACCUCCUACGGCUCC
E05-4	GGACCUUCUGCUGAAGCGGUGAUGGAGAGGAGCAAGGUUAACCUCCUCGGGGUCC
E05-5	GGAAGCUCCGAGAUAGCGGCGAUGGGGAGUCGCAAGGAUAACCGACUCAGUUUCC
E12-1	GGAGCCUCAGCGAAAGCUGUGAUGGAGAGGCACAGGGUUAACUACCUCAGGCUCC
E12-2	GGAACACCCGCGAUAGCGGUGAAGGAGCGGCGCAAGGUUAACUGCCGCAUGUUCC
E12-3	GGAACCUGCGAGAAAGCGCUGAAGGAGAUGCGCAAUGAUAACCGCGUCAGGCUCC
F01-1	GGAACCUCUUCGAAAGCAGUGAUGGAGAGUCGCAAGGUUAACUGACUCAGGUUCC
F03-1	GGACCGUCCAGAUAACGGUGAGGGANANGCGCAAUGNUAACCGCAUCACGGUCC
F05-1	GGANCCUCUGCUAAAGAGUGAAGGAGAGGCACNAUGNUAACUGNCUCAGGUUCCC
F06-1	GGAAAUUCCUCGAAAGAGGUGAAGGAGACGCGCAACGUUAACCUCGUCAGUUUCC
F07-1	GGAAAUUCCUCGAAAGAGGUGAAGGAGACGCGCAACGUUAACCUCGUCAGUUUCC
F09-1	NGAACGUGCCAGAAAGGGUUGAAGGAGCGACGCAAGGUUAACCGUUGCAGGUUCC
F11-1	GGAAGGUCCGCGAAAGGGNUGAAGGAAAGGCGGAUGGUUUAAUGCCUCANGUUCC
G03-1	GGAACCNCCGCGAAAGUGGKGAANGAGAGUCGCAAGGUNAACCCCCUCANAUUCC
G04-1	GGAAACUUCCGCGAUGGCGGUGAAGGAGAAGCUCAAGGAUAACAGCUUCAGGUUCC
G08-1	GGAACCUCCGUGAAAACGAUGAACGACAGUCGCAAGGUUACCGGCUGAGGCUCC
G09-1	GGAACWUGCGGAAAAGAAGUGAAGGAGAGUGGCAAGGUUAACCCACUCAUGUUCC
G10-1	GGAGCCUGCGCCAAAACGAAGAAGGAGAGGCACAAUGUUAACUGCCUCAGGUUCC
H03-1	GGAACCUCCGCGCAAGCGGUGAAGGAGAGGGGCAACGUUAACCUCCUCAGGCUCC
H06-1	GGAAUCUCCGCAAAAGCGUUGAAGGAGCCGUUCUAGGUGAACCUCCCCAAGUUCC
H06-2	GGAAACUCCGCGAAUGCGGUGAAGGAGAGCCGCAAUGGUAACCGUCUCAGUUUCC
H08-1	GGAACGUGCGUGAAAACGCUGAAGUAGGGGCGAAAAGUUAACCGUCUCACGUUCC
H08-2	GGAAGCUCCACGAUAGUGGUGAGGGAGAGGCACAACGUUAACUGCCUUAGCUUCC
H12-1	GGAACGUGCGUGAAAACGCUGAAGUAGGGGCGAAAAGUUAACCGUCUCACGUUCC
A02-1´	GGAACCACUGCGAUUGCAGUGAAGGAGAAUCACAAGGUAACUGAUUCUGGCUCC

Name	Sequence
A04-1´	GGACCCUGAAGCGGUUAGCCGUGCGCUUCUCCUUCACCGCUUUCGCGGAG
A09-1´	GGAAGCUACGCAUUAGCGUUGAUGGAGAUGCGCAAGGGUAACCGCGUCAAGUUCC
A12-1´	GGAACCUGCGCGAAAGCGCUGAAGGAAAGUCACAAGGUUAACUGACUUAGCGUCC
B03-1´	GGAACUUCCGUGAAAACGGUGAAGGAGAAGCGCAACGUUAACCGCUUCAGGUUCC
B08-1´	AGGAGGCUCUGCGAAAGCAGUGAAGGAGUGGCGCAAUGUUAACCGCCCCAGCGUCC
B12-1´	GGAACGUCCCCGAAAGGGGUGAAGGAGUGACGCAACGUUAACUGUCACACGUUCC
C06-1´	GGAUUCUCGGCGAAAGCCGUGAAGGAGAGACGCAAGGUUAACCGUCUCAGGAUCC
C10-1´	GGGACGUCGGCGAGAGCUGUGAUGGAAAGGCGCAUCGUUAACCGCCUUACGUUCC
C10-2´	GGAACCUACGAGAAAGCGUUGAAGGAGGGGCCCAAUGUUAACGGCCUCAGGUUCC
C12-1´	GGGACCUCCUCGAAAAAGGUGAAGGAGACACGCAAGGUUAACCGUUUCAGGUUCC
D02-1´	GGAACAUCCGUGAAAACGGUGAAGGAGAGCCGCAAGGUUAACCGUCUCAUGUUCC
D04-1´	GGAAACUCCGCGCACGCGGUGAAGGAGAGGCGCAAUGGUAACCGUCUCAGAUUCC
D11-1´	GGAACNUCCUCGAANGGGGUGAAGGAGAGGCGCAAKGUUAACCACCUCANGUUCC
D12-1´	GGAAUCGCCUAGAAAGUGGUGCAGGAGAGGACCAAGAUUAUCCUCCUCAUGUUCC
E02-1´	GGAACCUUCGCGCAAGCGAUGAAGGAGAGACGCAAGGCUAACCGUUUCAGGUUCC
E08-1´	GGAGCCUCAGCGAAAGCUGUGAAGGAGAGGUGCAAGGGUAACCACCUCAGACUCC
E09-1´	GGAACCUCCUCGAAAGAGGUGAAGGAGAGACACAAGGUUAACUGUCUCAGGUUCC
E10-1´	GGAACGUCCUCAAAAGUGGUGAAGGAGAAGCGCAAGGGUAACUGCUUCACGUUCC
E11-1´	GGACACAGCGCCAAAGGGGUGAAGGAGAUGAGCAAGGCUAACCUCAUCAGGUUCC
E11-2´	GGAAGCCCCGCGAAUGCGGCGAAGGGGAGGCACAACGUUAACUGUCUCAGCUUCC
E11-3´	AGAACGUCCGCGAAUGCGUUGAAGGCCAGGCGCAUGUUGAUCCGCCUAGGUUCC
G05-1´	GGAACUGCCGAGAAAUCGGUGAGGGAGUGGCGCAACGUUAACCGUCUCCAGUUCC
G05-2´	CGAACCUCCGCGAAAGCGGUGAAGGACAGUUGCAAGGUUAACCAACUGAGGCUCC
G05-3´	GGAAUCUACGUGAAACCGUUGAAGGAGUGGCGCAAGGCUAACCGCCGCAGGUUCC
G06-1´	GGAGCGUCCCCGAAAUCGGUGAAGGAGAGGCACAACGUUAACUGCCUCACGCUCC
H02-1´	GGAGCGUGAGGCAGUUAACGUUGUGCCUCUCCUUCACCGAUUUCGGGGACGCUCC
H02-2´	GGAACGUUCGCGAAAGCGAUGAAGGAGAGGUGCAAGGCUAACCACCUCACGUUCC
H02-3´	GGAAACUCCGCGCAAGCGGUGAUGGAGAGGAACAAGGUUAACUUCCUCAGGCUCC
H10-1´	GGAACCUCCGCGAACGCGGUGAAGGAGAUGCUCAAUGUUAACAGCGUCAGGUUCC
H11-1´	GGAACCUCCACGAAAGGGGUGAAGGAGAGUCACAAUGUUAACUGACUCAGGUUCC

Supplementary Table 7: Truncated and aligned sequences of the mutants obtained in SELEX and RhoBAST. The stem and loop regions of RhoBAST are marked. Nucleotides not according to the RhoBAST-sequence are given in bold. The percentage of occurrence per nucleotide (nt) is calculated and the rate of mutation is quatified.

Name	Aligned Sequences																																																										
		Stem 1							Stem 2					Loop 1				Stem 2				Loop 2								Stem 3						Loop 3											Stem 3						Stem 1							
#nt		1	2	3	4	5	6	7	8	9		10	11	12	13	14	15	16	17	18	19	20	21		22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39		40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	
RhoBAST	-	G	G	A	A	C	C	T	C	C	-	G	C	G	A	A	A	G	C	G	G	T	G	-	A	A	G	G	A	G	A	G	G	C	G	C	A	A	G	G	T	T	-	A	A	C	C	G	C	C	T	C	A	G	G	T	T	C	C	-
A01-1	-	G	G	A	**T**	C	C	T	**A**	C	-	G	**T**	G	A	A	A	G	C	G	G	T	**T**	**G**	A	A	G	G	A	G	A	G	G	**G**	G	C	A	A	G	G	T	T	-	A	A	C	C	**T**	C	C	T	C	A	G	G	**A**	T	C	C	-
A01-2	-	G	G	A	**G**	C	**G**	T	C	C	-	**T**	**A**	**T**	A	**G**	A	G	**A**	G	G	T	G	-	A	A	G	G	A	G	A	G	**A**	C	G	C	A	A	**T**	G	T	T	-	A	A	C	C	G	C	C	T	C	A	**C**	G	**C**	T	C	C	-
A02-1	-	G	G	A	**C**	C	C	T	C	C	-	G	**T**	**A**	A	A	A	**T**	C	G	G	T	G	-	A	A	G	G	A	G	A	**C**	G	C	G	C	A	A	**T**	G	**C**	T	-	A	A	C	C	G	C	**A**	T	C	A	G	G	**G**	T	C	C	-
A02-2	-	G	G	A	A	**A**	C	T	C	C	-	G	C	G	A	A	A	G	C	G	G	T	G	-	A	A	G	G	A	G	A	G	G	**A**	G	C	**G**	A	G	G	T	T	**T**	A	A	C	C	**T**	C	C	T	C	A	G	**T**	**C**	T	C	C	-
A02-3	-	G	G	A	A	C	**G**	T	C	C	-	G	C	G	A	**G**	A	G	C	G	G	T	G	-	A	A	G	G	A	G	A	**T**	G	C	**C**	C	A	A	G	G	T	T	-	A	A	C	**G**	G	**T**	**T**	T	C	A	**C**	G	T	T	C	C	-
A02-4	-	**-**	**-**	**-**	**-**	**-**	**-**	T	C	C	-	G	C	G	**T**	A	A	**C**	C	G	G	T	G	**C**	A	**G**	G	G	A	G	**G**	G	G	C	G	C	A	**T**	**T**	G	T	T	-	A	A	C	C	G	C	C	T	C	A	**C**	G	**G**	T	C	C	-
A04-1	-	G	G	A	A	**A**	C	T	C	C	-	**A**	C	G	A	A	A	G	**T**	G	G	T	G	-	A	**C**	G	G	A	G	A	G	**A**	**T**	G	C	A	A	G	G	**G**	T	-	A	A	C	C	**A**	**T**	C	T	C	A	G	G	T	T	C	C	-
A04-2	-	G	G	A	A	**A**	C	T	**A**	C	-	G	**T**	G	A	A	A	**A**	C	G	**T**	T	G	-	A	A	G	G	A	G	A	G	G	**A**	G	C	A	A	G	G	T	T	-	A	A	C	C	**T**	C	C	T	C	A	G	**C**	T	T	C	C	-
A05-1	-	G	G	A	A	**G**	**G**	T	**T**	C	-	**T**	C	**A**	A	A	A	G	C	**T**	**T**	T	G	-	**T**	A	**T**	G	A	**T**	A	G	**T**	**A**	G	C	A	**G**	**T**	**C**	T	T	-	A	A	C	C	G	C	C	T	C	A	G	G	T	**C**	C	**-**	-
A06-1	-	G	G	A	A	C	**G**	T	C	**G**	-	G	C	G	A	A	A	G	C	**C**	G	T	G	-	A	A	G	G	A	G	A	G	G	**A**	G	C	A	A	G	G	T	T	-	A	A	C	C	**C**	C	C	T	C	A	**C**	G	T	T	C	C	-
A10-1	-	G	G	A	A	C	**G**	T	C	C	-	**T**	C	G	A	A	A	G	**A**	G	G	T	G	-	A	A	G	G	A	G	A	**T**	G	C	**C**	C	A	A	G	G	**A**	T	-	A	A	C	C	G	C	C	T	C	A	**C**	G	T	T	C	C	-
A10-2	-	G	G	A	A	**G**	C	T	**A**	C	-	G	C	G	A	A	A	G	C	G	G	T	G	-	A	A	G	G	A	**A**	A	G	G	**T**	**A**	C	A	A	G	G	**G**	T	-	A	A	C	**T**	G	C	C	T	**T**	A	G	**C**	T	T	C	C	-
A11-1	-	**-**	G	**G**	A	C	C	T	**G**	C	-	G	C	G	**T**	A	A	G	C	G	**T**	T	G	-	A	A	**T**	G	A	G	A	G	**T**	C	G	**G**	**G**	**T**	G	G	T	T	-	A	A	**T**	C	**C**	C	C	T	C	**T**	G	G	T	T	C	C	-
B02-1	-	G	G	A	**T**	C	C	T	**G**	**T**	-	G	**T**	G	A	A	A	**T**	C	**T**	G	T	**C**	-	A	**C**	G	G	**G**	G	A	G	G	**T**	G	C	A	**G**	**T**	G	T	T	-	**C**	A	C	C	G	C	C	**C**	C	A	**A**	G	T	T	C	C	-
B02-2	-	G	G	A	A	C	**T**	T	C	C	-	G	C	G	**G**	A	**T**	G	C	G	G	T	G	-	A	**T**	G	G	A	G	A	G	G	C	G	C	A	**C**	**T**	G	T	T	-	A	A	C	C	G	**T**	C	T	C	A	**T**	G	T	T	C	C	-
B02-3	-	G	G	A	**T**	C	C	T	**A**	C	-	G	**T**	**A**	A	**T**	A	**A**	C	G	**T**	T	G	-	A	A	G	G	A	G	A	G	G	**A**	G	C	A	A	**A**	G	T	T	-	A	A	C	C	**T**	C	C	**A**	C	A	G	G	**G**	T	C	C	-
B02-4	-	G	G	A	A	**A**	C	**A**	C	C	-	G	C	**A**	A	A	A	**T**	C	G	**C**	T	G	-	A	A	G	G	A	G	A	G	G	C	G	C	A	A	G	G	T	T	-	A	A	C	**T**	G	C	C	T	C	**G**	G	**T**	**G**	T	C	C	-
B02-5	-	G	G	A	A	**A**	C	**A**	C	C	-	G	C	**A**	A	A	A	**T**	C	G	**C**	T	G	-	A	A	G	G	A	G	A	G	G	C	G	C	A	A	G	G	T	T	-	A	A	C	**T**	G	C	C	T	C	**G**	G	**T**	**G**	T	C	C	-
B06-1	-	G	G	A	A	C	**G**	T	C	C	-	G	**A**	G	A	A	A	G	**T**	G	**C**	T	G	-	A	A	G	G	A	**C**	A	G	**T**	C	G	C	**T**	A	G	G	**A**	**G**	-	**T**	**T**	**T**	C	G	C	C	T	C	A	G	G	T	T	C	C	-
B06-2	-	G	G	A	A	**G**	**T**	T	**A**	C	-	**T**	**T**	G	A	A	A	**C**	**T**	G	G	T	G	-	**C**	**C**	G	G	A	G	**T**	**A**	G	**T**	G	**T**	A	A	G	**T**	T	T	-	A	A	C	C	**C**	C	C	T	C	A	G	**C**	**C**	T	C	C	-
B06-3	-	G	G	A	A	**G**	C	T	**G**	C	-	**C**	C	G	A	A	A	G	C	G	**C**	T	G	-	A	A	G	G	A	G	A	**T**	G	C	G	C	A	A	G	G	T	T	-	A	A	C	C	G	C	**A**	T	C	A	G	**C**	T	T	C	C	-
B07-1	-	G	G	A	A	**G**	C	T	**A**	C	-	G	C	**C**	A	A	A	G	C	G	G	T	G	-	A	A	**A**	**T**	**G**	**A**	A	**-**	G	C	G	C	A	A	G	**C**	T	T	-	**C**	A	C	C	**T**	C	C	T	C	A	G	G	T	T	C	C	-
B09-1	-	G	G	A	A	C	**A**	T	C	**T**	-	G	C	G	A	**T**	A	**A**	C	**A**	G	T	G	-	A	A	G	G	A	G	A	G	G	**A**	G	C	A	A	G	G	**C**	T	-	A	A	C	C	**T**	C	C	T	C	A	**T**	G	T	T	C	C	-
B09-2	-	G	G	A	A	C	**G**	T	C	C	-	G	**T**	G	A	A	A	G	C	G	G	**C**	G	-	A	**C**	G	G	A	**A**	A	G	G	C	**A**	C	A	A	G	G	T	T	-	A	A	C	**T**	G	C	C	T	C	A	**C**	G	T	T	C	C	-
B10-1	-	G	G	A	A	C	**A**	T	C	C	-	G	C	G	**T**	A	A	G	C	G	G	T	G	-	A	**T**	G	G	A	G	A	G	G	**T**	G	C	A	A	G	G	T	T	-	A	A	C	C	**T**	C	C	T	C	A	**T**	G	T	T	C	C	-
B10-2	-	G	G	A	A	C	**G**	T	C	**T**	-	G	C	G	A	**G**	A	G	C	G	G	T	G	-	A	**C**	G	G	A	G	A	G	**A**	**T**	G	C	A	A	G	G	**A**	T	-	A	A	C	C	**A**	**T**	C	T	C	A	**C**	G	T	T	C	C	-
C01-1	-	G	G	A	A	C	**A**	T	**G**	C	-	G	C	G	A	A	A	G	C	G	**C**	T	G	-	A	A	G	G	A	G	**C**	G	G	C	**A**	C	A	A	**C**	G	T	T	-	A	A	C	**T**	G	C	C	**C**	C	A	**T**	G	T	T	C	C	-
C02-1	-	G	G	A	A	C	C	T	C	C	-	G	**T**	G	A	A	A	**A**	C	G	G	T	G	-	A	A	G	G	A	G	A	**A**	G	C	G	C	A	A	**C**	G	T	T	-	A	A	C	**T**	G	C	**T**	T	C	A	G	G	T	T	C	C	-
C04-1	-	G	G	A	A	C	C	**A**	C	C	-	**T**	C	G	A	**T**	A	G	C	G	G	T	G	-	A	**T**	G	G	A	G	A	G	G	**A**	G	C	A	A	**A**	G	T	T	-	A	A	C	C	**T**	C	C	T	C	**T**	G	G	T	T	C	C	-
C06-1	-	G	G	A	A	C	**G**	**A**	C	C	-	G	C	G	**C**	A	A	**A**	C	G	G	T	G	-	A	A	G	G	A	**C**	A	G	G	C	**A**	C	A	A	G	G	**G**	T	-	A	A	C	**T**	G	C	C	T	**G**	A	**C**	G	T	T	C	C	-
C07-1	-	G	G	A	A	**T**	**G**	T	C	C	-	**C**	C	G	A	A	A	G	**G**	G	G	T	G	-	A	A	G	G	A	G	A	G	G	C	G	C	A	A	**T**	G	T	T	-	A	A	C	C	G	C	**T**	T	C	A	**T**	G	T	T	C	C	-
C09-1	-	G	G	A	A	**T**	C	T	C	C	-	**T**	C	**A**	A	A	**T**	G	C	G	G	T	G	-	**C**	A	**A**	G	A	G	A	G	**A**	C	G	C	**G**	A	**T**	G	T	**G**	-	A	A	C	C	G	C	C	T	C	A	G	G	**C**	T	C	C	-
D01-1	-	G	G	A	A	C	**A**	T	**G**	C	-	G	C	G	A	A	A	G	C	G	**C**	T	G		A	A	G	G	A	G	**C**	G	G	C	**A**	C	A	A	**C**	G	T	T	-	A	A	C	**T**	G	C	C	**C**	C	A	**T**	G	T	T	C	C	-
D03-1	-	G	G	A	A	C	C	T	**G**	C	-	G	**T**	G	A	A	A	G	C	G	**C**	T	G	-	A	A	G	G	A	G	A	**T**	G	**A**	G	C	A	A	**T**	G	T	T	-	A	A	C	C	**T**	C	**A**	T	C	**T**	G	G	**C**	T	C	C	-
D03-2	-	G	G	A	A	C	C	T	**G**	C	-	G	C	G	**C**	A	A	G	C	G	**C**	T	G	-	A	A	G	G	A	G	A	G	**A**	**T**	G	C	A	A	G	G	T	T	-	A	A	C	C	**A**	C	C	T	C	A	G	G	T	T	C	C	-
D05-1	-	G	G	A	**G**	**T**	C	T	C	C	-	G	C	G	A	A	A	G	C	G	G	T	G	-	A	**T**	G	G	A	G	**T**	G	G	C	G	C	A	A	**C**	G	T	T	-	A	A	C	C	G	C	C	T	C	A	G	G	T	T	C	C	-
D06-1	-	G	G	A	A	C	C	T	C	C	-	G	C	G	**T**	A	A	G	C	G	G	T	G	-	A	A	G	**A**	A	G	A	G	G	**T**	**A**	**T**	A	A	G	G	T	T	-	A	A	C	**T**	**A**	C	C	T	C	A	G	G	**C**	T	C	C	-
D06-2	-	G	G	A	A	**T**	C	T	**T**	**A**	-	G	C	G	**T**	A	A	G	C	**-**	G	T	G	-	A	A	G	G	A	G	A	G	**C**	C	**A**	C	**W**	A	G	G	T	T	-	A	A	C	**T**	G	**G**	C	T	C	A	**C**	G	T	T	C	C	**C**
D07-1	-	G	G	A	A	**A**	C	T	C	C	-	**T**	C	G	**G**	A	A	G	**A**	G	G	T	G	-	A	**C**	G	G	A	G	A	G	G	C	G	C	A	A	**T**	G	T	T	-	A	A	C	C	G	C	C	T	C	A	G	**A**	T	T	C	C	-
D08-1	-	G	G	A	A	C	**A**	T	C	C	-	G	C	G	A	A	A	G	C	G	G	T	G	-	A	**C**	G	G	A	G	A	G	G	C	G	C	A	A	G	G	**C**	T	-	A	A	C	C	G	C	C	T	C	A	**T**	G	T	T	C	C	-
D08-2	-	G	G	A	**C**	C	C	T	C	C	-	G	C	G	A	**G**	**G**	**C**	C	G	G	T	G		A	A	G	G	A	G	**G**	**T**	**A**	**-**	**-**	**-**	**-**	A	G	**T**	T	T	-	A	A	C	C	G	C	C	**C**	C	A	**A**	G	T	T	C	C	-
D09-1	-	G	G	A	A	C	C	T	**N**	C	-	**N**	C	**C**	A	A	A	G	**N**	**N**	G	T	G	-	A	**N**	G	G	A	G	A	**N**	G	C	**A**	C	A	A	G	G	**N**	T	-	A	A	C	**T**	G	C	**A**	T	C	A	G	G	T	T	C	C	-

Name	Aligned Sequences																																																											
		Stem 1							Stem 2					Loop 1				Stem 2				Loop 2								Stem 3						Loop 3											Stem 3						Stem 1							
#nt		1	2	3	4	5	6	7	8	9		10	11	12	13	14	15	16	17	18	19	20	21		22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39		40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	
RhoBAST	-	G	G	A	A	C	C	T	C	C	-	G	C	G	A	A	A	G	C	G	G	T	G	-	A	A	G	G	A	G	A	G	G	C	G	C	A	A	G	G	T	T	-	A	A	C	C	G	C	C	T	C	A	G	G	T	T	C	C	-
D10-1	-	G	G	A	A	C	**T**	T	C	**M**	-	G	C	G	A	A	A	G	C	**K**	G	T	G	-	A	A	G	G	A	G	A	**A**	G	C	G	C	A	A	**T**	G	T	T	-	A	A	C	C	G	C	**T**	T	C	**R**	G	G	T	T	C	C	-
D10-2	-	G	G	A	A	**G**	C	T	C	C	-	G	**T**	G	**T**	A	A	**T**	C	G	G	T	G	-	A	**T**	G	G	A	G	A	G	G	**G**	G	C	A	A	G	G	**G**	T	-	A	A	C	C	**T**	C	C	T	C	A	G	**C**	T	T	C	C	-
D12-1	-	G	G	A	A	C	C	T	C	C	-	**T**	C	**T**	A	A	A	**T**	**A**	G	G	T	G	-	A	A	G	G	A	**T**	A	G	G	C	G	C	A	A	G	G	**G**	T	-	A	A	C	C	G	C	C	T	**A**	A	G	G	**C**	T	C	C	-
D12-2	-	G	G	A	A	**A**	C	T	C	C	-	**A**	C	G	A	A	A	G	**T**	G	G	T	G	-	A	A	G	G	A	G	A	G	**T**	C	**A**	C	A	A	**C**	G	T	T	-	A	A	C	**T**	G	C	C	T	C	A	G	**C**	T	T	C	**-**	-
D12-3	-	G	G	A	A	**G**	C	T	C	C	-	**A**	C	G	A	**T**	A	G	**T**	G	G	T	G	-	A	**G**	G	G	A	G	A	G	G	C	**A**	C	A	A	**C**	G	T	T	-	A	A	C	**T**	G	C	C	T	**T**	A	G	**C**	T	T	C	C	-
E05-1	-	G	G	A	A	C	**T**	T	**T**	C	-	G	C	G	A	A	A	G	C	**T**	**A**	T	G		A	**T**	G	G	A	G	**T**	G	G	C	**A**	C	A	**C**	G	G	T	T	-	A	A	C	**T**	G	C	**T**	**A**	C	A	**A**	G	T	T	C	C	-
E05-2	-	G	G	A	A	C	**G**	T	**T**	C	-	G	C	G	A	A	A	G	C	G	**A**	T	G	-	A	A	G	G	A	G	A	G	G	**A**	G	C	A	**C**	G	G	T	T	-	A	A	C	C	**T**	C	C	T	C	A	**C**	G	T	T	C	C	-
E05-3	-	G	G	A	A	C	C	**G**	**G**	C	-	G	C	G	A	A	A	G	C	G	**T**	T	G	-	A	**T**	G	G	A	**T**	A	G	G	**A**	G	C	A	A	G	G	T	T	-	A	A	C	C	**T**	C	C	T	**A**	**C**	G	G	**C**	T	C	C	-
E05-4	-	**-**	G	**G**	A	C	C	T	**T**	C	**T**	G	C	**T**	**G**	A	A	G	C	G	G	T	G	-	A	**T**	G	G	A	G	A	G	G	**A**	G	C	A	A	G	G	T	T	-	A	A	C	C	**T**	C	C	T	C	**G**	G	G	**G**	T	C	C	-
E05-5	-	G	G	A	A	**G**	C	T	C	C	-	G	**A**	G	A	**T**	A	G	C	G	G	**C**	G	-	A	**T**	G	G	**G**	G	A	G	**T**	C	G	C	A	A	G	G	**A**	T	-	A	A	C	C	G	**A**	C	T	C	A	G	**T**	T	T	C	C	-
E12-1	-	G	G	A	**G**	C	C	T	C	**A**	-	G	C	G	A	A	A	G	C	**T**	G	T	G	-	A	**T**	G	G	A	G	A	G	G	C	**A**	C	A	**G**	G	G	T	T	-	A	A	C	**T**	**A**	C	C	T	C	A	G	G	**C**	T	C	C	-
E12-2	-	G	G	A	A	C	**A**	**C**	C	C	-	G	C	G	A	**T**	A	G	C	G	G	T	G	-	A	A	G	G	A	G	**C**	G	G	C	G	C	A	A	G	G	T	T	-	A	A	C	**T**	G	C	C	**G**	C	A	**T**	G	T	T	C	C	-
E12-3	-	G	G	A	A	C	C	T	**G**	C	-	G	**A**	G	A	A	A	G	C	G	**C**	T	G	-	A	A	G	G	A	G	A	**T**	G	C	G	C	A	A	**T**	G	**A**	T	-	A	A	C	C	G	C	**G**	T	C	A	G	G	**C**	T	C	C	-
F01-1	-	G	G	A	A	C	C	T	C	**T**	-	**T**	C	G	A	A	A	G	C	**A**	G	T	G	-	A	**T**	G	G	A	G	A	G	**T**	C	G	C	A	A	G	G	T	T	-	A	A	C	**T**	G	**A**	C	T	C	A	G	G	T	T	C	C	-
F03-1	-	G	G	A	**C**	C	**G**	T	C	C	-	**A**	**G**	**A**	**T**	A	A	**-**	C	G	G	T	G	-	A	**G**	G	G	A	**N**	A	**N**	G	C	G	C	A	A	**T**	G	**N**	T	-	A	A	C	C	G	C	**A**	T	C	A	**C**	G	**G**	T	C	C	-
F05-1	-	G	G	A	**N**	C	C	T	C	**T**	-	G	C	**T**	A	A	A	G	**A**	**-**	G	T	G	-	A	A	G	G	A	G	A	G	G	C	**A**	C	**N**	A	**T**	G	**N**	T	-	A	A	C	**T**	G	**N**	C	T	C	A	G	G	T	T	C	C	**C**
F06-1	-	G	G	A	A	**A**	**T**	T	C	C	-	**T**	C	G	A	A	A	G	**A**	G	G	T	G	-	A	A	G	G	A	G	A	**C**	G	C	G	C	A	A	**C**	G	T	T	-	A	A	C	C	**T**	C	**G**	T	C	A	G	**T**	T	T	C	C	-
F07-1	-	G	G	A	A	**A**	**T**	T	C	C	-	**T**	C	G	A	A	A	G	**A**	G	G	T	G	-	A	A	G	G	A	G	A	**C**	G	C	G	C	A	A	**C**	G	T	T	-	A	A	C	C	**T**	C	**G**	T	C	A	G	**T**	T	T	C	C	-
F09-1	-	**N**	G	A	A	C	**G**	T	**G**	C	-	**C**	**A**	G	A	A	A	G	**G**	G	**T**	T	G	-	A	A	G	G	A	G	**C**	G	**A**	C	G	C	A	A	G	G	T	T	-	A	A	C	C	G	**T**	**T**	**G**	C	A	G	G	T	T	C	C	-
F11-1	-	G	G	A	A	**G**	**G**	T	C	C	-	G	C	G	A	A	A	G	**G**	G	**N**	T	G	-	A	A	G	G	A	**A**	A	G	G	C	G	**G**	A	**T**	G	G	T	T	-	**T**	A	**A**	**T**	G	C	C	T	C	A	**N**	G	T	T	C	C	-
G03-1	-	G	G	A	A	C	C	**N**	C	C	-	G	C	G	A	A	A	G	**T**	G	G	**K**	G	-	A	A	**N**	G	A	G	A	G	**T**	C	G	C	A	A	G	G	T	**N**	-	A	A	C	C	**C**	C	C	T	C	A	**N**	**A**	T	T	C	C	-
G04-1	**G**	G	**A**	A	A	C	**T**	T	C	C	-	G	C	G	A	**T**	**G**	G	C	G	G	T	G	-	A	A	G	G	A	G	A	**A**	G	C	**T**	C	A	A	G	G	**A**	T	-	A	A	C	**A**	G	C	**T**	T	C	A	G	G	T	T	C	C	-
G08-1	-	G	G	A	A	C	C	T	C	C	-	G	**T**	G	A	A	A	**A**	C	G	**A**	T	G	-	A	A	**C**	G	A	**C**	A	G	**T**	C	G	C	A	A	G	G	T	T	-	A	**-**	C	C	G	**G**	C	T	**G**	A	G	G	**C**	T	C	C	-
G09-1	-	G	G	A	A	C	**W**	T	**G**	C	-	G	**G**	**A**	A	A	A	G	**A**	**A**	G	T	G	-	A	A	G	G	A	G	A	G	**T**	**G**	G	C	A	A	G	G	T	T	-	A	A	C	C	**C**	**A**	C	T	C	A	**T**	G	T	T	C	C	-
G10-1	-	G	G	A	**G**	C	C	T	**G**	C	-	G	C	**C**	A	A	A	**A**	C	G	**A**	**A**	G	-	A	A	G	G	A	G	A	G	G	C	**A**	C	A	A	**T**	G	T	T	-	A	A	C	**T**	G	C	C	T	C	A	G	G	T	T	C	C	-
H03-1	-	G	G	A	A	C	C	T	C	C	-	G	C	G	**C**	A	A	G	C	G	G	T	G	-	A	A	G	G	A	G	A	G	G	**G**	G	C	A	A	**C**	G	T	T	-	A	A	C	C	**T**	C	C	T	C	A	G	G	**C**	T	C	C	-
H06-1	-	G	G	A	A	**T**	C	T	C	C	-	G	C	**A**	A	A	A	G	C	G	**T**	T	G	-	A	A	G	G	A	G	**C**	**C**	G	**T**	**T**	C	**T**	A	G	G	T	**G**	-	A	A	C	**T**	**C**	C	C	**C**	C	A	**A**	G	T	T	C	C	-
H06-2	-	G	G	A	A	**A**	C	T	C	C	-	G	C	G	A	A	**T**	G	C	G	G	T	G	-	A	A	G	G	A	G	A	G	**C**	C	G	C	A	A	**T**	G	**G**	T	-	A	A	C	C	G	**T**	C	T	C	A	G	**T**	T	T	C	C	-
H08-1	-	G	G	A	A	C	**G**	T	**G**	C	-	G	**T**	G	A	A	A	**A**	C	G	**C**	T	G	-	A	A	G	**T**	A	G	**G**	G	G	C	G	**A**	A	A	**A**	G	T	T	-	A	A	C	C	G	**T**	C	T	C	A	**C**	G	T	T	C	C	-
H08-2	-	G	G	A	A	**G**	C	T	C	C	-	**A**	C	G	A	**T**	A	G	**T**	G	G	T	G	-	A	**G**	G	G	A	G	A	G	G	C	**A**	C	A	A	**C**	G	T	T	-	A	A	C	**T**	G	C	C	T	**T**	A	G	**C**	T	T	C	C	-
H12-1	-	G	G	A	A	C	**G**	T	**G**	C	-	G	**T**	G	A	A	A	**A**	C	G	**C**	T	G	-	A	A	G	**T**	A	G	**G**	G	G	C	G	**A**	A	A	**A**	G	T	T	-	A	A	C	C	G	**T**	C	T	C	A	**C**	G	T	T	C	C	-
A02-1′	-	G	G	A	A	C	C	**A**	C	**U**	-	G	C	G	A	**U**	**U**	G	C	**A**	G	**U**	G	-	A	A	G	G	A	G	A	**A**	**U**	C	**A**	C	A	A	G	G	**U**	**A**	-	A	A	C	**U**	G	**A**	**U**	**U**	C	**U**	G	G	**C**	**U**	C	C	
A04-1 ′	-	**-**	**-**	**-**	**-**	**-**	C	T	C	C	-	G	C	G	A	A	A	G	C	G	G	T	G	-	A	A	G	G	A	G	A	**A**	G	C	G	C	A	**C**	G	G	**C**	T	-	A	A	C	C	G	C	**T**	T	C	A	G	G	**G**	T	C	C	-
A09-1′	-	G	G	A	A	**G**	C	T	**A**	C	-	G	C	**A**	**T**	**T**	A	G	C	G	**T**	T	G	-	A	**T**	G	G	A	G	A	**T**	G	C	G	C	A	A	G	G	**G**	T	-	A	A	C	C	G	C	**G**	T	C	A	**A**	G	T	T	C	C	-
A12-1′	-	G	G	A	A	C	C	T	**G**	C	-	G	C	G	A	A	A	G	C	G	**C**	T	G	-	A	A	G	G	A	**A**	A	G	**T**	C	**A**	C	A	A	G	G	T	T	-	A	A	C	**T**	G	**A**	C	T	**T**	A	G	**C**	**G**	T	C	C	-
B03-1′	-	G	G	A	A	C	**T**	T	C	C	-	G	**T**	G	A	A	A	**A**	C	G	G	T	G	-	A	A	G	G	A	G	A	**A**	G	C	G	C	A	A	**C**	G	T	T	-	A	A	C	C	G	C	**T**	T	C	A	G	G	T	T	C	C	-
B08-1′	**A**	G	G	A	**G**	**G**	C	T	C	**T**	-	G	C	G	A	A	A	G	C	**A**	G	T	G	-	A	A	G	G	A	G	**T**	G	G	C	G	C	A	A	**T**	G	T	T	-	A	A	C	C	G	C	C	**C**	C	A	G	**C**	**G**	T	C	C	-
B12-1′	-	G	G	A	A	C	**G**	T	C	C	-	**C**	C	G	A	A	A	G	**G**	G	G	T	G	-	A	A	G	G	A	G	**T**	G	**A**	C	G	C	A	A	**C**	G	T	T	-	A	A	C	**T**	G	**T**	C	**A**	C	A	**C**	G	T	T	C	C	-
C06-1′	-	G	G	A	**T**	**T**	C	T	C	**G**	-	G	C	G	A	A	A	G	C	**C**	G	T	G	-	A	A	G	G	A	G	A	G	**A**	C	G	C	A	A	G	G	T	T	-	A	A	C	C	G	**T**	C	T	C	A	G	G	**A**	T	C	C	-
C10-1′	-	G	G	**G**	A	C	**G**	T	C	**G**	-	G	C	G	A	**G**	A	G	C	**T**	G	T	G	-	A	**T**	G	G	A	**A**	A	G	G	C	G	C	A	**T**	**C**	G	T	T	-	A	A	C	C	G	C	C	T	**T**	A	**C**	G	T	T	C	C	-
C10-2′	-	G	G	A	A	C	C	T	**A**	C	-	G	**A**	G	A	A	A	G	C	G	**T**	T	G		A	A	G	G	A	G	**G**	G	G	C	**C**	C	A	A	**T**	G	T	T	-	A	A	C	**G**	G	C	C	T	C	A	G	G	T	T	C	C	-
C12-1′	-	G	G	**G**	A	C	C	T	C	C	-	**T**	C	G	A	A	A	**A**	**A**	G	G	T	G	-	A	A	G	G	A	G	A	**C**	**A**	C	G	C	A	A	G	G	T	T	-	A	A	C	C	G	**T**	**T**	T	C	A	G	G	T	T	C	C	-
D02-1′	-	G	G	A	A	C	**A**	T	C	C	-	G	**T**	G	A	A	A	**A**	C	G	G	T	G	-	A	A	G	G	A	G	A	G	**C**	C	G	C	A	A	G	G	T	T	-	A	A	C	C	G	**T**	C	T	C	A	**T**	G	T	T	C	C	-
D04-1′	-	G	G	A	A	**A**	C	T	C	C	-	G	C	G	**C**	A	**C**	G	C	G	G	T	G	-	A	A	G	G	A	G	A	G	G	C	G	C	A	A	**T**	G	**G**	T	-	A	A	C	C	G	**T**	C	T	C	A	G	**A**	T	T	C	C	-
D11-1′	-	G	G	A	A	C	**N**	T	C	C	-	**T**	C	G	A	A	**N**	G	**G**	G	G	T	G	-	A	A	G	G	A	G	A	G	G	C	G	C	A	A	**-**	G	T	T	-	A	A	C	C	**A**	C	C	T	C	A	**N**	G	T	T	C	C	-
D12-1′	-	G	G	A	A	**T**	C	**G**	C	C	-	**T**	**A**	G	A	A	A	G	**T**	G	G	T	G	-	**C**	A	G	G	A	G	A	G	G	**A**	**C**	C	A	A	G	**A**	T	T	-	A	**T**	C	C	**T**	C	C	T	C	A	**T**	G	T	T	C	C	-
E02-1′	-	G	G	A	A	C	C	T	**T**	C	-	G	C	G	**C**	A	A	G	C	G	**A**	T	G	-	A	A	G	G	A	G	A	G	**A**	C	G	C	A	A	G	G	**C**	T	-	A	A	C	C	G	**T**	**T**	T	C	A	G	G	T	T	C	C	-
E08-1′	-	G	G	A	**G**	C	C	T	C	**A**	-	G	C	G	A	A	A	G	C	**T**	G	T	G	-	A	A	G	G	A	G	A	G	G	**T**	G	C	A	A	G	G	**G**	T	-	A	A	C	C	**A**	C	C	T	C	A	G	**A**	**C**	T	C	C	-

Name	Aligned Sequences																																																										
		Stem 1							Stem 2					Loop 1				Stem 2				Loop 2								Stem 3						Loop 3											Stem 3						Stem 1							
#nt		1	2	3	4	5	6	7	8	9		10	11	12	13	14	15	16	17	18	19	20	21		22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39		40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	
RhoBAST	-	G	G	A	A	C	C	T	C	C	-	G	C	G	A	A	A	G	C	G	G	T	G	-	A	A	G	G	A	G	A	G	G	C	G	C	A	A	G	G	T	T	-	A	A	C	C	G	C	C	T	C	A	G	G	T	T	C	C	-
E09-1´	-	G	G	A	A	C	C	T	C	C	-	**T**	C	G	A	A	A	G	**A**	G	G	T	G	-	A	A	G	G	A	G	A	G	**A**	C	**A**	C	A	A	G	G	T	T	-	A	A	C	**T**	G	**T**	C	T	C	A	G	G	T	T	C	C	-
E10-1´	-	G	G	A	A	C	**G**	T	C	C	-	**T**	C	**A**	A	A	A	G	**T**	G	G	T	G	-	A	A	G	G	A	G	A	**A**	G	C	G	C	A	A	G	G	**G**	T	-	A	A	C	**T**	G	C	**T**	T	C	A	**C**	G	T	T	C	C	-
E11-1´	-	G	G	A	**C**	**A**	C	**A**	**G**	C	-	G	C	**C**	A	A	A	G	**G**	G	G	T	G	-	A	A	G	G	A	G	A	**T**	G	**A**	G	C	A	A	G	G	**C**	T	-	A	A	C	C	**T**	C	**A**	T	C	A	G	G	T	T	C	C	-
E11-2´	-	G	G	A	A	**G**	C	**C**	C	C	-	G	C	G	A	A	**T**	G	C	G	G	**C**	G	-	A	A	G	G	**G**	G	A	G	G	C	**A**	C	A	A	**C**	G	T	T	-	A	A	C	**T**	G	**T**	C	T	C	A	G	**C**	T	T	C	C	-
E11-3´	-	**A**	G	A	A	C	**G**	T	C	C	-	G	C	G	A	A	**T**	G	C	G	**T**	T	G	-	A	A	G	G	**C**	**C**	A	G	G	C	G	C	A	**T**	G	**T**	T	**G**	-	A	**T**	C	C	G	C	C	T	**-**	A	G	G	T	T	C	C	-
G05-1´	-	G	G	A	A	C	**T**	**G**	C	C	-	G	**A**	G	A	A	A	**T**	C	G	G	T	G	-	A	**G**	G	G	A	G	**T**	G	G	C	G	C	A	A	**C**	G	T	T	-	A	A	C	C	G	**T**	C	T	C	**C**	**A**	G	T	T	C	C	-
G05-2´	-	**C**	G	A	A	C	C	T	C	C	-	G	C	G	A	A	A	G	C	G	G	T	G	-	A	A	G	G	A	**C**	A	G	**T**	**T**	G	C	A	A	G	G	T	T	-	A	A	C	C	**A**	**A**	C	T	**G**	A	G	G	**C**	T	C	C	-
G05-3´	-	G	G	A	A	**T**	C	T	**A**	C	-	G	**T**	G	A	A	A	**C**	C	G	**T**	T	G	-	A	A	G	G	A	G	**T**	G	G	C	G	C	A	A	G	G	**C**	T	-	A	A	C	C	G	C	C	**G**	C	A	G	G	T	T	C	C	-
G06-1´	-	G	G	A	**G**	C	**G**	T	C	C	-	**C**	C	G	A	A	A	**T**	C	G	G	T	G	-	A	A	G	G	A	G	A	G	G	C	**A**	C	A	A	**C**	G	T	T	-	A	A	C	**T**	G	C	C	T	C	A	**C**	G	**C**	T	C	C	-
H02-1´	-	G	G	A	**G**	C	**G**	T	C	C	-	**C**	C	G	A	A	A	**T**	C	G	G	T	G	-	A	A	G	G	A	G	A	G	G	C	**A**	C	A	A	**C**	G	T	T	-	A	A	C	**T**	G	C	C	T	C	A	**C**	G	**C**	T	C	C	-
H02-2´	-	G	G	A	A	C	**G**	T	**T**	C	-	G	C	G	A	A	A	G	C	G	**A**	T	G	-	A	A	G	G	A	G	A	G	G	**T**	G	C	A	A	G	G	**C**	T	-	A	A	C	C	**A**	C	C	T	C	A	**C**	G	T	T	C	C	-
H02-3´	-	G	G	A	A	**A**	C	T	C	C	-	G	C	G	**C**	A	A	G	C	G	G	T	G	-	A	**T**	G	G	A	G	A	G	G	**A**	**A**	C	A	A	G	G	T	T	-	A	A	C	**T**	**T**	C	C	T	C	A	G	G	**C**	T	C	C	-
H10-1´	-	G	G	A	A	C	C	T	C	C	-	G	C	G	A	A	**C**	G	C	G	G	T	G	-	A	A	G	G	A	G	A	**T**	G	C	**T**	C	A	A	**T**	G	T	T	-	A	A	C	**A**	G	C	**G**	T	C	A	G	G	T	T	C	C	-
H11-1´	-	G	G	A	A	C	C	T	C	C	-	**A**	C	G	A	A	A	G	**G**	G	G	T	G	-	A	A	G	G	A	G	A	G	**T**	C	**A**	C	A	A	**T**	G	T	T	-	A	A	C	**T**	G	**A**	C	T	C	A	G	G	T	T	C	C	-

		Stem 1							Stem 2					Loop 1				Stem 2				Loop 2								Stem 3						Loop 3											Stem 3						Stem 1							
#nt		1	2	3	4	5	6	7	8	9		10	11	12	13	14	15	16	17	18	19	20	21		22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39		40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	
RhoBAST	-	G	G	A	A	C	C	T	C	C	-	G	C	G	A	A	A	G	C	G	G	T	G	-	A	A	G	G	A	G	A	G	G	C	G	C	A	A	G	G	T	T	-	A	A	C	C	G	C	C	T	C	A	G	G	T	T	C	C	-

nt	percentage of occurrence per nucleotide																																																										
G	1	93	97	4	8	12	22	3	15	3	0	72	2	82	3	5	2	75	7	84	70	2	89	10	0	5	94	96	4	86	5	76	73	4	70	2	3	3	59	94	10	4	0	0	0	0	2	68	2	5	3	3	3	63	79	10	0	0	0	0
A	1	1	1	93	81	12	7	6	9	3	0	6	8	10	83	85	89	11	10	5	5	2	0	0	95	73	2	1	94	6	83	8	11	13	22	2	91	88	4	1	6	0	0	95	95	1	2	9	7	6	3	2	90	6	4	2	0	0	0	0
C	0	1	0	0	4	64	58	2	68	85	0	6	74	4	6	0	2	4	72	2	10	5	1	1	3	7	1	0	1	5	5	5	3	70	4	91	0	4	16	2	8	0	0	2	0	95	64	6	72	75	6	88	2	18	10	17	1	98	96	2
T	0	0	0	0	3	8	9	86	7	7	1	14	14	3	8	10	6	9	9	6	6	88	10	0	1	13	2	3	0	3	7	9	12	11	3	2	2	5	19	3	71	91	1	2	3	2	30	17	16	12	86	5	4	10	7	70	96	0	0	0

Rate of mutation	#	percentage of occurrence of original nucleotide
Highly conserved	9	>95
Conserved	17	>85, <95
Moderately mutated	11	>75, <85
Highly mutated	18	>50, <75

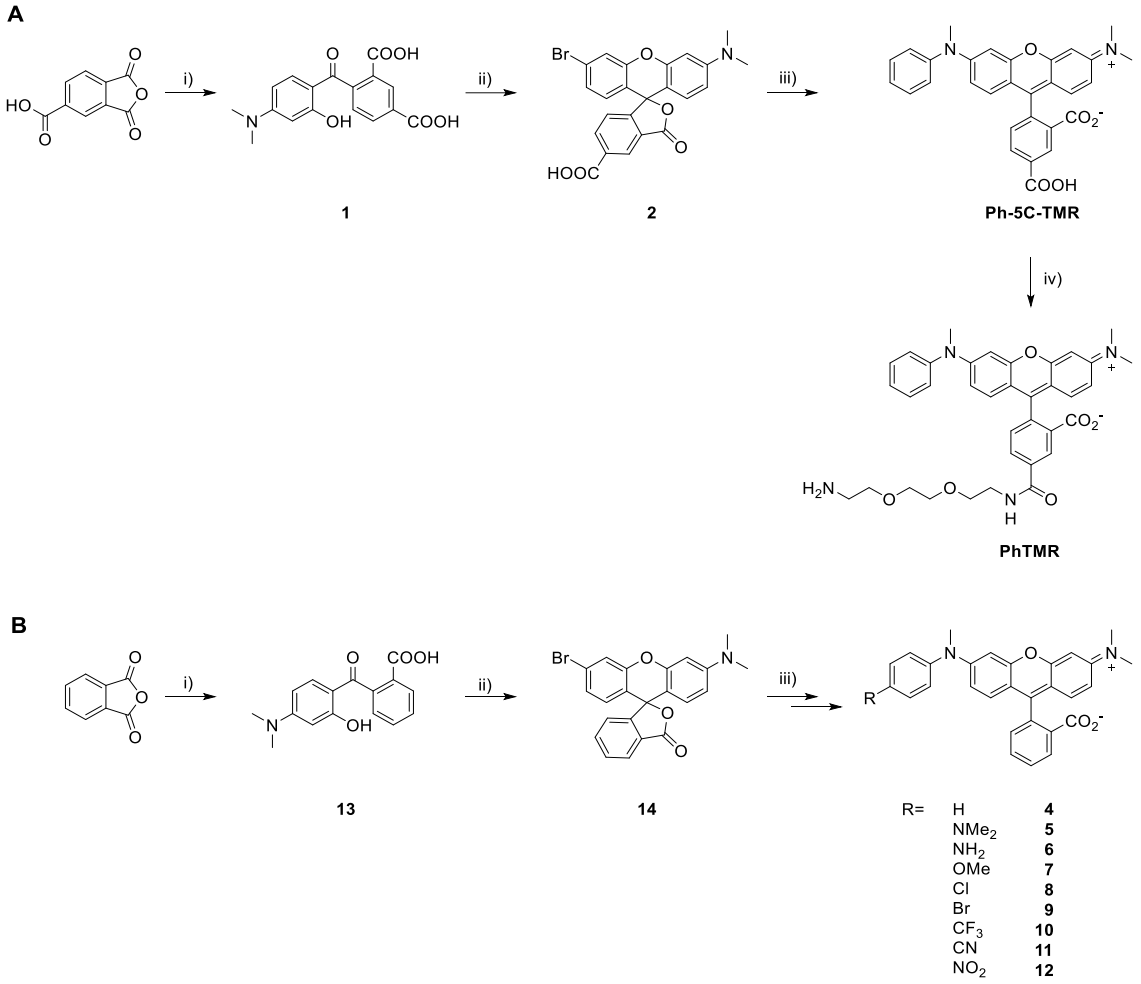
Supplementary Table 8: Truncated and aligned sequences of the mutants exhibiting a similar secondary structure like RhoBAST. The nucleotides which are not in accordance with the RhoBAST sequence are color-coded. The percentage of occurrence per nucleotide (nt) was calculated.

Name	Aligned Sequences																																																						
RhoBAST	G	G	A	A	C	C	T	C	C	G	C	G	A	A	A	G	C	G	G	T	G	-	A	A	G	G	A	G	A	G	G	C	G	C	A	A	G	G	T	T	A	A	C	C	G	C	C	T	C	A	G	G	T	T	C	C
# nt	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55
	Stem 1							Stem 2				Loop 1				Stem 2				Loop 2								Stem 3						Loop 3										Stem 3						Stem 1						
A01-1	G	G	A	T	C	C	T	A	C	G	T	G	A	A	A	G	C	G	G	T	T	G	A	A	G	G	A	G	A	G	G	G	G	C	A	A	G	G	T	T	A	A	C	C	T	C	C	T	C	A	G	G	A	T	C	C
A04-1	G	G	A	A	A	C	T	C	C	A	C	G	A	A	A	G	T	G	G	T	G	-	A	C	G	G	A	G	A	G	A	T	G	C	A	A	G	G	G	T	A	A	C	C	A	T	C	T	C	A	G	G	T	T	C	C
A06-1	G	G	A	A	C	G	T	C	G	G	C	G	A	A	A	G	C	C	G	T	G	-	A	A	G	G	A	G	A	G	G	A	G	C	A	A	G	G	T	T	A	A	C	C	C	C	C	T	C	A	C	G	T	T	C	C
A10-1	G	G	A	A	C	G	T	C	C	T	C	G	A	A	A	G	A	G	G	T	G	-	A	A	G	G	A	G	A	T	G	C	C	C	A	A	G	G	A	T	A	A	C	C	G	C	C	T	C	A	C	G	T	T	C	C
B02-3	G	G	A	T	C	C	T	A	C	G	T	A	A	T	A	A	C	G	T	T	G		A	A	G	G	A	G	A	G	G	A	G	C	A	A	A	G	T	T	A	A	C	C	T	C	C	A	C	A	G	G	G	T	C	C
B06-3	G	G	A	A	G	C	T	G	C	C	C	G	A	A	A	G	C	G	C	T	G	-	A	A	G	G	A	G	A	T	G	C	G	C	A	A	G	G	T	T	A	A	C	C	G	C	A	T	C	A	G	C	T	T	C	C
B09-2	G	G	A	A	C	G	T	C	C	G	T	G	A	A	A	G	C	G	G	C	G	-	A	C	G	G	A	A	A	G	G	C	A	C	A	A	G	G	T	T	A	A	C	T	G	C	C	T	C	A	C	G	T	T	C	C
B10-2	G	G	A	A	C	G	T	C	T	G	C	G	A	G	A	G	C	G	G	T	G	-	A	C	G	G	A	G	A	G	A	T	G	C	A	A	G	G	A	T	A	A	C	C	A	T	C	T	C	A	C	G	T	T	C	C
C01-1	G	G	A	A	C	A	T	G	C	G	C	G	A	A	A	G	C	G	C	T	G		A	A	G	G	A	G	C	G	G	C	A	C	A	A	C	G	T	T	A	A	C	T	G	C	C	C	C	A	T	G	T	T	C	C
C04-1	G	G	A	A	C	C	A	C	C	T	C	G	A	T	A	G	C	G	G	T	G	-	A	T	G	G	A	G	A	G	G	A	G	C	A	A	A	G	T	T	A	A	C	C	T	C	C	T	C	T	G	G	T	T	C	C
C07-1	G	G	A	A	T	G	T	C	C	C	C	G	A	A	A	G	G	G	G	T	G	-	A	A	G	G	A	G	A	G	G	C	G	C	A	A	T	G	T	T	A	A	C	C	G	C	T	T	C	A	T	G	T	T	C	C
D01-1	G	G	A	A	C	A	T	G	C	G	C	G	A	A	A	G	C	G	C	T	G		A	A	G	G	A	G	C	G	G	C	A	C	A	A	C	G	T	T	A	A	C	T	G	C	C	C	C	A	T	G	T	T	C	C
D03-1	G	G	A	A	C	C	T	G	C	G	T	G	A	A	A	G	C	G	C	T	G	-	A	A	G	G	A	G	A	T	G	A	G	C	A	A	T	G	T	T	A	A	C	C	T	C	A	T	C	T	G	G	C	T	C	C
D03-2	G	G	A	A	C	C	T	G	C	G	C	G	C	A	A	G	C	G	C	T	G	-	A	A	G	G	A	G	A	G	A	T	G	C	A	A	G	G	T	T	A	A	C	C	A	C	C	T	C	A	G	G	T	T	C	C
D06-1	G	G	A	A	C	C	T	C	C	G	C	G	T	A	A	G	C	G	G	T	G	-	A	A	G	A	A	G	A	G	G	T	A	T	A	A	G	G	T	T	A	A	C	T	A	C	C	T	C	A	G	G	C	T	C	C
D08-1	G	G	A	A	C	A	T	C	C	G	C	G	A	A	A	G	C	G	G	T	G	-	A	C	G	G	A	G	A	G	G	C	G	C	A	A	G	G	C	T	A	A	C	C	G	C	C	T	C	A	T	G	T	T	C	C
E05-1	G	G	A	A	C	T	T	T	C	G	C	G	A	A	A	G	C	T	A	T	G		A	T	G	G	A	G	T	G	G	C	A	C	A	C	G	G	T	T	A	A	C	T	G	C	T	A	C	A	A	G	T	T	C	C
E05-2	G	G	A	A	C	G	T	T	C	G	C	G	A	A	A	G	C	G	A	T	G	-	A	A	G	G	A	G	A	G	G	A	G	C	A	C	G	G	T	T	A	A	C	C	T	C	C	T	C	A	C	G	T	T	C	C
E12-2	G	G	A	A	C	A	C	C	C	G	C	G	A	T	A	G	C	G	G	T	G		A	A	G	G	A	G	C	G	G	C	G	C	A	A	G	G	T	T	A	A	C	T	G	C	C	G	C	A	T	G	T	T	C	C
G08-1	G	G	A	A	C	C	T	C	C	G	T	G	A	A	A	A	C	G	A	T	G	-	A	A	C	G	A	C	A	G	T	C	G	C	A	A	G	G	T	T	A	-	C	C	G	G	C	T	G	A	G	G	C	T	C	C
G09-1	G	G	A	A	C	W	T	G	C	G	G	A	A	A	A	G	A	A	G	T	G	-	A	A	G	G	A	G	A	G	T	G	G	C	A	A	G	G	T	T	A	A	C	C	C	A	C	T	C	A	T	G	T	T	C	C
G10-1	G	G	A	G	C	C	T	G	C	G	C	C	A	A	A	A	C	G	A	A	G	-	A	A	G	G	A	G	A	G	G	C	A	C	A	A	T	G	T	T	A	A	C	T	G	C	C	T	C	A	G	G	T	T	C	C
H03-1	G	G	A	A	C	C	T	C	C	G	C	G	C	A	A	G	C	G	G	T	G	-	A	A	G	G	A	G	A	G	G	G	G	C	A	A	C	G	T	T	A	A	C	C	T	C	C	T	C	A	G	G	C	T	C	C
H08-1	G	G	A	A	C	G	T	G	C	G	T	G	A	A	A	A	C	G	C	T	G	-	A	A	G	T	A	G	G	G	G	C	G	A	A	A	A	G	T	T	A	A	C	C	G	T	C	T	C	A	C	G	T	T	C	C
A02-1′	G	G	A	A	C	C	A	C	T	G	C	G	A	T	T	G	C	A	G	T	G		A	A	G	G	A	G	A	A	T	C	A	C	A	A	G	G	T	A	A	A	C	T	G	A	T	T	C	T	G	G	C	T	C	C
A09-1′	G	G	A	A	G	C	T	A	C	G	C	A	T	T	A	G	C	G	T	T	G	-	A	T	G	G	A	G	A	T	G	C	G	C	A	A	G	G	G	T	A	A	C	C	G	C	G	T	C	A	A	G	T	T	C	C
B03-1′	G	G	A	A	C	T	T	C	C	G	T	G	A	A	A	A	C	G	G	T	G	-	A	A	G	G	A	G	A	A	G	C	G	C	A	A	C	G	T	T	A	A	C	C	G	C	T	T	C	A	G	G	T	T	C	C
B08-1′	G	G	A	G	G	C	T	C	T	G	C	G	A	A	A	G	C	A	G	T	G	-	A	A	G	G	A	G	T	G	G	C	G	C	A	A	T	G	T	T	A	A	C	C	G	C	C	C	C	A	G	C	G	T	C	C
B12-1′	G	G	A	A	C	G	T	C	C	C	C	G	A	A	A	G	G	G	G	T	G	-	A	A	G	G	A	G	T	G	A	C	G	C	A	A	C	G	T	T	A	A	C	T	G	T	C	A	C	A	C	G	T	T	C	C
C06-1′	G	G	A	T	T	C	T	C	G	G	C	G	A	A	A	G	C	C	G	T	G	-	A	A	G	G	A	G	A	G	A	C	G	C	A	A	G	G	T	T	A	A	C	C	G	T	C	T	C	A	G	G	A	T	C	C
C10-1′	G	G	G	A	C	G	T	C	G	G	C	G	A	G	A	G	C	T	G	T	G	-	A	T	G	G	A	A	A	G	G	C	G	C	A	T	C	G	T	T	A	A	C	C	G	C	C	T	T	A	C	G	T	T	C	C
D04-1′	G	G	A	A	A	C	T	C	C	G	C	G	C	A	C	G	C	G	G	T	G	-	A	A	G	G	A	G	A	G	G	C	G	C	A	A	T	G	G	T	A	A	C	C	G	T	C	T	C	A	G	A	T	T	C	C
E02-1′	G	G	A	A	C	C	T	T	C	G	C	G	C	A	A	G	C	G	A	T	G	-	A	A	G	G	A	G	A	G	A	C	G	C	A	A	G	G	C	T	A	A	C	C	G	T	T	T	C	A	G	G	T	T	C	C
E09-1′	G	G	A	A	C	C	T	C	C	T	C	G	A	A	A	G	A	G	G	T	G	-	A	A	G	G	A	G	A	G	A	C	A	C	A	A	G	G	T	T	A	A	C	T	G	T	C	T	C	A	G	G	T	T	C	C
E11-2′	G	G	A	A	G	C	C	C	C	G	C	G	A	A	T	G	C	G	G	C	G	-	A	A	G	G	G	G	A	G	G	C	A	C	A	A	C	G	T	T	A	A	C	T	G	T	C	T	C	A	G	C	T	T	C	C
G05-2′	C	G	A	A	C	C	T	C	C	G	C	G	A	A	A	G	C	G	G	T	G	-	A	A	G	G	A	C	A	G	T	T	G	C	A	A	G	G	T	T	A	A	C	C	A	A	C	T	G	A	G	G	C	T	C	C
G06-1′	G	G	A	G	C	G	T	C	C	C	C	G	A	A	A	T	C	G	G	T	G	-	A	A	G	G	A	G	A	G	G	C	A	C	A	A	C	G	T	T	A	A	C	T	G	C	C	T	C	A	C	G	C	T	C	C
H02-1′	G	G	A	G	C	G	T	C	C	C	C	G	A	A	A	T	C	G	G	T	G	-	A	A	G	G	A	G	A	G	G	C	A	C	A	A	C	G	T	T	A	A	C	T	G	C	C	T	C	A	C	G	C	T	C	C
H02-2′	G	G	A	A	C	G	T	T	C	G	C	G	A	A	A	G	C	G	A	T	G	-	A	A	G	G	A	G	A	G	G	T	G	C	A	A	G	G	C	T	A	A	C	C	A	C	C	T	C	A	C	G	T	T	C	C
H10-1′	G	G	A	A	C	C	T	C	C	G	C	G	A	A	C	G	C	G	G	T	G	-	A	A	G	G	A	G	A	T	G	C	T	C	A	A	T	G	T	T	A	A	C	A	G	C	G	T	C	A	G	G	T	T	C	C
H11-1′	G	G	A	A	C	C	T	C	C	A	C	G	A	A	A	G	G	G	G	T	G	-	A	A	G	G	A	G	A	G	T	C	A	C	A	A	T	G	T	T	A	A	C	T	G	A	C	T	C	A	G	G	T	T	C	C

RhoBAST	G	G	A	A	C	C	T	C	C	G	C	G	A	A	A	G	C	G	G	T	G	-	A	A	G	G	A	G	A	G	G	C	G	C	A	A	G	G	T	T	A	A	C	C	G	C	C	T	C	A	G	G	T	T	C	C
# nt	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55
	Stem 1							Stem 2				Loop 1				Stem 2				Loop 2								Stem 3						Loop 3										Stem 3						Stem 1						

nt	percentage of occurrence per nucleotide																																																						
G	98	100	2	10	10	29	0	20	7	76	2	90	0	5	0	83	7	83	66	0	98	2	0	0	98	95	2	90	2	83	71	7	66	0	0	0	54	100	7	0	0	0	0	0	66	2	5	2	5	0	54	90	5	0	0	0
A	0	0	98	83	5	10	5	7	0	5	0	7	85	83	90	12	7	7	15	2	0	0	100	80	0	2	98	5	83	5	17	12	29	2	100	93	7	0	5	2	100	98	0	2	15	10	5	7	0	93	5	2	5	0	0	0
C	2	0	0	0	80	54	5	63	85	12	80	2	10	0	5	0	83	5	15	5	0	0	0	10	2	0	0	5	7	0	0	66	2	95	0	5	22	0	7	0	0	0	100	63	5	66	78	7	93	0	27	7	20	0	100	100
T	0	0	0	7	5	5	90	10	7	7	17	0	5	12	5	5	2	5	5	93	2	0	0	10	0	2	0	0	7	12	12	15	2	2	0	2	17	0	80	98	0	0	0	34	15	22	12	83	2	7	15	0	71	100	0	0

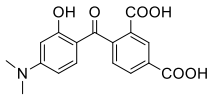
Chemical Synthesis



Supplementary Scheme S1: Synthesis strategy.

A) Synthesis of **PhTMR**. i) 3-Dimethylaminophenol, toluene. ii) 3-Bromophenol, TFA, $MeSO_3H$. iii) *N*-Methylaniline, $ZnCl_2$.
B) Synthesis of aryl-substituted derivatives. i) 3-Dimethylaminophenol, toluene. ii) 3-Bromophenol, TFA, $MeSO_3H$. iii) Either zinc-mediated aromatic substitution or Buchwald-Hartwig reaction.

4-(4-(Dimethylamino)-2-hydroxybenzoyl)isophthalic acid (1)



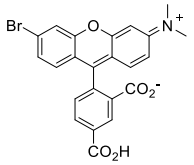
$C_{17}H_{15}NO_6$
329.31 g/mol

In a 250 mL flask, 3-dimethylaminophenol (2.98 g, 21.7 mmol, 1.00 eq) was dissolved in toluene (60 mL) at 60°C and trimellitic anhydride (5.00 g, 26.0 mmol, 1.20 eq) was added. The reaction mixture was refluxed for 24 h. After allowing the mixture to cool to room temperature, the precipitate was filtered off and washed with toluene (30 mL). The residue was dissolved in MeOH (60 mL) and refluxed for 10 min. Next, acetic acid (20 mL) was added to the mixture and solvents were removed under reduced pressure. The residue was dissolved in MeOH (40 mL) and refluxed for 2 h. The mixture was stored at 4°C overnight and the precipitate was filtered off and washed with cold MeOH (10 mL). The product was obtained as violet solid (1.60 g, 4.86 mmol, 22%). The recorded spectrum is in accordance with literature.[10]

^{1}H NMR (300 MHz, $(CD_3)_2SO$): δ = 13.40 (bs, 2H), 12.36 (s, 1H), 8.48 (d, *J* = 1.6 Hz, 1H), 8.20 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.52 (d, *J* = 7.9 Hz, 1H), 6.81 (d, *J* = 9.2 Hz, 1H), 6.22 (dd, *J* = 9.2, 2.5 Hz, 1H), 6.11 (d, *J* = 2.5 Hz, 1H), 3.01 (s, 6H) ppm.

2-(6-Bromo-3-(dimethylimino)-3H-xanthen-9-yl)-5-carboxybenzoate (2)

2-(6-bromo-3-(dimethyliminio)-3*H*-xanthen-9-yl)-5-carboxybenzoate



$C_{23}H_{16}BrNO_5$
466.29 g/mol

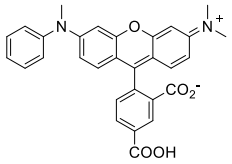
Benzophenone **1** (500 mg, 2.52 mmol, 1.00 eq.) and 3-bromophenol (394 mg, 2.28 mmol, 1.50 eq.) were dissolved in methanesulfonic acid (3 mL). Next, trifluoroacetic acid (3 mL) was added and the reaction was stirred at 80°C for 24 h. The reaction mixture was poured into water (150 mL) and the precipitate was filtered off. The aqueous solution was additionally extracted with chloroform (2 × 50 mL) and the organic phase was dried over magnesium sulfate, filtered and the solvent was evaporated under reduced pressure. The precipitate and the obtained residue were combined and purified by silica column chromatography ($CHCl_3$:MeOH = 50:1 → 20:1). The product was obtained as a red solid (422 mg, 905 µmol, 60%). The synthesis protocol was adapted from C.C. Woodroofe *et al.*[11]

^{1}H NMR (500 MHz, $(CD_3)_2SO$): δ = 8.42 (d, *J* = 0.6 Hz, 1H), 8.30 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.64 (d, *J* = 2.0 Hz, 1H), 7.41 (d, *J* = 7.9 Hz, 1H), 7.27 (dd, *J* = 8.4, 2.0 Hz, 1H), 6.81 (d, *J* = 8.6 Hz, 1H), 6.62 - 6.59 (m, 1H), 6.55 - 6.51 (m, 2H), 2.95 (s, 6H) ppm.

^{13}C NMR (126 MHz, $(CD_3)_2SO$): δ = 167.7, 165.9, 155.8, 152.2, 151.5, 151.3, 136.2, 133.0, 130.0, 128.5, 126.9, 126.5, 125.6, 124.6, 123.4, 119.7, 118.0, 109.7, 104.2, 97.9, 82.6, 39.8 ppm.

MS (HR-ESI, pos): meas. *m/z* = 466.0273, calc. *m/z* = 466.0285 for $C_{23}H_{17}BrNO_5$ $[M+H]^+$.

5-Carboxy-2-(3-(dimethylimino)-6-(methyl(phenyl)amino)-3*H*-xanthen-9-yl)benzo-ate (Ph-5C-TMR)



$C_{30}H_{24}N_2O_5$
492.53 g/mol

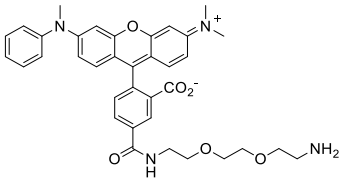
The starting material bromo-substituted 5-carboxy rhodamine **2** (500 mg, 1.07 mmol, 1.00 eq.), *N*-methylaniline (575 mg, 5.36 mmol, 5.00 eq.) and zinc (II) chloride (365 mg, 2.68 mmol, 2.50 eq.) were heated at 170°C for 4 h in a 10 mL round-bottom flask without stirring. After cooling to room temperature, adding concentrated hydrochloric acid (70 mL) resulted in a red solution. The solution was diluted with water (50 mL), whereby a purple solid precipitated as the product. The crude product was obtained by filtration. Column chromatography was used (gradient 10:1:0 to 5:1:0 to 50:10:1 $CHCl_3$:MeOH:H_2O) to obtain the purple product **6** in quantitative yield (530 mg, 1.07 mmol, quant.).

^{1}H-NMR (300 MHz, CD_3OD): δ = 8.80 (d, *J* = 1.6 Hz, 1H), 8.28 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.53 (t, *J* = 7.6, 2H), 7.40 (dd, *J* = 10.6 Hz, 7.6 Hz, 2H), 7.37 – 7.27 (m, 2H), 7.16 (d, *J* = 9.5 Hz, 1H), 7.12 – 6.98 (m, 2H), 6.91 (dd, *J* = 6.8 Hz, 2.4 Hz, 2H), 6.79 (dd, *J* = 9.3 Hz, 2.4 Hz, 1H), 3.54 (s, 3H), 3.28 (s, 6H).

^{13}C-NMR (126 MHz, CD_3OD): δ = 170.9, 169-8, 158.9, 158.8, 158.4, 157.9, 146.7, 139.3, 138.1, 136.0, 132.7, 132.4, 132.1, 131.9, 131.6, 130.4, 128.9, 127.8, 116.0, 115.3, 115.0, 114.7, 99.3, 97.5, 41.5, 40.9.

MS (HR-ESI, pos): meas. *m/z* = 493.1769, calc. *m/z* = 493.1758 for $C_{30}H_{25}N_2O_5$ $[M+H]^+$.

5-((2-(2-(2-Aminoethoxy)ethoxy)ethyl)carbamoyl) phenyl-trimethyl rhodamine (PhTMR)



$C_{36}H_{38}N_4O_6$
622.72 g/mol

A *Schlenk* flask was charged with rhodamine **6** (55.0 mg, 111 µmol, 1.00 eq.) and PyBOP (75.6 mg, 145 µmol, 1.30 eq.), evacuated and backfilled with argon (3×). Anhydrous DMF (10 mL) and DIPEA (25.3 µL, 145 µmol, 1.30 eq.) were added and the mixture was stirred for 1 h under argon atmosphere. Next, *tert*-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (33.3 mg, 223 µmol, 1.20 eq.) was dissolved in anhydrous *N, N*-dimethylformamide (5 mL) and added into the reaction mixture. The mixture was stirred at room temperature for 3 h. The solvent was evaporated under reduced pressure and 4 M hydrochloric acid in dioxane (2 mL) was added. The reaction mixture was stirred at room temperature for 3 h. The solvent was evaporated under reduced pressure and the crude product was purified by silica column chromatography ($CHCl_3$:MeOH:H_2O = 20:1:0 → 2:1:0.02). The product was obtained as violet solid (51.0 mg, 73%).

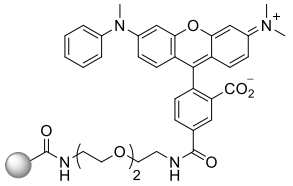
^{1}H NMR (500 MHz, CD_3OD): *δ* = 8.52 (d, *J* = 1.8 Hz, 1H), 8.09 (dd, *J* = 7.9, 1.8 Hz, 1H), 7.55 (t, *J* = 7.8 Hz, 2H), 7.46 – 7.37 (m 2H), 7.37 – 7.31 (m, 2H), 7.22 (d, *J* = 9.5 Hz, 1H), 7.14 (d, *J* = 9.4 Hz, 1H), 7.06 (dd, *J* = 9.5, 2.5 Hz, 1H), 6.95 (dd, *J* = 10.2, 2.5 Hz, 2H), 6.81 (dd, *J* = 9.4, 2.4 Hz, 1H), 3.77 – 3.71 (m, 8H), 3.66 (t, *J* = 5.6 Hz, 2H), 3.56 (s, 3H), 3.30 (s, 6H), 3.14 – 3.12 (m, 2H) ppm.

^{13}C NMR (126 MHz, CD_3OD): *δ* = 171.0, 169.2, 159.2, 159.0, 158.7, 153.1, 145.0*, 138.6*, 137.3, 134.6, 134.0, 132.5, 132.0, 131.6, 130.2, 129.1, 127.8, 127.8, 125.8, 117.3, 116.1, 115.5, 115.2, 99.1, 95.9*, 71.4, 71.4, 70.6, 67.9, 41.6, 40.9, 40.9, 40.7 ppm.

Note: Highlighted peaks (*) were only detected in HSQC or HMBC experiments.

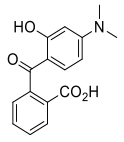
MS (HR-ESI, pos): meas. *m/z* = 623.2878, calc. *m/z* = 623.2864 for $C_{36}H_{39}N_4O_6$ $[M+H]^+$.

Immobilized PhTMR (3)



First, the dispersion of the NHS-activated Sepharose 4 Fast Flow resin (GE HEALTHCARE) in isopropanol (4 mL, 50% v/v) was centrifuged and the supernatant discarded. The resin was washed twice with 1 mM aqueous HCl (1 mL) and afterwards resuspended in HEPES buffer (1 mL, 100 mM, pH 7.4). Next, **PhTMR** (5.5 mg, 8.83 µmol) was dissolved in DMSO (100 µL) and diluted with HEPES buffer (1 mL, 100 mM, pH 7.4). The resin was incubated with the prepared solution of target **PhTMR** (1.1 mL, 8 mM) at 25°C for 18 h while shaking. The unreacted NHS groups were blocked by the addition of ethanolamine (42 µL, c_{final}=0.2 M). The functionalized resin was washed seven times with HEPES buffer (8 mL, 50 mM, pH 7.4). The yield (98%) of target loaded onto the resin was determined by absorption measurements of the washing solution. The final concentration of the immobilized PhTMR-PEG_2-NH_2 onto the Sepharose resin was 4.4 mM.

2-(4-(Dimethylamino)-2-hydroxybenzoyl)benzoic acid (13)

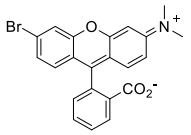


$C_{16}H_{15}NO_4$
285.30 g/mol

Phthalic anhydride (3.00 g, 20.3 mmol, 1.00 eq) and 3-dimethylaminophenol (2.78 g, 20.3 mmol, 1.00 eq) were dissolved in toluene (80 mL) and heated at reflux for 18 h. The solvent was removed under reduced pressure and the crude mixture was purified by silica column chromatography (cyclohexane:EtOAc = 5:1 → 2:1). The product was obtained as colorless solid (1.36 g, 24%). The recorded spectrum is in accordance with literature.[12]

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 12.48 (s, 1H), 8.13-8.08 (m, 1H), 7.64 (dt, *J* = 7.5, 1.1 Hz, 1H), 7.54 (dt, *J* = 7.5, 1.1 Hz, 1H), 7.40-7.33 (m, 1H), 6.91 (d, *J* = 9.2 Hz, 1H), 6.20 (s, 1H), 6.14-6.08 (m, 1H), 3.04 (s, 6H) ppm.

2-(6-Bromo-3-(dimethylimino)-3H-xanthen-9-yl)benzoate (14).



$C_{22}H_{16}BrNO_3$
422.28 g/mol

Benzophenone **13** (500 mg, 1.75 mmol, 1.00 eq.), 3-bromophenol (455 mg, 2.63 mmol, 1.50 eq) were dissolved in methanesulfonic acid (3 mL) and trifluoroacetic acid (3 mL) was added. The reaction was stirred at 80°C for 24 hours. The reaction mixture was allowed to cool down to room temperature und was poured into water (150 mL). The resulting

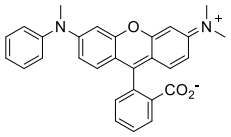
precipitate was isolated by filtration. The mother liquor was made basified with 1 M aqueous sodium hydroxide (10 mL) and extracted with chloroform (3 × 50 mL). The combined organic phases were concentrated under reduced pressure. The crude product was purified by recrystallisation in ethanol (50 mL) and the product was obtained as red solid (530 mg, 1.26 mmol, 72 %). The synthesis protocol was adapted from C.C. Woodroofe *et al.*[11]

^{1}H-NMR (300 MHz, $CDCl_3$): *δ* = 8.04-8.00 (m, 1H), 7.45 (d, *J* = 1.8 Hz, 1H), 7.18-7.09 (m, 2H), 6.68-6.59 (t, *J* = 8.4 Hz, 2H), 6.54 (d, *J* = 2.6 Hz, 1H), 6.45 (dd, *J* = 8.8, 2.6 Hz, 1H), 3.00 (s, 6H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): *δ* = 169.4, 153.0, 152.2, 152.1, 152.1, 135.0, 129.7, 129.4, 128.6, 126.8, 126.6, 125.0, 123.9, 123.6, 120.2, 118,6, 109.2, 105.6, 98.5, 83.1, 40.2 ppm.

MS (HR-ESI, pos): meas. *m/z* = 422.0391 calc. *m/z* = 422.0386 for $C_{22}H_{17}BrNO_3$ $[M+H]^+$.

Phenyl-trimethyl rhodamine (4)



$C_{29}H_{24}N_2O_3$
448.52 g/mol

A small round bottom flask was charged with **14** (50.0 mg, 118µmol, 1.00 eq), N-methylaniline (64.1 µL, 592 µmol, 5.00 eq), and $ZnCl_2$ (40.3 mg, 296 µmol, 2.50 eq). The mixture was stirred at 170 °C for 4 h. The crude material was purified by silica column chromatography ($CHCl_3$:MeOH = 20:1 → 10:1) and the product was obtained as violet solid (45.0 mg, 85 %).

^{1}H-NMR (500 MHz, $CDCl_3$): *δ* = 8.01 (d, *J* = 7.6 Hz, 1H), 7.68 - 7.62 (m, 1H), 7.62 - 7.56 (m, 1H), 7.40 - 7.33 (m, 2H), 7.23 - 7.18 (m, H), 7.17 - 7.12 (m, 1H), 6.69 (d, *J* = 2.2 Hz, 1H), 6.62 (d, *J* = 8.8 Hz, 1H), 6.58 (d, *J* = 8.8 Hz, 1H), 6.52 - 6.47 (m, 2H), 6.41 (dd, *J* = 8.9, 2.4 Hz, 1H), 3.34 (s, 3H), 2.98 (s, 6H) ppm.

^{13}C NMR (126 MHz, $CDCl_3$): *δ* = 196.6, 152.9, 152.8, 152.7, 152.2, 151.0, 147.7, 134.5, 129.6, 129.3, 128.8, 128.6, 127.6, 125.0, 124.9, 124.6, 124.1, 112.2, 109.4, 108.8, 102.3, 98.4, 87.1*, 40.2. ppm.

Note: Highlighted peaks (*) were only detected in HMBC experiments.

MS (HR-ESI, pos): meas. *m/z* = 449.1865 calc. *m/z* = 449.1860 for $C_{29}H_{25}N_2O_3$ $[M+H]^+$.

General procedure (GP1) for the synthesis of asymmetric rhodamine dyes using a zinc-mediated aromatic substitution reaction:

The following procedure is exemplary for the nucleophilic aromatic substitution reaction of the bromo-precursor **14** and arylamines to obtain asymmetric aryl-substituted rhodamines.

A small round bottom flask was charged with bromo-precursor **14** (1.00 eq), the arylamine (5.00 eq), and $ZnCl_2$ (2.50 eq). The mixture was stirred at 170 °C for 4 h and the crude material was purified by silica column chromatography.

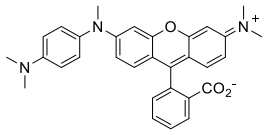
General Procedure (GP2) for the synthesis of asymmetric rhodamine dyes using a *Buchwald-Hartwig* reaction:

The following procedure is exemplary for the *Buchwald-Hartwig* coupling of the bromo-precursor **14** and arylamines to obtain asymmetric aryl-substituted rhodamines.

A reaction vessel was charged with bromo-precursor **14** (1.00 eq), arylamine (2.00 eq), Cs_2CO_3 (1.40 eq), $Pd_2(dba)_3$ (0.10 eq) and 2-dicyclohexyl-phosphino-2′,4′,6′-triisopropyl-biphenyl (XPhos, 0.30 eq), sealed, evacuated and backfilled with

argon (3×). The solids were dissolved in dry dioxane (50 mM solution of **14**) and the mixture was stirred at 100 °C for 18 h. The solvent was removed under reduced pressure and the crude mixture was purified by silica column chromatography.

(4-(Dimethylamino)phenyl)-trimethyl rhodamine (5)



$C_{31}H_{29}N_3O_3$
491.59 g/mol

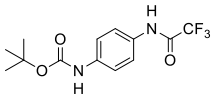
Rhodamine derivative **5** was synthesized from precursor **14** and N^1,N^1,N^4-trimethylbenzene-1,4-diamine according to general procedure **GP2**. The crude product was purified by silica column chromatography ($CHCl_3$:MeOH = 20:1 → 2:1) and the product was obtained as violet solid (54.0 mg, 93%).

^{1}H NMR (500 MHz, CD_3OD): δ = 8.12 (dd, J = 7.5, 1.5 Hz, 1H), 7.68 – 7-61 (m, 2H), 7.26 - 7.21 (m, 2 H), 7.14 - 7.09 (m, 3H), 6.99 (dd, J = 9.5, 2.5 Hz, 1H), 6.88 (d, J = 2.4 Hz, 1H), 6.83 - 6.87 (m, 2H), 6.82 (d, J = 2.4 Hz, 1H), 6.77 (dd, J = 9.4, 2.3 Hz, 1H), 3.49 (s, 3H), 3.26 (s, 6H), 2.99 (s, 6H) ppm.

^{13}C NMR (126 MHz, CD_3OD): δ = 172.7, 160.2, 159.1, 158.9, 158.8, 151.8, 139.8, 135.4, 135.2, 132.8, 132.4, 131.4, 131.1,131.0, 130.4, 128.3, 116.2, 115.4, 115.1, 115.1, 114.8, 98.7, 97.5, 41.9, 41.0, 40.9 ppm.

MS (HR-ESI, pos): meas. m/z = 492.2269, calc. m/z = 492.2282 for $C_{31}H_{30}N_3O_3$ $[M+H]^+$.

***tert*-Butyl (4-(2,2,2-trifluoroacetamido)phenyl)carbamate (15)**

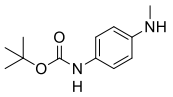


$C_{13}H_{15}F_3N_2O_3$
304.27 g/mol

Tert-butyl (4-aminophenyl) carbamate (1.00 g, 4.80 mmol, 1.00 eq) was dissolved in dry dichloromethane (40 mL) under argon atmosphere. The solution was cooled to -78 °C and trifluoroacetic anhydride (0.95 mL, 6.72 mmol, 1.40 eq) and triethylamine (1.86 mL, 13.4 mmol, 2.80 eq) were added dropwise. The reaction mixture was stirred at room temperature for 30 min. EtOAc (50 mL) was added and the mixture was washed with 1M HCl (30 mL) and brine (2 × 30 mL). The organic phase was dried over anhydrous magnesium sulfate, filtered and the solvent was removed under reduced pressure. The product was obtained as brown solid (1.38 g, 4.54 mmol, 95%). The recorded spectrum is in accordance with literature.[13]

^{1}H NMR (300 MHz, $(CD_3)_2CO$): δ = 8.49 (s, 1H), 7.65-7.56 (m, 4H), 1.48 (s, 9H) ppm.

***tert*-Butyl (4-(methylamino)phenyl)carbamate (16)**



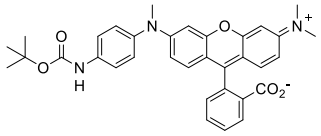
$C_{12}H_{18}N_2O_2$
222.29 g/mol

A *Schlenk* flask was charged with **15** (1.30 g, 4.27 mmol, 1.00 eq), methyl iodide (267 µL, 4.27 mmol, 1.00 eq) and KO^{t}Bu (479 mg, 4.27 mmol, 1.00 eq), evacuated and backfilled with argon (3×). The solids were dissolved in dry THF (70 mL) and

the reaction mixture was stirred at 50 °C for 5 min. Next, a solution of KO^{t}Bu (743 mg, 6.62 mmol, 1.55 eq) in dry THF (70 mL) was added dropwise over 1 h and the reaction was stirred at 50°C for additional 1 h. The solvent was removed under reduced pressure and the residue was resuspended in ethyl acetate (30 mL). The suspension was filtered, and the filtrate concentrated under reduced pressure. The crude product was purified by silica column chromatography (cyclohexane:EtOAc =10:1 → 2:1) and the product was obtained as brown oil (0.33 g, 1.47 mmol, 34%). The recorded spectrum is in accordance with literature.[13]

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.17 (d, *J* = 8.7 Hz, 2H), 6.62 (d, *J* = 8.7 Hz, 2H), 6.25 (s, 1H), 2.82 (s, 3H), 1.50 (s, 9H) ppm.

(4-((Tert-butoxycarbonyl)amino)phenyl)-trimethyl rhodamine (**17**)



$C_{34}H_{33}N_3O_5$
563.65 g/mol

Rhodamine derivative **17** was synthesized from precursor **14** and *tert*-butyl (4-(methylamino)phenyl)carbamate **(16)** according to general procedure **GP2**. The crude product was purified by silica column chromatography ($CHCl_3$:MeOH = 100:1 → 6:1) and the product was obtained as violet solid (32.0 mg, 48%).

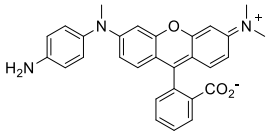
^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.02 (d, *J* = 7.6 Hz, 1H), 7.63 (dt, *J* = 7.5, 1.1 Hz, 1H), 7.57 (dt, *J* = 7.6, 1.0 Hz, 1H), 7.37 (d, *J* = 8.4 Hz, 2H), 7.17 (d, *J* = 7.5 Hz, 1H), 7.12-7.08 (m, 2H), 6.70 (s, 1H), 6.64 (d, *J* = 8.9 Hz, 1H), 6.59-6.54 (m, 2H), 6.47 (d, *J* = 2.5 Hz, 1H), 6.43-6.39 (m, 2H), 3.28 (s, 3H), 2.98 (s, 6H), 1.52 (s, 9H) ppm.

^{13}C NMR (126 MHz, $CDCl_3$): δ = 169.7, 153.5, 153.4, 153.0, 152.7, 151.9, 151.3, 142.6, 136.0, 134.4, 129.5, 129.2, 128.9, 128.2, 126.8, 125.6, 124.8, 120.2, 111.7, 109.4, 109.3, 107.6, 98.5, 93.4*,80.8, 40.6, 40.4, 28.5 ppm.

Note: Highlighted peaks (*) were only detected in HMBC experiments.

MS (HR-ESI, pos): meas. *m/z* = 546.2505, calc. *m/z* = 546.2493 for $C_{34}H_{33}N_3O_5$ $[M+H]^+$.

(4-Aminophenyl)-trimethyl rhodamine (**6**)



$C_{29}H_{25}N_3O_3$
463.54 g/mol

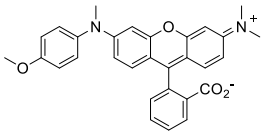
Rhodamine **15** (28.0 mg, 49.7 µmol, 1.00 eq) was dissolved in 4 M HCl in dioxane (993 µL, 3.97 mmol, 80.0 eq) and the mixture was stirred at room temperature for 1 h. The solution was removed under reduced pressure and the residue was purified by silica column chromatography ($CHCl_3$:MeOH = 10:1 → 3:1). The product was obtained as violet solid (23.0 mg, >99 %).

^{1}HNMR (500 MHz, $CDCl_3$): δ = 8.01 (d, *J* = 7.5 Hz, 1H), 7.62 (t, *J* = 7.5 Hz, 1H), 7.57 (t, *J* = 7.5 Hz, 1H), 7.17 (d, *J* = 7.5 Hz, 1H), 6.98 (d, *J* = 8.1 Hz, 2H), 6.69 (d, *J* = 8.1 Hz, 2H), 6.64 (dd, *J* = 9.1, 1.6 Hz, 1H), 6.55 (dd, *J* = 8.8, 1.6 Hz, 1H), 6.51 (d, *J* = 1.6 Hz, 1H), 6.47 (d, *J* = 1.6 Hz, 1H), 6.42 (d, *J* = 9.1 Hz, 1H), 6.35 (d, *J* = 8.8 Hz, 1H), 3.26 (s, 3H), 2.99 (s, 6H) ppm.

^{13}C NMR (126 MHz, $CDCl_3$): δ = 169.8, 153.5, 153.4, 152.7, 152.5, 151.6, 151.3, 144.8, 138.7, 134.4, 129.5, 129.2, 128.8, 128.3, 128.2, 125.6, 124.7, 116.4, 110.9, 109.3, 108.3, 107.7, 99.8, 98.5, 40.7, 40.5 ppm.

MS (HR-ESI, pos): meas. *m/z* = 464.1960, calc. *m/z* = 464.1969 for $C_{29}H_{25}N_3O_3$ $[M+H]^+$.

(4-Methoxyphenyl)-trimethyl rhodamine (**7**)



$C_{30}H_{26}N_2O_4$
478.5480 g/mol

Rhodamine derivative **7** was synthesized from precursor **14** and 4-methoxy-*N*-methylaniline according to general procedure **GP2**. The crude product was purified by silica column chromatography ($CHCl_3$:MeOH = 20:1 → 2:1) and the product was obtained as violet solid (54.0 mg, 95%).

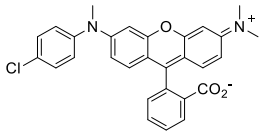
^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.00 (d, *J* = 7.5 Hz, 1H), 7.62 (dt, *J* = 7.4, 1.1 Hz, 1H), 7.57 (dt, *J* = 7.5, 0.9 Hz, 1H), 7.17 (d, *J* = 7.4 Hz, 1H), 7.12 (td, *J* = 8.9, 2.9 Hz, 2H), 6.91 (td, *J* = 8.9, 2.9 Hz, 2H), 6.62 (d, *J* = 8.9 Hz, 1H), 6.55 (d, *J* = 8.9 Hz, 1H), 6.53 (d, *J* = 2.2 Hz, 1H), 6.47 (d, *J* = 2.2 Hz, 2H), 6.40 (dd, *J*=8.9, 2.2 Hz, 1H), 6.36 (dd, *J* = 8.9, 2.2 Hz, 1H), 3.81 (s, 3H), 3.27 (s, 3H), 2.97 (s, 6H) ppm.

^{13}C NMR (126 MHz, $CDCl_3$): δ = 169.8, 157.6, 153.3, 153.1, 152.4, 152.2, 151.9, 140.6, 134.5, 129.4, 129.0, 128.7, 128.0, 128.0, 125.2, 124.5, 115.2, 110.8, 109.1, 108.4, 107.3, 100.3, 98.5, 89.8*, 55.6, 40.6, 40.4 ppm.

Note: Highlighted peaks (*) were only detected in HMBC experiments.

MS (HR-ESI, pos): meas. *m/z* = 479.1954, calc. *m/z* = 479.1965 for $C_{30}H_{26}N_2O_4$ $[M+H]^+$.

(4-Chlorophenyl)-trimethyl rhodamine (**8**)



$C_{29}H_{23}ClN_2O_3$
482.96 g/mol

Rhodamine derivative **8** was synthesized from precursor **14** and 4-chloro-*N*-methylaniline according to general procedure **GP1**. The crude product was purified by silica column chromatography ($CHCl_3$:MeOH = 30:1 → 7:1) and the product was obtained as violet solid (55.0 mg, 96%).

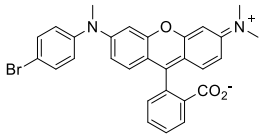
^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.00 (d, *J* = 7.4 Hz, 1H), 7.64 (dt, *J* = 7.5, 0.9 Hz, 1H), 7.59 (t, *J* = 7.4 Hz, 1H), 7.30 (td, *J* = 8.7, 2.5 Hz, 2H), 7.19 (d, *J* = 7.5 Hz, 1H), 7.09 (td, *J* = 8.7, 2.5 Hz, 2H), 6.69 (d, *J* = 2.4 Hz, 1H), 6.61 (d, *J* = 8.9 Hz, 1H), 6.58 (d, *J* = 8.8 Hz, 1H), 6.50 (dd, *J* = 8.8, 2.4 Hz, 1H), 6.46 (d, *J* = 2.5 Hz, 1H), 6.40 (dd, *J* = 8.9, 2.5 Hz, 1H), 3.30 (s, 3H), 2.97 (s, 6H) ppm.

^{13}C NMR (126 MHz, $CDCl_3$): δ = 169.8, 153.1, 153.0, 152.9, 152.3, 150.7, 146.6, 134.8, 129.8, 129.6, 129.4, 128.9, 128.9, 127.6, 125.7, 125.1, 124.2, 113.0, 110.4, 109.0, 106.8, 103.6, 98.6, 85.5*, 40.4, 40.4 ppm.

Note: Highlighted peaks (*) were only detected in HMBC experiments.

MS (HR-ESI, pos): meas. *m/z* = 483.1485, calc. *m/z* = 483.1470 for $C_{29}H_{23}ClN_2O_3$ $[M+H]^+$.

(4-Bromophenyl)-trimethyl rhodamine (**9**)



$C_{29}H_{23}BrN_2O_3$
527.42 g/mol

Rhodamine derivative **9** was synthesized from precursor **14** and 4-bromo-*N*-methylaniline according to general procedure **GP1**. The crude product was purified by silica column chromatography ($CHCl_3$:MeOH = 20:1) and the product was obtained as violet solid (40.0 mg, 64 %).

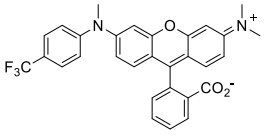
^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.02 (d, *J* = 7.5 Hz, 1H), 7.65 (dt, *J* = 7.4, 1.2 Hz, 1H), 7.59 (dt, *J* = 7.5, 1.0 Hz, 1H), 7.44 (m, 2H), 7.19 (d, *J* = 7.4 Hz, 1H), 7.02 - 7.04 (m, 2H), 6.71 (d, *J* = 2.4 Hz, 1H), 6.63 (d, *J* = 8.9 Hz, 1H), 6.61 (d, *J* = 8.8 Hz, 1H), 6.53 (dd, *J* = 8.8, 2.4 Hz, 1H), 6.47 (d, *J* = 2.5 Hz, 1H), 6.42 (dd, *J* = 8.9, 2.5 Hz, 1H), 3.31 (s, 3H), 2.98 (s, 6H) ppm.

^{13}C NMR (126 MHz, $CDCl_3$): δ = 169.7, 153.2, 153.1, 152.5, 152.3, 150.8, 147.0, 134.7, 132.8, 129.6, 129.1, 129.0, 127.8, 125.9, 125.4, 124.4, 117.0, 113.4, 110.9, 109.3, 107.1, 103.9, 98.6, 88.4*, 40.4 ppm.

Note: Highlighted peaks (*) were only detected in HMBC experiments.

MS (HR-ESI, pos): meas. *m/z* = 527.0982, calc. *m/z* = 527.0965 for $C_{29}H_{23}BrN_2O_3$ $[M+H]^+$.

(4-(Trifluoromethyl)phenyl)-trimethyl rhodamine (**10**)



$C_{30}H_{23}F_3N_2O_3$
516.52 g/mol

Rhodamine derivative **10** was synthesized from precursor **14** and 4-trifluoromethyl-*N*-methylaniline according to general procedure **GP2**. The crude product was purified by silica column chromatography ($CHCl_3$:MeOH = 100:1 → 20:1) and the product was obtained as violet solid (60.0 mg, 98%).

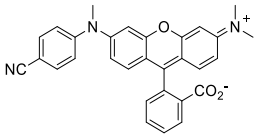
^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.02 (d, *J* = 7.6 Hz, 1H), 7.67 (dt, *J* = 7.6, 1.1 Hz, 1H), 7.62 (dt, *J* = 7.6, 0.9 Hz, 1H), 7.52 (d, *J* = 8.5 Hz, 2H), 7.22 (d, *J* = 7.6 Hz, 1H), 7.10 (d, *J* = 8.5 Hz, 2H), 6.94 (d, *J* = 2.1 Hz, 1H), 6.73-6.68 (m, 2H), 6.63 (d, *J* = 8.9 Hz, 1H), 6.49 (d, *J* = 2.6 Hz, 1H), 6.42 (dd, *J* = 8.9, 2.6 Hz, 1H), 3.37 (s, 3H), 2.98 (s, 6H) ppm.

^{13}C -NMR (500 MHz, $CDCl_3$): δ = 169.7, 153.0, 152.9, 152.8, 152.4, 150.9, 149.9, 134.9, 129.7, 129.2, 128.9, 127.5, 126.6 (q, $^3J_{C\text{-}F}$= 3.8 Hz, CH), 125.1, 124.6 (q, $^1J_{C\text{-}F}$= 264.3 Hz, CF_3), 124.2, 123.2 (q, $^2J_{C\text{-}F}$= 32.8 Hz, C_q), 119.6, 117.0, 113.6, 109.2, 108.6, 106.4, 98.6, 85.0*, 40.3, 40.2 ppm.

Note: Highlighted peaks (*) were only detected in HMBC experiments.

MS (HR-ESI, pos): meas. *m/z* = 517.1744, calc. *m/z* = 517.1734 for $C_{30}H_{23}F_3N_2O_3$ $[M+H]^+$.

(4-Cyanophenyl)-trimethyl rhodamine (**11**)



$C_{30}H_{23}N_3O_3$
473.53 g/mol

Rhodamine derivative **11** was synthesized from precursor **14** and 4-(methylamino)benzonitrile according to general procedure **GP2**. The crude product was purified by silica column chromatography ($CHCl_3$:MeOH = 100:1 → 30:1) and the product was obtained as violet solid (52.0 mg, 93%).

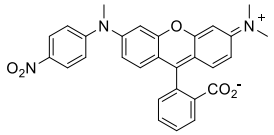
^{1}H NMR (500 MHz, $CDCl_3$): *δ* = 8.03 (d, *J* = 7.6 Hz, 1H), 7.69 (dt, *J* = 7.4, 1.1 Hz, 1H), 7.63 (dt, *J* = 7.6, 0.9 Hz, 1H), 7.47 (td, *J* = 9.0, 2.3 Hz, 2H), 7.23 (d, *J* = 7.4 Hz, 1H), 7.05 (d, *J* = 2.2 Hz, 1H), 6.92 (td, *J* = 9.0, 2.3 Hz, 2H), 6.80 (dd, *J* = 8.5, 2.2 Hz, 1H), 6.76 (d, *J* = 8.5 Hz, 1H), 6.63 (d, *J* = 8.9 Hz, 1H), 6.48 (d, *J* = 2.5 Hz, 1H), 6.43 (dd, *J* = 8.9, 2.5 Hz, 1H), 3.37 (s, 3H), 2.99 (s, 6H) ppm.

^{13}C NMR (126 MHz, $CDCl_3$): *δ* = 169.6, 153.0, 152.8*, 152.7, 152.4, 151.5, 148.9, 135.1, 133.5, 129.8, 129.6, 128.9, 127.3, 125.2, 124.2, 120.0, 119.7, 116.6, 116.1, 112.3, 109.3, 106.2, 101.8, 98.6, 84.1*, 40.4, 40.2 ppm.

Note: Highlighted peaks (*) were only detected in HMBC experiments.

MS (HR-ESI, pos): meas. *m/z* = 474.1816, calc. *m/z* = 474.1812 for $C_{30}H_{23}N_3O_3$ $[M+H]^+$.

(4-Nitrophenyl)-trimethyl rhodamine (**12**)



$C_{29}H_{23}N_3O_5$
493.52 g/mol

Rhodamine derivative **12** was synthesized from precursor **14** and 4-nitro-*N*-methylaniline according to general procedure **GP2**. The crude product was purified by silica column chromatography ($CHCl_3$:MeOH = 200:1 → 50:1) and the product was obtained as violet solid (53.0 mg, 91%).

^{1}H-NMR (500 MHz, $CDCl_3$): *δ* = 8.11- 8.07 (m, 2H), 8.02 (d, *J* = 7.6 Hz, 1H), 7.70 (dt, *J* = 7.6, 1.1 Hz, 1H), 7.64 (dt, *J* = 7.6, 0.8 Hz, 1H), 7.24 (d, *J* = 7.6 Hz, 1H), 7.12 (d, *J* = 2.1 Hz, 1H), 6.87-6.80 (m, 4H), 6.63 (d, *J* = 8.8 Hz, 1H), 6.49 (d, *J* = 2.4 Hz, 1H), 6.44 (dd, *J* = 8.8, 2.4 Hz, 1H), 3.42 (s, 3H), 2.99 (s, 6H) ppm.

^{13}C -NMR (126 MHz, $CDCl_3$): *δ* = 169.6, 153.3, 153.0, 152.9, 152.6, 152.4, 148.5, 139.5, 135.1, 129.9, 129.8, 128.9, 127.3, 125.9, 125.3, 124.2, 120.7, 117.2, 114.5, 113.6, 109.4, 106.1, 98.6, 83.9*, 40.6, 40.4 ppm

Note: Highlighted peaks (*) were only detected in HMBC experiments.

MS (HR-ESI, pos): meas. m/z = 494.1715, calc. m/z = 494.1710 for $C_{29}H_{23}N_3O_5$ $[M+H]^+$.

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