iScience, Volume *24*

Supplemental information

Kinetic analysis of Cas12a and Cas13a RNA-Guided nucleases for development of improved CRISPR-Based diagnostics

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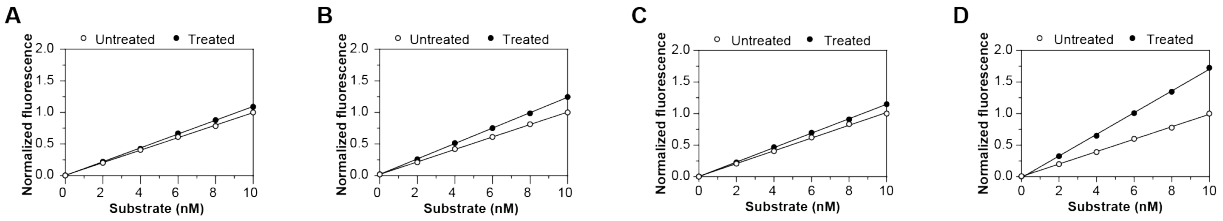

Figure S1. Effect of Nuclease Digestion on Fluorescence of Reporter Probes (Related to Figures 2 – 6)

RNA and DNA target and *trans*-substrates used in substrate-capture assay were digested with nucleases. Fluorescence of digested products was recorded and normalized to that of untreated material (symbols). Lines show linear fit yielding slopes listed in Supplement Table 2. RNase A-treatment of *trans*-substrate U_{10} (A) or Fluorescent Target RNA-1 (B). DNase I-treatment of *trans*-substrate C_{10} (C) or Fluorescent Target DNA-1 (D).

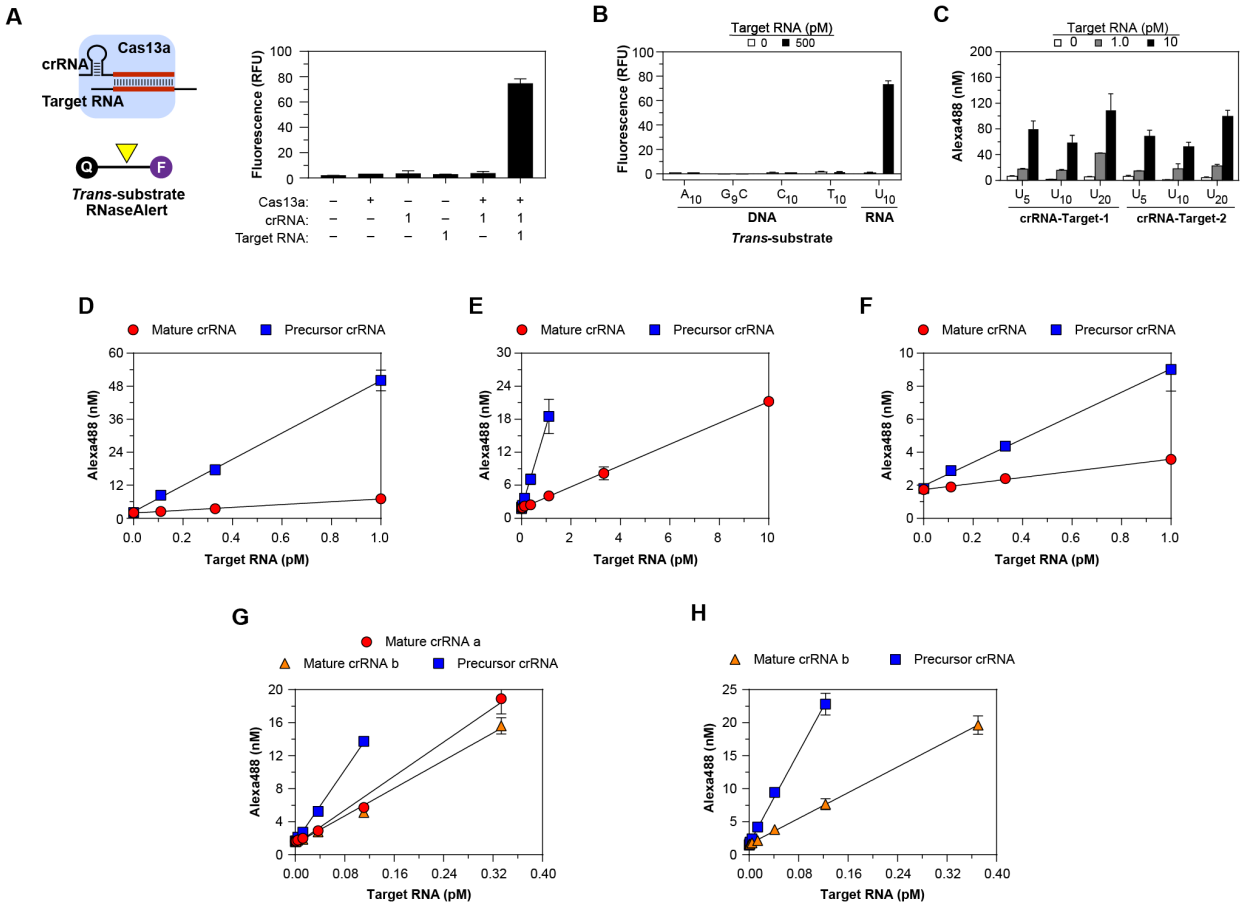

Figure S2. Cleavage of *Trans*-Substrates by Cas13a (Related to Figures 2 and 3)

(A) *Trans*-cleavage assay used to test components on RNaseAlert. Cas13a-crRNA was pre-reacted with target, then reacted with RNaseAlert. Cleavage of this substrate un-tethers fluorescein (F) from a quencher molecule (Q), resulting in increased fluorescence.

(B) Specificity for RNA *trans*-substrates tested with Cas13a, crRNA-3, Target RNA-3, and different Alexa488-labeled DNA or RNA *trans*-substrates.

(C) *Trans*-substrate length-preference tested with two different Cas13a-crRNA reacted with varied targets and poly-U *trans*-substrates of differing lengths.

(D – H) Cas13a bound to mature or precursor crRNA was reacted with varied target RNA and *trans*-substrate U_{10}. Lines show linear fit to yield k_{app} (Table S3) for: (D) mature or precursor crRNA-1, Target RNA-1-a, WT Cas13a; (E) mature or precursor crRNA-2, Target RNA-2, WT Cas13a; (F) mature or precursor crRNA-2, Target RNA-2-a, WT Cas13a; (G) mature crRNA-3-a or -b or precursor crRNA-3, Target RNA-3, WT Cas13a; (H) mature crRNA-3 b or precursor crRNA-3, Target RNA-3, K1082A Cas13a. In all panels data represent mean ± SD.

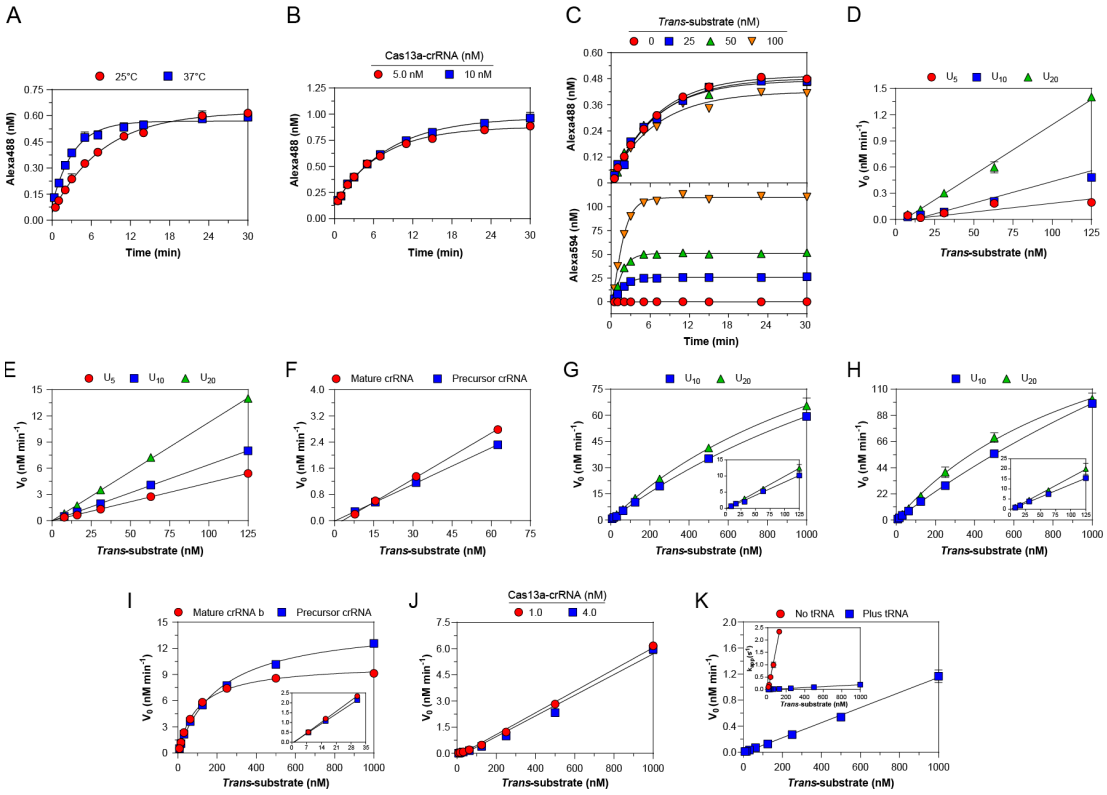

Figure S3. Kinetics of Target and *Trans*-Substrate Cleavage by Cas13a (Related to Figures 2 and 3)

(A) Single-turnover reactions carried out using 2.5 nM Cas13a-crRNA-1 reacted with 1.0 nM Fluorescent Target RNA-1 at varied temperature. Lines show single-exponential fit of control-adjusted signals (symbols) to yield k_{obs} (± SE) of 0.0021 (± 0.0001) s^{-1} (25°C) and 0.0051 (± 0.0005) s^{-1} (37°C). (B) Single-turnover reactions carried out using varied Cas13a-crRNA-1 reacted with 1.0 nM Fluorescent Target RNA-1 at 25°C and quenched with a denaturing solution containing guanidinium chloride and EDTA. Lines show single-exponential fit of control-adjusted signals (symbols) to yield k_{obs} (± SE) of 0.0024 (± 0.0002) s^{-1} and 0.0021 (± 0.0001) s^{-1} for 5.0 and 10 nM ribonucleoprotein, respectively. (C) Simultaneously-recorded single-turnover cleavage of target (*top*) and multiple-turnover cleavage of *trans*-substrate (*bottom*), carried out using 5.0 nM Cas13a-crRNA reacted with a mixture containing 1.0 nM of Fluorescent Target RNA-1a, a modified form of Fluorescent Target RNA-1 with additional U_{10} residues at its 5' end, and varied Alexa594-labeled *trans*-substrate U_{10}. Lines show single-exponential fit (where applicable) of control-adjusted signals (symbols) to yield k_{obs} ranging between 0.0024 – 0.0025 s^{-1} (target cleavage) and 0.010 – 0.011 s^{-1} (*trans*-cleavage). (D – F) Estimation of kinetic constants from data collected at low substrate (from Figures 3E, 3F, and 3I). Symbols (± SE) show V_0; lines show linear fit to yield estimates (Table 1 and Table S4). Cas13a was activated by: mature (D) and precursor (E) forms of crRNA-1 with Target RNA-1, then reacted with varied poly-U *trans*-substrates; (F) mature and precursor forms of crRNA-1 and Target RNA-1, then reacted with RNaseAlert. (G – I) Steady-state kinetics for *trans*-cleavage by Cas13 activated by a different crRNA-target combination. Cas13a-crRNA was pre-reacted with Target RNA-3, then reacted with varied *trans*-substrate. Symbols show V_0 (± SE); lines show Michaelis-Menten fit to yield kinetic constants (Table 1). Cas13a was bound to: mature b (G) and precursor (H) forms of crRNA-Target-3 reacted with poly-U *trans*-substrates; (I) mature b and precursor forms of crRNA-Target-3 reacted with RNaseAlert. (*Insets*) Linear fit at low substrate concentrations to yield estimates of kinetic constants (Table S4). (J) Steady-state kinetics for *trans*-cleavage using varied Cas13a-crRNA concentrations. Either 1.0 or 4.0 nM Cas13a-crRNA-1 was pre-reacted with Target RNA-1, then reacted with varied *trans*-substrate U_{10}. Results obtained with 1.0 nM Cas13a-crRNA are taken from Figure 3E and used in Table 1. Symbols show V_0 (± SE); lines show linear fit to yield a slope of 0.0059 (± 0.0003) min^{-1} allowing estimation for k_{cat}/K_M of 9.9 (± 0.5) x 10^6 M^{-1} s^{-1} (4.0 nM Cas13a-crRNA). (K) Effect of tRNA on Cas13a *trans*-nuclease. Cas13a-crRNA-1 was pre-reacted with Target RNA-1, then reacted with varied *trans*-substrate U_{20} in the presence of 100 µg/mL yeast tRNA. Symbols show V_0 (± SE); line shows linear fit to yield slope of 0.00116 (± 0.00002) min^{-1} and an estimate for k_{cat}/K_M of 1.94 (± 0.03) x 10^5 M^{-1} s^{-1}, ~100-fold lower than that obtained in the absence of tRNA. (*Inset*) Comparison of k_{app}. In panels (A, B, and D – K) data represent mean ± SD.

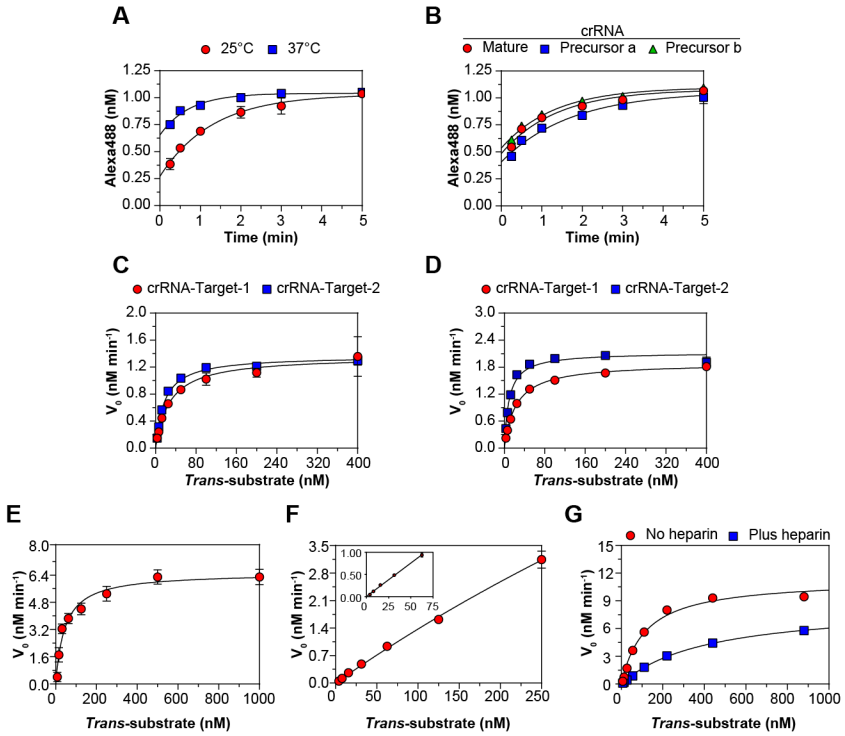

Figure S4. Kinetics of Target and *Trans*-Substrate Cleavage by Cas12a (Related to Figures 4 and 5)

(A) Single-turnover reactions carried out at different temperatures using 5.0 nM Cas12a-crRNA-1 reacted with 1.0 nM Fluorescent Target DNA-1. Lines show single-exponential fit of control-adjusted signals (symbols) to yield k_{obs} (± SE) of 0.013 (± 0.002) s^{-1} (25°C) and 0.023 (± 0.007) s^{-1} (37°C).

(B) Single-turnover reactions using 5.0 nM Cas12a bound to mature and precursor crRNA reacted with 1.0 nM Fluorescent Target DNA-1. Lines show single-exponential fit of control-adjusted signals (symbols) to yield k_{obs} (± SE): 0.012 (± 0.004) s^{-1} (mature crRNA); 0.010 (± 0.002) s^{-1} (precursor crRNA a); 0.013 (± 0.002) s^{-1} (precursor crRNA b).

(C and D) Steady-state kinetics of *trans*-cleavage activated by two different crRNA-target pairs, using 1.0 nM (C) or 4.0 nM (D) Cas12a-crRNA reacted with varied C_{20} concentrations. Symbols show V_0 (± SE) calculated from time courses; lines show Michaelis-Menten fit to yield kinetic constants. Results for reactions shown in (C) are listed in Table 1. Results for reactions shown in (D): k_{cat} = 3.15 (± 0.07) s^{-1}, K_M = 23.4 (± 0.8) nM, and k_{cat}/K_M = 1.35 (± 0.05) x 10^8 M^{-1} s^{-1} (crRNA-Target-1); k_{cat} = 3.55 (± 0.07) s^{-1}, K_M = 9.6 (± 1.3) nM, and k_{cat}/K_M = 3.70 (± 0.13) x 10^8 M^{-1} s^{-1} (crRNA-Target-2).

(E) Application of quenching method in measuring steady-state cleavage of DNaseAlert by Cas12a. Cleavage of DNaseAlert by Cas12a activated by crRNA-Target-1 as in Figure 5E except that after 1 min intervals for 5 min, reactions were quenched with the salt-EDTA quench solution, and fluorescence values were recorded. Symbols show V_0 (± SE) calculated from time courses; line shows Michaelis-Menten fit to yield K_M = 45 (± 8) nM, V_{max} = 6.5 (± 0.3) nM min^{-1}, k_{cat} of 1.05 (± 0.05) s^{-1} and k_{cat}/K_M of 2.4 (± 0.5) x 10^7 M^{-1} s^{-1}.

(F) Steady-state kinetics for *trans*-cleavage of C_{10} by Cas12a activated by crRNA-Target-3 carried out in the presence of 50 µg/mL heparin. Symbols show V_0 (± SE) calculated from time courses; line shows Michaelis-Menten fit to yield K_M of 1400 (± 500) nM, V_{max} of 21 (± 6) nM min^{-1}, k_{cat} of 3.5 (± 1.0) s^{-1} and k_{cat}/K_M = 2.5 (± 1.1) x 10^6 M^{-1} s^{-1}. (*Inset*) Linear fit of V_0 at low substrate concentrations to yield an estimate for k_{cat}/K_M of 2.50 (± 0.06) x 10^6 M^{-1} s^{-1}. Assay was performed in an alternative buffer composed of 5% glycerol, 20 mM Tris (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 100 µg/mL BSA.

(G) Steady-state kinetics for cleavage of DNaseAlert by Cas12a activated by crRNA-Target-6 was carried out in the absence or presence of 100 µg/mL heparin. Symbols show V_0 (± SE) calculated from time courses; lines show Michaelis-Menten fit to yield: K_M of 120 (± 20) nM, V_{max} of 11.3 (± 0.6) nM min^{-1}, k_{cat} of 1.89 (± 0.10) s^{-1}, and k_{cat}/K_M of 1.6 (± 0.3) x 10^7 M^{-1} s^{-1} (no heparin); K_M of 420 (± 30) nM, V_{max} of 8.6 (± 0.3) nM min^{-1}, k_{cat} of 1.43 (± 0.05) s^{-1}, and k_{cat}/K_M of 3.5 (± 0.3) x 10^6 M^{-1} s^{-1} (heparin). Assay was performed in an alternative buffer composed of 5% glycerol, 20 mM Tris (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 100 µg/mL BSA. In panels (A, B, and C – G) data represent mean ± SD.

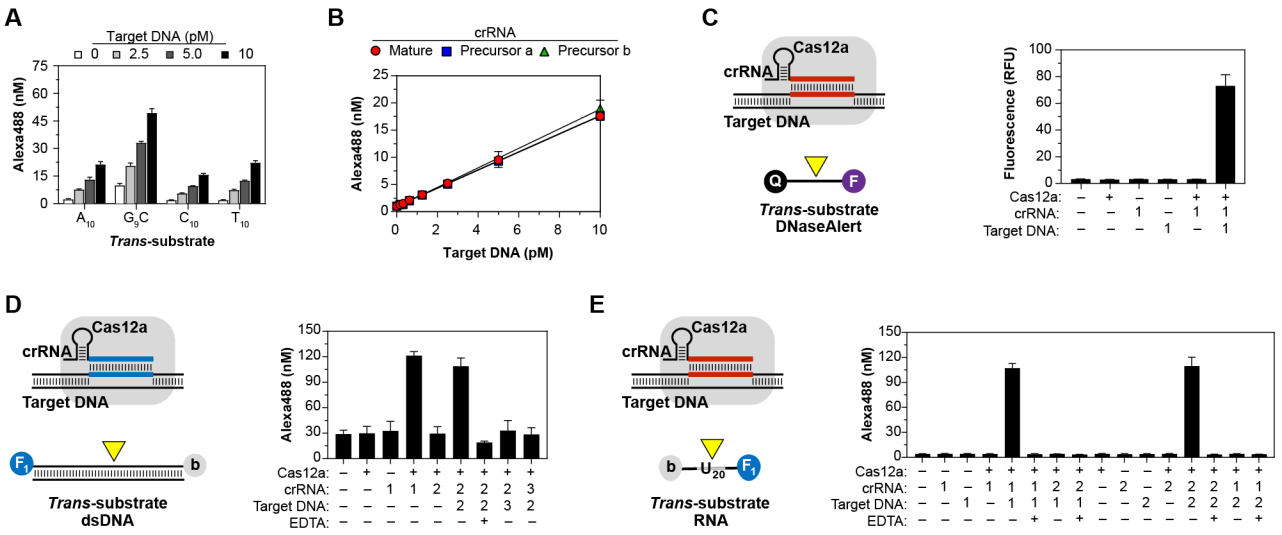

Figure S5. Cleavage of *Trans*-Substrates by Cas12a (Related to Figures 4 and 5)

(A) Nucleobase-preference for AsCas12a-crRNA-1 reacted with varied Target DNA-1 concentrations and *trans*-ssDNA substrate of differing sequence.

(B) Cas12a bound to mature or two precursor forms of crRNA-1 was reacted with varied Target DNA-1 and *trans*-substrate C_{10}. Lines show linear fitting to yield k_{app} (± SE): 0.922 (± 0.006) s^{-1} (mature crRNA-1); 0.922 (± 0.006) s^{-1} (precursor crRNA-1a); 0.994 (± 0.011) s^{-1} (precursor crRNA-1b).

(C) *Trans*-DNase assay using components tested on DNaseAlert. Cleavage increases fluorescence by untethering HEX (F) from a quencher (Q).

(D) *Trans*-dsDNA cleavage assay using fluorescent dsDNA *trans*-substrate labeled with biotin and Alexa488 at 3' and 5' ends, respectively, on one of its strands. Components tested under multi-turnover conditions on Fluorescent Target DNA-1 containing protospacer for crRNA-1 but not for crRNA-2 and -3.

(E) *Trans*-RNA cleavage assay using components tested on RNA substrate U_{20} labeled with biotin and Alexa488.

In all panels data represent mean ± SD.

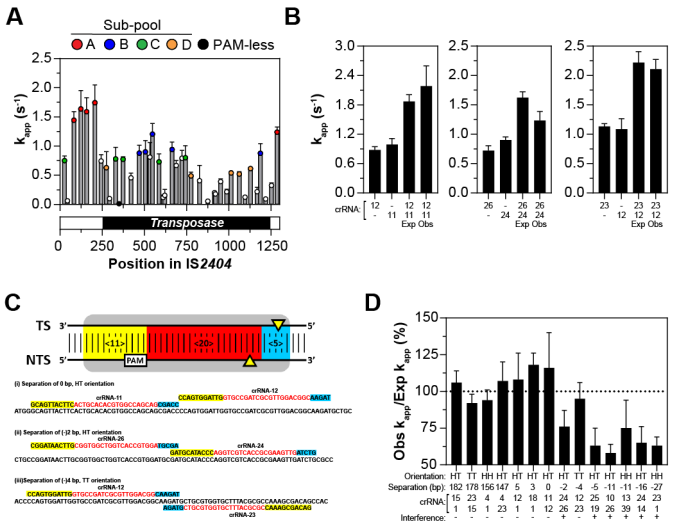

Figure S6. Selection of Cas12a crRNA Specific for *M. ulcerans IS2404* (Related to Figure 6)

(A) *Trans*-DNase activity of Cas12a bound to individual crRNA, then reacted with IS*2404* and *trans*-substrate C_{10} (taken from Figure 6E). Colors indicate activities of 20 individual crRNA selected for sub-pooling; open symbols show those omitted because of low activity or interference with other crRNA (see panel D). The black symbol shows activity of enzyme guided by crRNA-4 to a protospacer lacking an adjacent PAM. Positions of protospacers are numbered according to its most 5' base. The transposase open reading frame within IS*2404* is indicated by a black bar below the main plot.

(B) *Trans*-DNase activity of Cas12a bound with individual or combined (Obs) crRNA, then reacted with IS*2404* and *trans*-substrate C_{10}. The expected activity (Exp) is based on the sum of activities for the individual crRNA reactions.

(C) (Top) 35-bp footprint of crRNA-Cas12a in vicinity of the protospacer region based on exonuclease mapping of *F. novicida* Cas12a (Swarts et al., 2017). Protected sequences include 20-bp (red) of the protospacer, 11-bp (yellow) of the PAM-proximal region (the Head-end), and 8-bp (cyan) of the PAM-distal region (the Tail-end), and include the Cas12a target endonuclease sites (yellow triangles). (Bottom) Examples of closely targeted protospacer regions included in this analysis. Separation between sites is defined as the number of base pairs intervening footprints; negative values indicate the number of base pairs in footprint overlaps (underlined). Only the TS sequence is shown.

(D) *Trans*-DNase activity of Cas12a bound to combined crRNA reacted with IS*2404* and *trans*-substrate C_{10} performed as in (B). Relative orientation and separation of Cas12a-crRNA complexes are defined in (C). Interference between crRNA pairs, in which greater than 25% reduction in the expected activity is observed, is indicated by "+."

In all panels data represent mean ± SD.

Table S2. Effect of Nuclease Treatment on Fluorescence of Alexa488-Labeled Substrates (Related to Figures 2 – 6)

Substrate [a]	Calibration slope (RFU/nM) [b]		Effect of nuclease treatment on fluorescence [c]
	Untreated	Treated	
U_5	7700 (± 80)	8230 (± 70)	1.07 (± 0.01)
U_{10} [d]	6230 (± 70)	6880 (± 70)	1.10 (± 0.02)
U_{20}	7550 (± 140)	8160 (± 160)	1.08 (± 0.03)
Fluorescent Target RNA-1 [d]	6770 (± 50)	8370 (± 60)	1.24 (± 0.01)
TTATT	7330 (± 150)	7600 (± 200)	1.04 (± 0.03)
$(TTATT)_2$	5000 (± 50)	5730 (± 60)	1.15 (± 0.02)
$(TTATT)_4$	5610 (± 80)	6410 (± 70)	1.14 (± 0.02)
A_{10}	3220 (± 30)	2870 (± 60)	0.89 (± 0.02)
T_{10}	5500 (± 60)	5910 (± 40)	1.07 (± 0.01)
C_{10} [d]	6760 (± 110)	7640 (± 60)	1.13 (± 0.02)
G_9C	1070 (± 10)	7840 (± 170)	7.31 (± 0.18)
Fluorescent Target DNA-1 [d]	2246 (± 29)	3910 (± 60)	1.74 (± 0.03)

[a] See Table S1.

[b] Slope (± SE) of calibration line, as described in Figure S1.

[c] Ratio (± SE) of calibration slopes for treated to untreated.

[d] Representative plot of normalized fluorescence shown in Figure S1.

Table S3. Estimation of Kinetic Constants for Cas13a *Trans*-Cleavage from Endpoint Data (Related to Figure 3 and Table 1)

Enzyme	crRNA (form)	Target RNA	Figure	k_{app} (s^{-1}) [a]	Estimated k_{cat}/K_M (M^{-1} s^{-1}) [b]	Measured k_{cat}/K_M (M^{-1} s^{-1}) [c]
Cas13a WT	1 (mature)	1	3B	1.69 (± 0.01)	1.69 (± 0.01) x 10^7	1.03 (± 0.03) x 10^7
		1a	S2D	1.42 (± 0.04)	1.42 (± 0.04) x 10^7	NT [d]
	1 (precursor)	1	3B	15.8 (± 0.1)	1.58 (± 0.01) x 10^8	1.1 (± 0.2) x 10^8
		1a	S2D	13.2 (± 0.7)	1.33 (± 0.07) x 10^8	NT
	2 (mature)	2	S2E	0.538 (± 0.003)	5.38 (± 0.03) x 10^6	NT
		2a	S2F	0.512 (± 0.019)	5.12 (± 0.19) x 10^6	NT
	2 (precursor)	2	S2E	4.16 (± 0.04)	4.16 (± 0.04) x 10^7	NT
		2a	S2F	1.97 (± 0.06)	1.97 (± 0.06) x 10^7	NT
	3a (mature)	3	S2G	14.3 (± 0.6)	1.43 (± 0.06) x 10^8	NT
	3b (mature)	3	S2G	11.6 (± 0.4)	1.16 (± 0.04) x 10^8	1.43 (± 0.08) x 10^8
	3 (precursor)	3	S2G	30.3 (± 0.6)	3.03 (± 0.06) x 10^8	2.1 (± 0.2) x 10^8
Cas13a K1082A	1 (mature)	1	3C	1.214 (± 0.008)	1.21 (± 0.01) x 10^7	NT
	1 (precursor)	1	3C	22.5 (± 0.3)	2.25 (± 0.03) x 10^8	NT
	3b (mature)	3	S2H	13.54 (± 0.08)	1.35 (± 0.01) x 10^8	NT
	3 (precursor)	3	S2H	48.0 (± 0.8)	4.80 (± 0.08) x 10^8	NT

[a] Taken from slope (±SE) of dependence of product released from 100 nM U_{10} as a function of target RNA, accounting for assay duration, from figures indicated.

[b] Assuming K_M for *trans*-substrate >> 100 nM.

[c] Ratio k_{cat}/K_M based on steady-state analysis (Table 1).

[d] NT = not tested.

Table S4. Estimation of Kinetic Constants for Cas13a *Trans*-Nuclease Activity at Low Substrate Concentrations (Related to Figure 3 and Table 1)

crRNA (form)	Target RNA	Figure	*Trans*-substrate	Slope (min^{-1}) [a]	Estimated k_{cat}/K_M (M^{-1} s^{-1}) [b]	Measured k_{cat}/K_M (M^{-1} s^{-1}) [d]
1 (mature)	1	3E, S3D	U_5	0.00200 (± 0.00009)	3.3 (± 0.2) x 10^6 [c]	ND
			U_{10}	0.00622 (± 0.00015)	1.03 (± 0.03) x 10^7 [c]	ND
		S3D	U_{20}	0.0115 (± 0.0005)	1.92 (± 0.08) x 10^7	2.2 (± 0.2) x 10^7
		S3F	RNaseAlert	0.0471 (± 0.0007)	7.85 (± 0.12) x 10^7	9.2 (± 0.5) x 10^7
1 (precursor)	1	S3E	U_5	0.0436 (± 0.0002)	7.27 (± 0.03) x 10^7	7.9 (± 0.5) x 10^7
			U_{10}	0.0640 (± 0.0036)	1.07 (± 0.06) x 10^8	1.1 (± 0.2) x 10^8
			U_{20}	0.113 (± 0.001)	1.88 (± 0.17) x 10^8	2.1 (± 0.1) x 10^8
		S3F	RNaseAlert	0.0374 (± 0.0002)	6.24 (± 0.03) x 10^7	7.8 (± 0.5) x 10^7
3 (mature)	3	S3G	U_{10}	0.082 (± 0.003)	1.36 (± 0.05) x 10^8	1.43 (± 0.08) x 10^8
			U_{20}	0.0994 (± 0.0010)	1.66 (± 0.02) x 10^8	1.82 (± 0.07) x 10^8
		S3I	RNaseAlert	0.0786 (± 0.0016)	1.31 (± 0.03) x 10^8	1.7 (± 0.1) x 10^8
3 (precursor)	3	S5H	U_{10}	0.1247 (± 0.0014)	2.08 (± 0.02) x 10^8	2.1 (± 0.2) x 10^8
			U_{20}	0.1612 (± 0.0032)	2.68 (± 0.05) x 10^8	3.2 (± 0.4) x 10^8
		S3I	RNaseAlert	0.07078 (± 0.00018)	1.118 (± 0.003) x 10^8	1.1 (± 0.1) x 10^8

[a] Slope for linear range of dependence of product released as a function of substrate concentration in the presence of 10 pM target RNA, taken from figures indicated.

[b] Assuming K_M for *trans*-substrate >> highest point used to determine slope.

[c] Estimate used for Table 1.

[d] Calculated from k_{cat} and K_M based on steady-state Michaelis-Menten analysis (Table 1).

Table S5. Limits of Detection for Single- and Pooled crRNA Cas Reactions (Related to Figure 6)

Enzyme	*Trans*-substrate	Target	Number of crRNA	crRNA	LOD (fM) [b]	k_{app} (s^{-1}) [c]	Figure
Cas13a [a]	100 nM U_{10}	1	1	Mature crRNA-1	156 (± 10)	1.69 (± 0.01)	3B
			1	Precursor crRNA-1	18 (± 5)	15.8 (± 0.1)	
	100 nM U_{20}	Pool of 13 [a]	1	Precursor crRNA-3	160 (± 50)	3.47 (± 0.06)	6B
			13	Precursor crRNA pool [d]	29 (± 3)	31.7 (± 0.4)	
	100 nM RNaseAlert	Pool of 13 [a]	1	Precursor crRNA-3	100 (± 60)	NC [e]	
			13	Precursor crRNA pool [d]	15 (± 3)		
Cas12a [b]	10 nM C_{10}	1	1	Mature crRNA-1	56 (± 6)	0.64 (± 0.02)	5C
		IS*2404*	20	Mature crRNA pool [f]	0.31 (± 0.08)	14.4 (± 0.2)	6G

[a] Pool of 13 equimolar target RNA specified by each crRNA in pool.
[b] Calculated based on total target concentrations as described in Methods.
[c] Calculated from slope of dependence of product on target concentration, accounting for assay time.
[d] Pool of 13 equimolar crRNA-3 to -5, 1-2, 2-2, -6 to -10, 11-2, 12-2, and 13.
[e] NC = not calculated.
[f] Pool of 20 equimolar crRNA-1 to -3, -5, and -7 to -22.