Supplemental Figure 1. *Ribose*-**614** cleavage is unaffected by the addition of 10% complementary strand. Addition of equimolar amounts of the complement greatly lowers cleavage rate and plateau. (A) Cleavage profile for *ribose*-**614** (100 nM)(triangles), or *ribose*-**614** (115 nM) plus complementary strand (11.5 nM)(squares). (B) Cleavage profile for *ribose*-**614** (222 nM)(triangles), or *ribose*-**614** (200 nM) plus equimolar complementary strand (circles). Unless otherwise stated, all kinetics were performed under standard reaction conditions, namely 1M NaCl, 1 mM $MgCl_2$, 50 mM HEPES, 25°C, and errors in percent cleaved are $\leq \pm 2$ percentage points.

Supplemental Figure 2. Cleavage products do not affect *ribose*-**614** cleavage, and only minimally alter *ribose*-**library** cleavage. *Ribose*-**614** or *ribose*-**library** were incubated either alone (400 nM), or with the addition of the 28-nt product of **614** cleavage (400 nM), the 78-nt product of **614** cleavage (400 nM), or both (400 nM each). Data are fit to a single exponential curve. Errors in percent cleaved are $\leq \pm 2$ percentage points.

Supplemental Figure 3. Gel-purification of *ribose*-**614** at cleavage plateau results in additional cleaveage, indicating a fraction of **614** is folded into an inactive conformation. *Ribose*-**614** (222 nM) was allowed to self-cleave for 140 hours, nearing cleavage plateau (triangles, top curve), at which point half the sample was resolved with denaturing PAGE. The fraction of *ribose*-**614** remaining uncleaved at 140 hours was gel-purified, resuspended in reaction buffer (to a final concentration of 100 nM) and incubated for additional time (diamonds, bottom curve; percentage cleaved as a fraction of the label in the gel purified product, not of initial substrate). Errors in percent cleaved are $\leq \pm 2$ percentage points.

Supplemental Figure 4. Reheating *ribose*-**614** results in additional cleavage, indicating a fraction of *ribose*-**614** is folded into an inactive conformation. *Ribose*-**614** (100 nM) was allowed to react until it reached cleavage plateau (141 h), at which point half of the sample was denatured by heating to 96°C for 3 min, and slowly cooled to 23°C over 10 min. Errors in percent cleaved are $\leq \pm 2$ percentage points.

Supplemental Figure 5. *Ribose*-**614** cleavage rate is concentration dependent. (A) Complete time course for *ribose*-**614** at various concentrations. Rates are estimated based on a fit to a single first-order exponential equation. (B) Linear initial phase of time course. Rates are estimated based on the slope of best fit line.

Supplemental Figure 6. *Cat+ribose* competes with *ribose*-**614** for cleavage. *Ribose*-**614** (100 nM) was incubated with and without *cat+ribose* (100 nM). *Cat+ribose* is not catalytic and when incubated alone is not cleaved. Data are fit to a single first-order exponential equation.

Supplemental Figure 7. Various substrates can compete with *ribose*-**614** for self-cleavage. A nine fold excess of unlabeled *deoxyribose*-**lib62**, *deoxyribose*-**614**, *deoxyribose*-**614ΔC72T**, or *cat+deoxyribose* was added to radiolabeled *ribose*-**614** (33 nM) at time zero. Errors in percent cleaved are $\leq \pm 2$ percentage points.

Supplemental Figure 8. The *cis*-cleavage rate of *ribose*-**614** is not affected by excess competitors. Cleavage assays of *ribose*-**614** (at a low concentration (0.33 nM) so as to favor *cis*-cleavage) were performed with and without the addition of a 9-fold excess of *deoxyribose*-**614** or *deoxyribose*-**lib6lo2**. Data are fit to a single first-order exponential equation.

Supplemental Figure 9. Cleavage rates of three different substrates reaches saturation at high concentrations of *deoxyribose*-**614**. Substrate saturation experiments were conducted by incubating individual substrates (4 nM) with increasing amounts of *deoxyribose*-**614** enzyme. Various substrates are (A) *cat+ribose,* (B) *ribose*-**614**, and (C) *ribose*-**lib62**. Data were fit using a Michaelis-Menten model using Prism software. Initial rates are estimates based on fitting the data to an exponential equation, and therefore correct for cleavage plateaus. Primary data are in Supplemental Fig. 10.

Supplemental Figure 10. Primary data used to derive initial rates reported in Supplemental Fig. 9. Cleavage profile for cleavage of *cat+ribose* (A), *ribose*-**614** (B), and *ribose*-**lib62** (C) by increasing amounts of *deoxyribose*-**614**. Data are fit to a single first-order exponential equation.

Supplemental Figure 11. Single-turnover kinetics for *deoxyribose*-**614** cleaving *cat+ribose* (A) or *ribose*-**lib62** (B) at levels of *deoxyribose*-**614** below saturation. Data were fit to first-order exponential curve.

Supplemental Figure 12. Compound *deoxyribose*-**614** cleaves substrates with multiple turnover, and cleavage is unaffected by the slow cooling protocol. Substrate (*ribose*-**lib62** in (A), or *cat+ribose* in (B)) were incubated in 4-fold excess of *deoxyribose*-**614** (at 33 and 100 nM). The number of turnovers of substrate was calculated by multiplying the percent of substrate cleaved by the initial concentration of substrate and dividing by the concentration of *deoxyribose*-**614**. Substrates and *deoxyribose*-**614** were resuspended in reaction buffer, and combined either before (pre-mix) or immediately after (post-mix) heating to 96°C and slowly cooling to 23°C over 10 minutes.

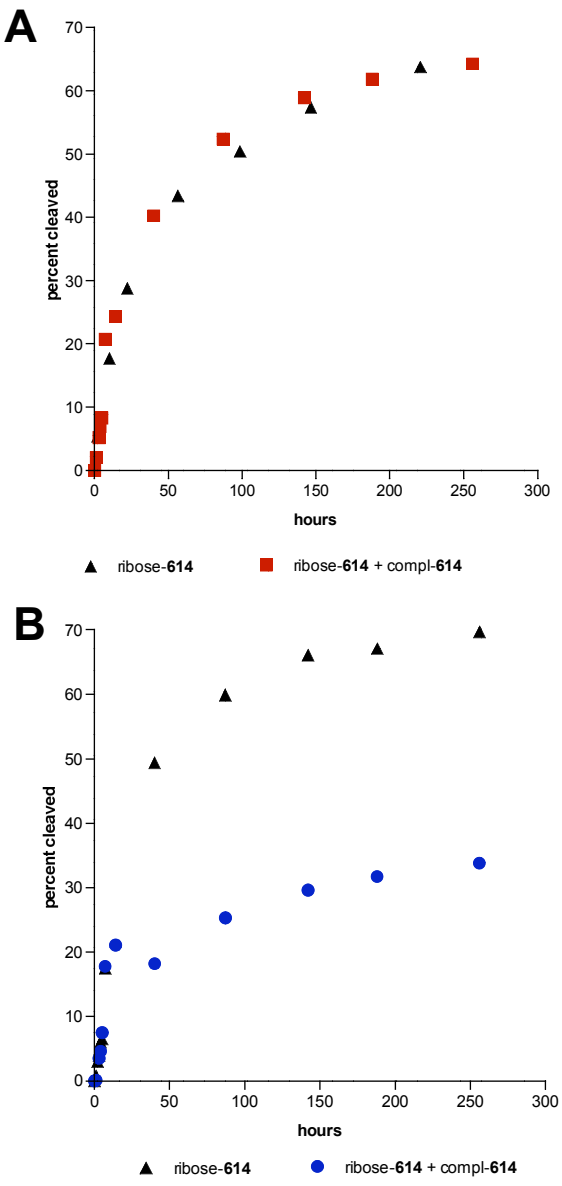
Supplemental Figure 13. The effect of the slow cooling protocol on intra-molecular and inter-molecular *ribose*-**614** cleavage rates. *Ribose*-**614** was incubated at high concentrations (200 nM) favoring *trans* cleavage (A), and low concentrations (2 nM) favoring *cis*-cleavage (B), with and without denaturing and folding using the slow cool protocol.

Supplemental Figure 14. The number of substrate turnovers remains linear beyond the initial turnover during multi-turnover kinetics of *deoxyribose*-**614** cleaving substrate at various concentrations. Compound *deoxyribose*-**614** (20 nM) was incubated with increasing concentrations of *cat+ribose* substate. The number of turnovers of *cat+ribose* was calculated by multiplying the percent of substrate cleaved by the initial concentration of substrate and dividing by the concentration of *deoxyribose*-**614**.

Supplemental Figure 15. The addition of a chase substrate reduces the residual *ribose*-**614** cleavage following the chase below that seen under predominately *cis*-cleavage conditions. The residual cleavage of *ribose*-**614** (200 nM) following the addition of chase (3 mM *deoxyribose*-**614**) was calculated by subtracting the time (1 h) and percent cleaved (ca. 3%) at the time the chase was added from the percent cleaved per time point for all subsequent time points. For comparison, data from *ribose*-**614** cleavage under predominately *cis*-cleaving conditions (1.1 nM) is plotted.

Supplemental Figure 16. The cleavage profile of *ribose*-**614** shows that *cis*-cleavage is unaffected by lowering the incubation temperature. *Ribose*-**614** was incubated at low concentration (2 nM, so as to favor *cis*-cleavage) at either 4°C or 25°C.

Supplemental Figure 17. The rate of *ribose*-**lib62** *trans*-cleavage by *deoxyribose*-**614** is increased at lower temperature. *Ribose*-**lib6lo2** substrate (1.5 nM) was incubated with *deoxyribose*-**614** (100 nM) at either 15°C or 25°C.



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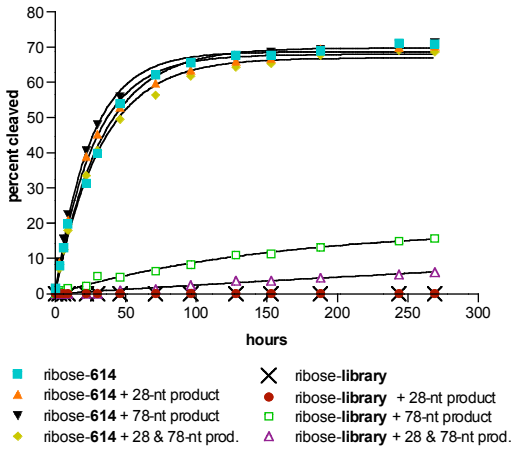

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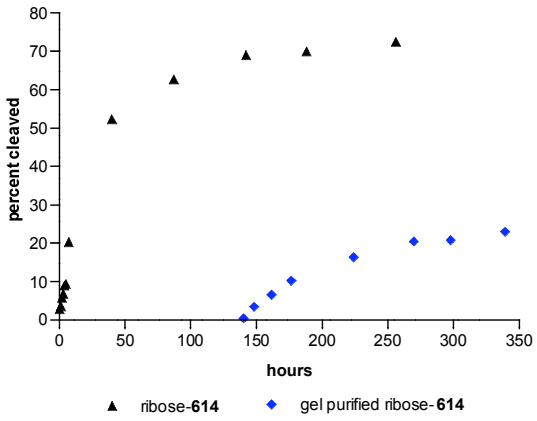

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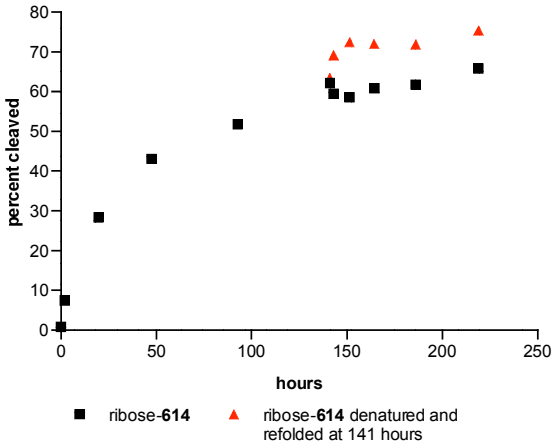

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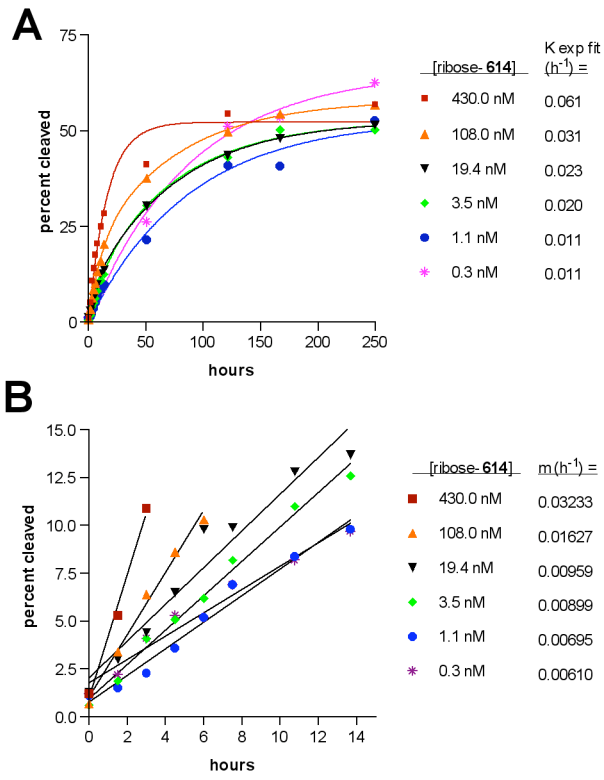

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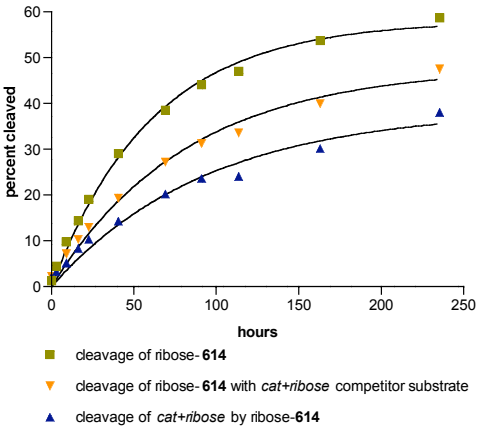

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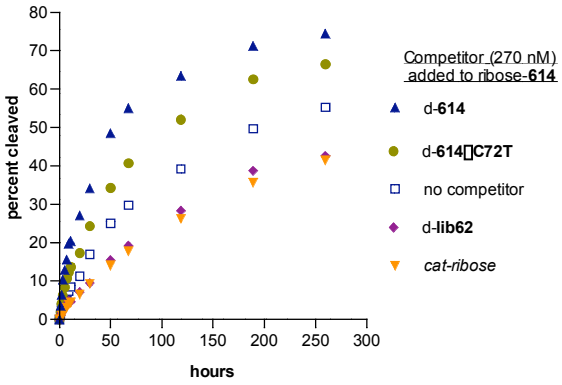

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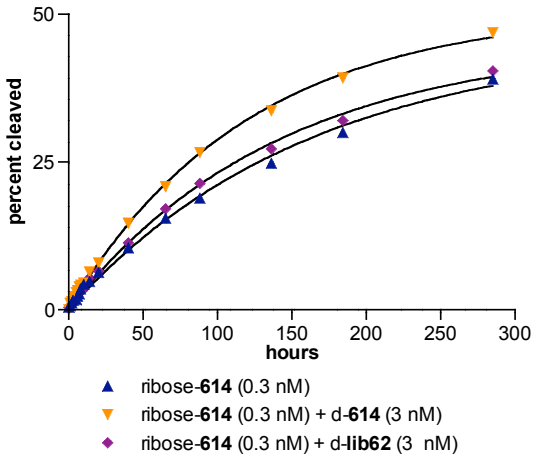

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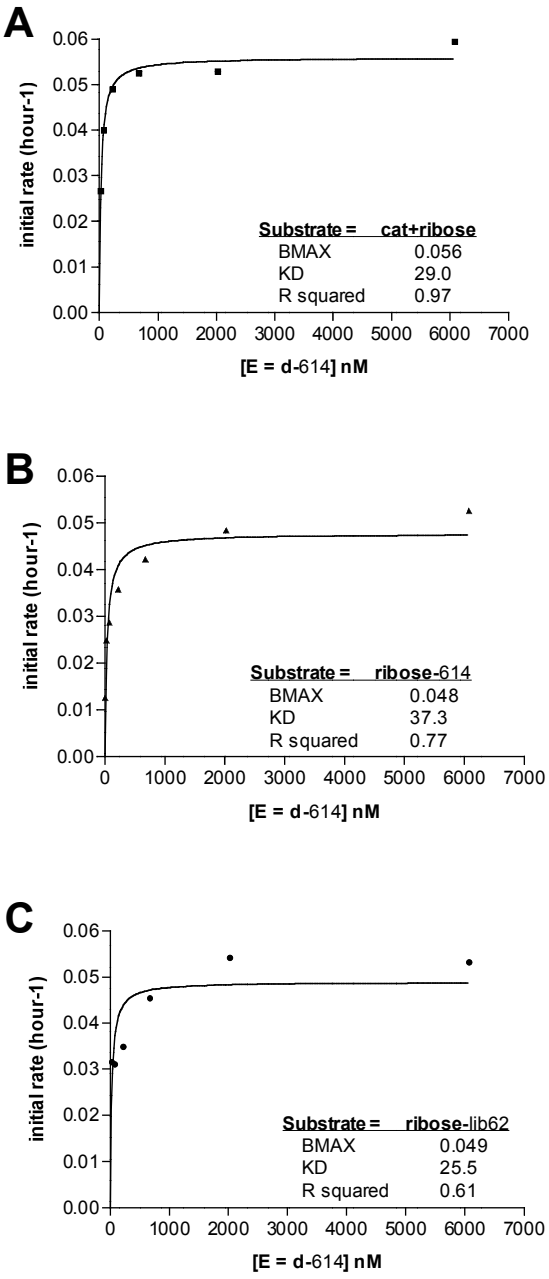

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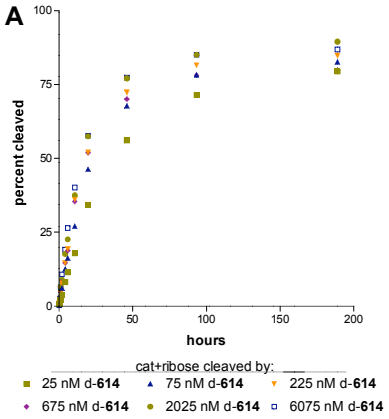

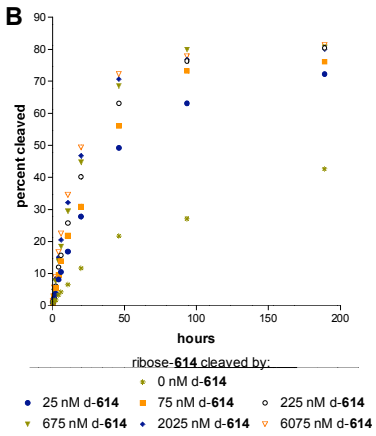

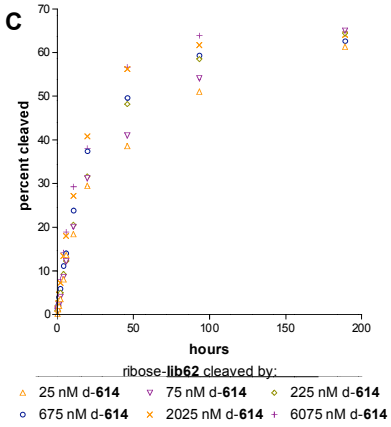

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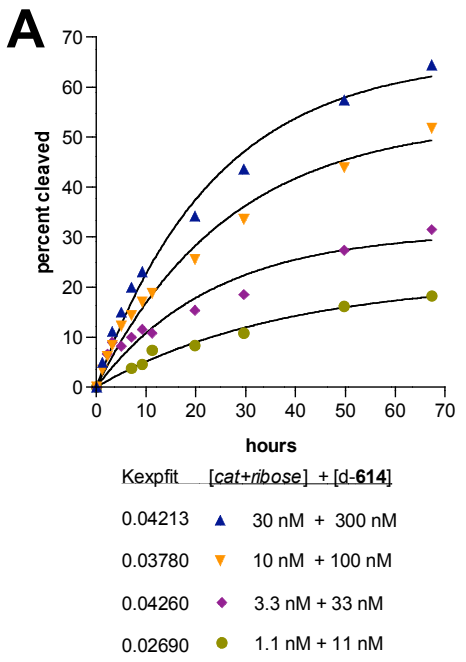

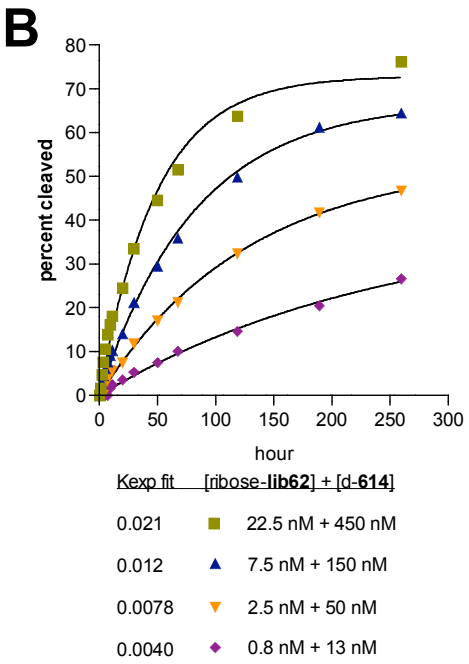

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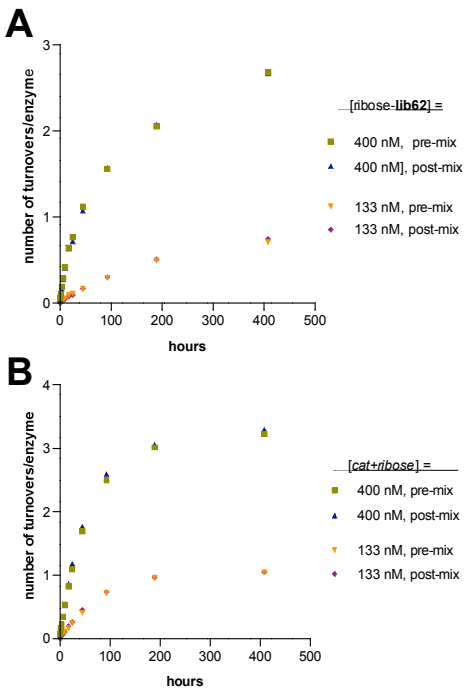

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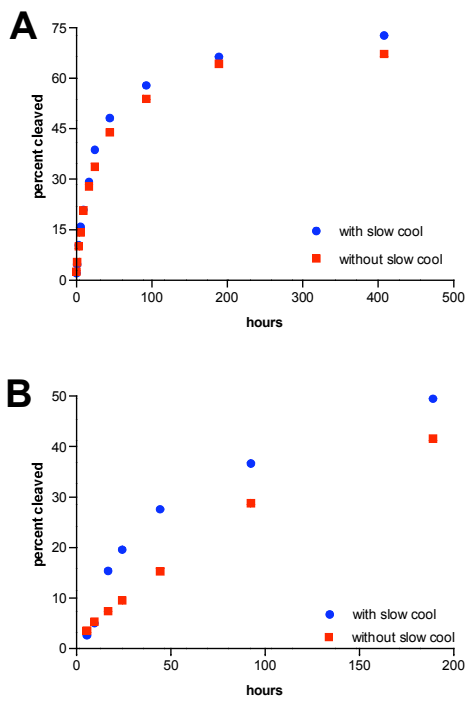

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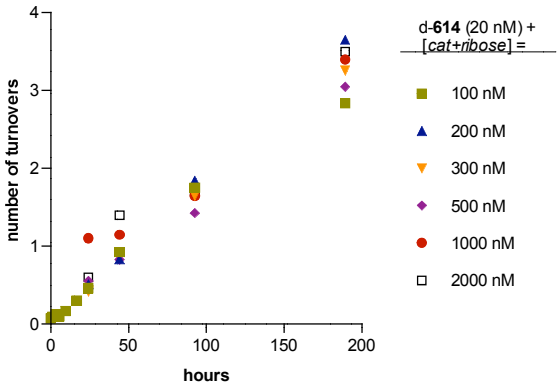

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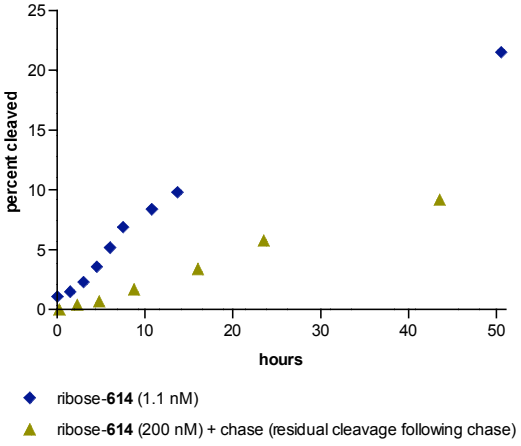

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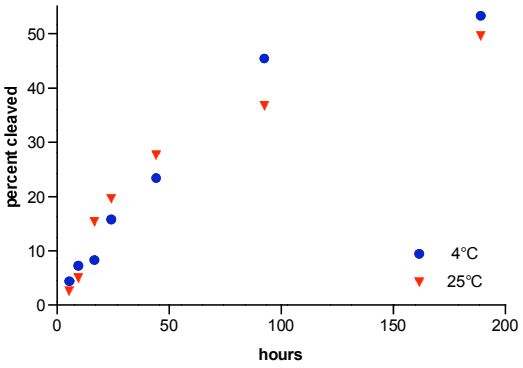

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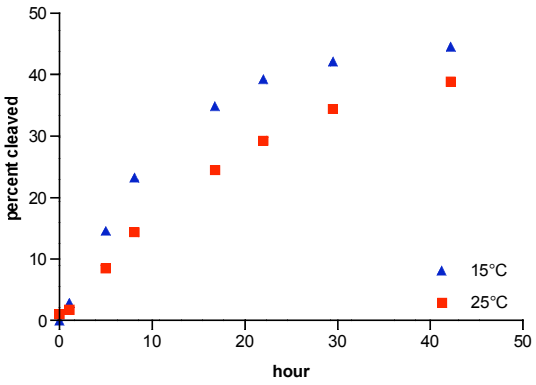

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