Extended Data Table 1. OD_{600} of *E. coli* cultures and relative copy number of plasmid (pINF or control pUC19) as determined by its molar ratio to pACS after 19 h of growth. **X** = **NaM** and **Y** = **5SICS**.

Extended Data Table 2. Relative quantification by LC-MS/MS using synthetic oligonucleotides containing d**5SICS** and d**NaM.**

Footnote for Extended Data Table 2:

*dA/d**5SICS** and dA/d**NaM** ratios were calculated assuming that randomized nucleotides (N) around the unnatural base are equally distributed.

Extended Data Table 3. Summary of the most successful extraction methods.

Footnotes for Extended Data Table 3:

*Recovery of all nucleotides (3P, 2P, 1P and nucleoside).

†Calculated as a ratio of 3P composition (%) before and after the extraction.

Extended Data Figure 1. a, Survey of reported substrate specificity **(**K_M, μM) of the NTTs assayed in this study. **b**, *Pt*NTT2 is significantly more active in the uptake of [α-^{32}P]-dATP compared to other nucleotide transporters. Raw (*left*) and processed (*right*) data are shown. Relative radioactivity corresponds to the total number of counts produced by each sample. Interestingly, both *Pam*NTT2 and *Pam*NTT5 also exhibit a measurable uptake of dATP although this activity was not reported before. This can possibly be explained by the fact that only competition experiments were carried out to characterize substrate specificity, and assay sensitivity might not have been adequate to detect this activity[15].

Extended Data Figure 2. Unnatural triphosphates (3P) of d**NaM** and d**5SICS** are degraded to diphosphates (2P), monophosphate (1P) and nucleoside (0P) in the growing bacterial culture. Potassium phosphate (KPi) significantly slows down the dephosphorylation of both unnatural triphosphates. **a**,

Representative HPLC traces and **b**, composition profiles are shown. d**NaM** and d**5SICS** nucleosides are eluted at approximately 40 min and not shown in panel **a**.

Extended Data Figure 3. a, KPi inhibits the uptake of [α-^{32}P]-dATP at concentrations above 100 mM. Raw (*left*) and processed (*right*) data from are shown. The NTT from *Rickettsia prowazekii* (*Rp*NTT2) does not mediate the uptake any of the dNTPs and was used as a negative control: its background signal was subtracted from those of *Pt*NTT2 (black bars) and *Tp*NTT2 (white bars). Relative radioactivity corresponds to the total number of counts produced by each sample. **b**, 50 mM KPi significantly stabilizes [α-^{32}P]-dATP in the media. Triphosphate stability in the media is not significantly affected by the nature of the NTT expressed. 3P, 2P and 1P correspond to triphosphate, diphosphate and monophosphate states, respectively. Errors represent s.d. of the mean, n=3.

Extended Data Figure 4. Growth curves and [α-^{32}P]-dATP uptake by bacterial cells transformed with pCDF-1b-*Pt*NTT2 (pACS) plasmid as a function of IPTG concentration. **a,** Total uptake of radioactive substrate (left) and total intracellular triphosphate content (right) are shown at two different time points. Relative radioactivity corresponds to the total number of counts produced by each sample. **b**, A stationary phase culture of C41(DE3)-pACS cells was diluted 100× into fresh 2×YT media containing 50 mM KPi, streptomycin, IPTG at the indicated concentrations and were grown at 37 °C. Errors represent s.d. of the mean, n=3.

Extended Data Figure 5. Stability and uptake of dATP in the presence of 50 mM KPi and 1 mM IPTG. Composition of [α-^{32}P]-dATP in the media (left) and cell lysate (right) as a function of time. TLC images and their quantifications are shown at the bottom and the top of each of the panels, respectively. 3P, 2P and 1P correspond to nucleoside triphosphate, diphosphate and monophosphate, respectively. M refers to a mixture of all three compounds that was used as a TLC standard. The position labeled “Start” corresponds to the position of sample spotting on the TLC plate.

Extended Data Figure 6. Calibration of the streptavidin shift (SAS) as a function of the fraction of template containing the UBP (**a**). Each sample was run in triplicate and representative data are shown in panel **b**. SA = streptavidin.

Extended Data Figure 7. a, Dephosphorylation of the unnatural nucleoside triphosphate and **b,** restriction analysis of pINF and pACS plasmids purified from *E. coli*, linearized with NdeI restriction endonuclease and separated on a 1% agarose gel. 3P, 2P, 1P and 0P correspond to triphosphate, diphosphate, monophosphate and nucleoside states, respectively. In panel **a**, the composition at the end of the 1 h recovery is shown at the right. In panel **b** (assembled from independent gel images), molar ratios of pINF/pACS plasmids are shown at the top of each lane. For each time point, triplicate data (average and s.d.) are shown in three lanes with untransformed control shown in the fourth, rightmost lane (see Methods). **c**, Number of pINF doublings as a function of time. The decrease starting at ~50 h is due to the loss of the pINF plasmid that also results in increased error. See section "pINF replication in *E. coli*" of the Methods for details. **d,** UBP retention (%) as a function of growth as determined by gel shift and Sanger sequencing (Fig. 3; sequencing traces are available as source data).