Supplementary information

Enzyme Cycling Method Using Hypoxanthine-Guanine Phosphoribosyltransferase: A Highly Sensitive Assay for Pyrophosphate

Shigeru Ueda,[ab] Yoshiaki Yasutake,[cd] Tatsuya Hirata,[e] Takehiko Sahara[f] and Shin-ichi Sakasegawa*[ef]

[a]*Japanese Committee for Clinical Laboratory Standards, Tokyo 101-0047, Japan.* [b]*Department of Health and Medical Sciences, Faculty of Risk and Crisis Management, Chiba Institute of Science, Choshi, 288-0025, Japan.* [c]*Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Sapporo 062-8517, Japan.* [d]*Computational Bio Big-Data Open Innovation Laboratory (CBBD-OIL), AIST, Tokyo 169-8555, Japan.* [e]*R&D group, Diagnostics Dept., Asahi Kasei Pharma Corporation, Izunokuni 410-2321, Japan.* [f]*Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8561, Japan.*

*Corresponding author. Shin-ichi Sakasegawa: E-mail, sakasegawa.sb@aist.go.jp.

Supplementary Table S1. Hypoxanthine-guanine phosphoribosyltransferase from *Hungateiclostridium thermocellum* (HtHGPRT) cycling reaction efficiency in comparison with the other enzyme cycling methods

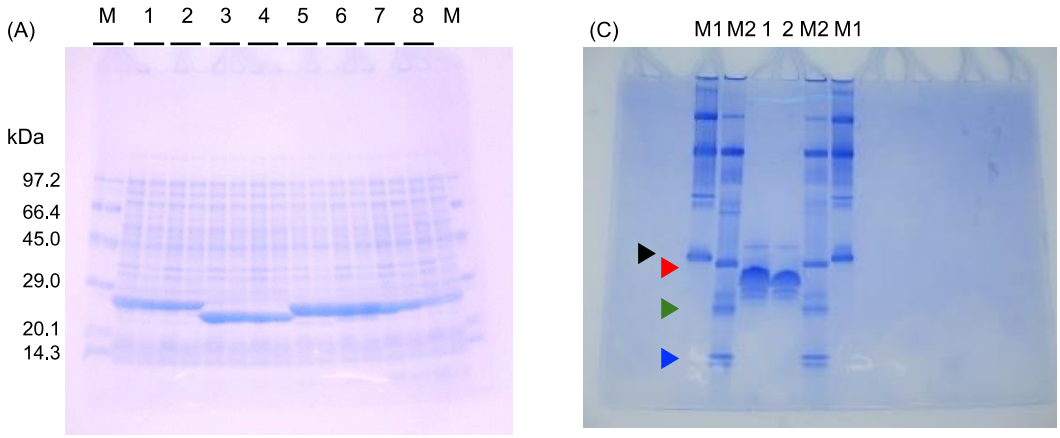
Mesured substrate	Enzyme	Enzyme Amount (U/mL)	k_c (min^{-1})	Ref.
PPi	HtHGPRT	1	60~	This study
Pi	Purine nucleoside kinase	1	2.5	(6)
Creatine	Creatine kinase	250	1.7	(10)
Pyruvate or Phosphoenolpyruvate	Pyruvate kinase	20	9	(11)
PPi	PPDK/NMNATase, DI/12α-HSD	10	7.2	(25)
NMN	DI/12α-HSD	~50	~20	(27)

PPDK, Pyruvate phosphate dikinase; NMNATase, nicotinamide mononucleotide adenylyltransferase; DI, diaphorase; 12α-HSD, 12α-hydroxysteroid dehydrogenase; NMN, nicotinamide mononucleotide.

Supplementary Table S2. Determination of pyrophosphate (PPi) concentrations in polymerase chain reaction (PCR) mixtures.

PCR cycle No.	PPi concentration (μM)		
	n = 1	n = 2	Average
5	0.0	0.0	0.0
10	0.0	0.0	0.0
15	8.7	9.1	8.9
20	26.0	30.0	28.0
30	218.5	232.1	225.3

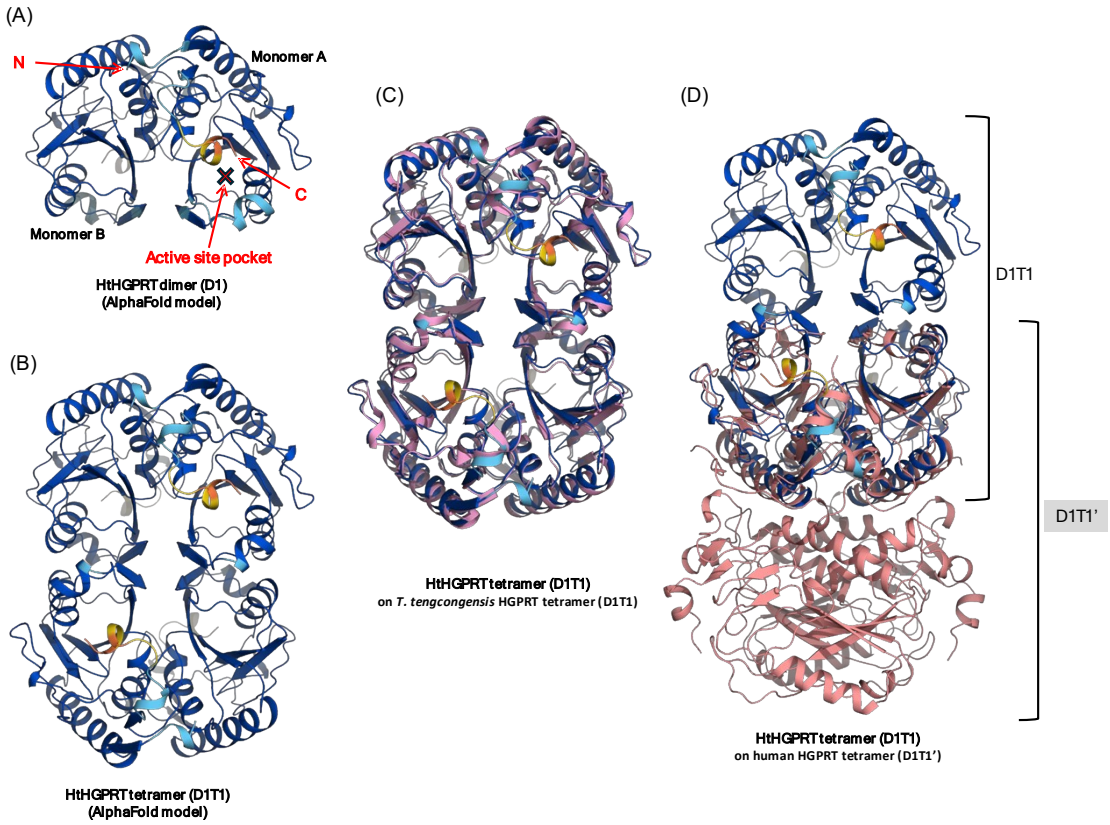
Fig. 5(A) is a plot of the averages presented in this table.



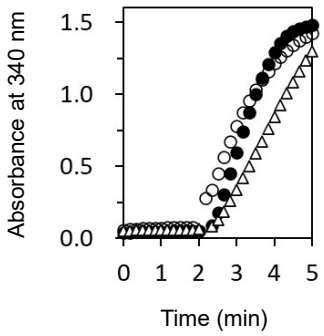
(B)

Enzyme	His-tag	Specific activity (U/mL)
AvHGPRT	N-terminus	0.37 ± 0.066
	C-terminus	0.27 ± 0.039
ChHGPRT	N-terminus	-0.04 ± 0.015
	C-terminus	-0.04 ± 0.019
HtHGPRT	N-terminus	1.52 ± 0.047
	C-terminus	1.43 ± 0.097
RmHGPRT	N-terminus	0.42 ± 0.019
	C-terminus	0.22 ± 0.043

Supplementary Fig. S1. Characterization of HGPRTs. (A) SDS-PAGE analysis of HGPRTs (n = 2). Lanes 5 and 6 correspond to HtHGPRT with His-tag added to the N-terminus and C-terminus, respectively. Approximately 5 µL of supernatant was SDS-treated and loaded onto a 5–20% gradient gel. All other lanes represent the following: lane 1, *Archaeoglobus veneficus* HGPRT (AvHGPRT) with N-terminal His-tag; lane 2, AvHGPRT with C-terminal His-tag; lane 3, *Carboxydothermus hydrogenoformans* HGPRT (ChHGPRT) with N-terminal His-tag; lane 4, ChHGPRT with C-terminal His-tag; lane 7, *Rhodothermus marinus* HGPRT (RmHGPRT) with N-terminal His-tag; and lane 8, RmHGPRT with C-terminal His-tag. M represents the molecular weight marker. (B) Specific activities of HGPRTs shown in (A) with either NAD or NADP as a cofactor. The HtHGPRT with N-terminal His-tag exhibited the highest activity and was selected for this study. (C) NATIVE-PAGE analysis of HtHGPRT with 5–20% gradient gel. Lanes 1 and 2 represent the 10 µL and 5 µL of the purified HtHGPRT, respectively. The molecular mass standard M1 (Cytiva HMW Native Marker) comprised tyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactase dehydrogenase (140 kDa), and albumin (66 kDa; indicated by black triangle). The molecular mass standard M2 (SERVA Native Marker Liquid Mix) comprised ferritin (720 kDa), urease (hexamer-545 kDa), ferritin (450 kDa), urease (trimer-272 kDa), lactate dehydrogenase (146 kDa), BSA (67 kDa; red triangle), ovalbumin (45 kDa; green triangle), and trypsin-Inhibitor (21 kDa; blue triangle). The results suggest that the HtHGPRT is most likely a homodimer.



Supplementary Fig. S2. 3D structural models of HtHGPRT homodimer and homotetramer. (A) AlphaFold model of HtHGPRT homodimer. The active site pocket, N-terminus, and C-terminus of monomer A are indicated by red arrows. The model is colored according to the per-residue confidence score (pLDDT) output by AlphaFold as follows: blue, pLDDT > 90 (high confident); cyan, 90 > pLDDT >70; yellow, 70 > pLDDT > 50; orange, pLDDT < 50 (low confident). (B) AlphaFold model of the HtHGPRT homotetramer. (C) Structural superimposition of the HtHGPRT homotetramer on the structurally most homologous *T. tengcongenesis* HGPRT homotetramer colored in pink (D1T1-type tetramer; PDB code, 1yfz) (see text). (D) Structural superimposition of the HtHGPRT homotetramer on the human HGPRT (D1T1′-type tetramer; PDB code, 1bzy), shown in salmon pink. The inter-dimer interface in the D1T1′ tetramer is positioned on the opposite side compared to that in the D1T1 tetramer.



Supplementary Fig. S3. pH dependency of the forward cycling reaction assessed using a U-3900/3900H spectrophotometer. The reaction mixture contained the following components: 40 mM Bicine-NaOH at three different pH values (8.0 [open circles], 8.25 [closed circles], and 8.5 [open triangles]), 2 mM nicotinamide adenine dinucleotide (NAD^+), 2 mM inosine 5'-monophosphate (IMP), 1 mM $MgCl_2$, 1.7 U/mL xanthine dehydrogenase (XDH), 0.14 mM guanine (Gua), and 0.6 U/mL of HtHGPRT. The reaction was initiated by adding 0.01 mL of 5 μM pyrophosphate (PPi) to 1 mL of the reagent mixture after a 2-min preincubation period.