

5 *Electronic Supplementary Information*

for

10 **Mouse Lactate Dehydrogenase X: A Promising Magnetic Resonance
Reporter Protein Using Hyperpolarized Pyruvic Acid Derivative Y**

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1. Supporting Figures

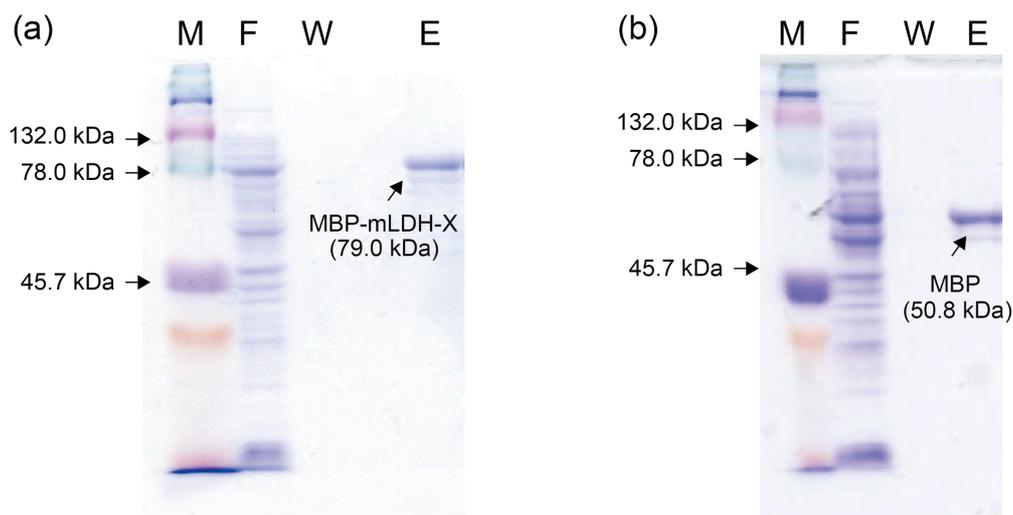


Fig. S1 SDS-PAGE analysis of (a) MBP-mLDH-X and (b) MBP purification using amylose resin. M: Marker proteins, F: Flow-through fraction, W: Wash fraction, E: Elution fraction.

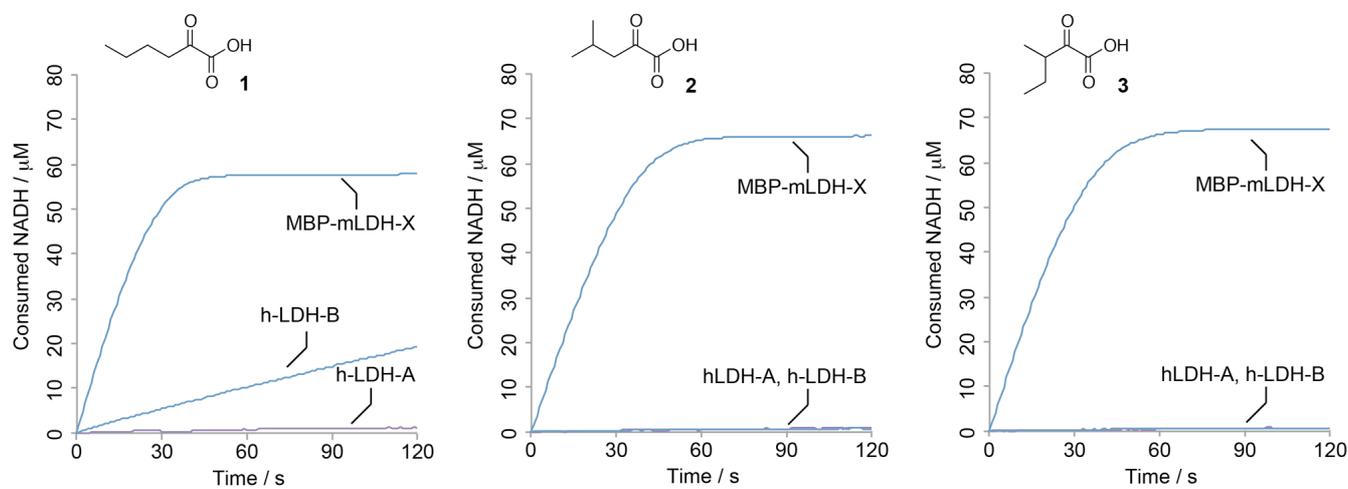


Fig. S2 Time course of substrate conversion from **1** (left), **2** (middle), or **3** (right) (200 μM) to each reductant in the presence of hLDH-A, hLDH-B, or MBP-mLDH-X (200 nM) in 100 mM potassium phosphate buffer (pH 7.5) containing NADH (100 μM).

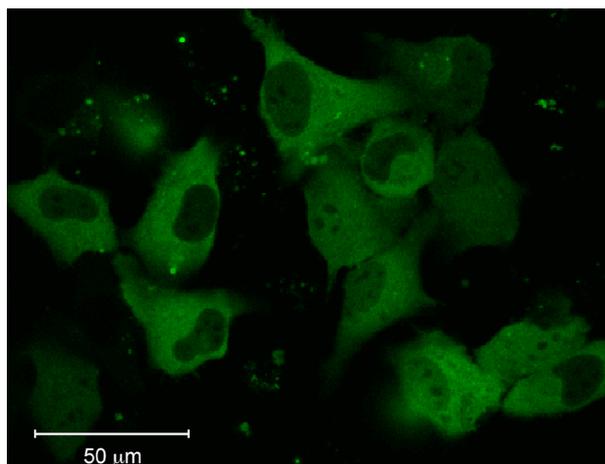


Fig. S3 Confocal fluorescence image of HeLa cells transfected with pAcGFP-mLDH-X.

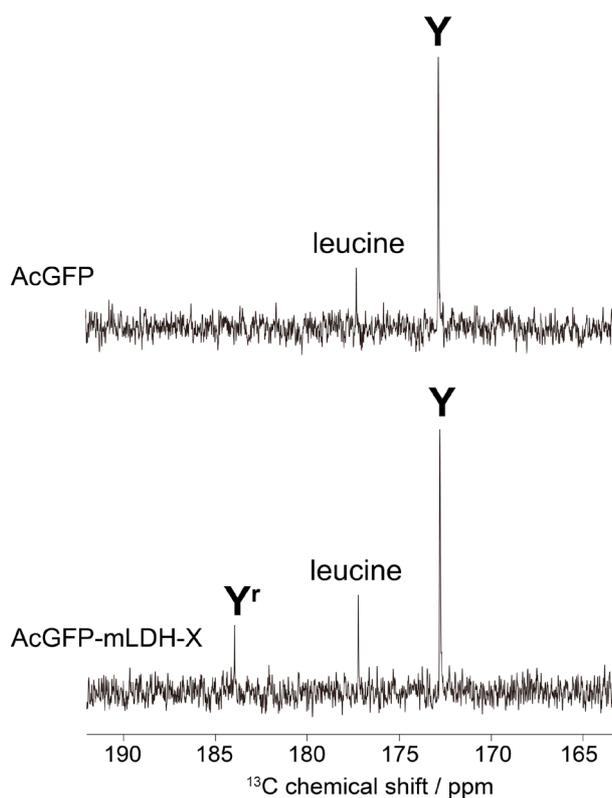


Fig. S4 ¹³C NMR measurement of **Y** to **Y^r** conversion by HeLa cells transfected with pAcGFP-mLDH-X (bottom) or pAcGFP (top). HeLa cells were incubated in DMEM with 10 mM **Y**. After 30 h of incubation, the supernatants were mixed with D₂O (10% v/v) and were subjected to ¹³C NMR measurements. The ¹³C NMR spectra were obtained using a 30° flip angle pulse (32 scans) with a Bruker AVANCE III (400 MHz, 9.4 T). Chemical shift of **Y** was set to 173 ppm.

2. Preparation of mLDH-X-fusion protein

Construction of maltose-binding protein (MBP)-mLDH-X plasmid. The full length cDNA clone of mLDH-X was amplified from pBluescript KS(+) encoding mLDH-X by PCR using primers designed to introduce restriction sites for *SphI* and *HindIII* at the 5' and 3' ends, respectively: forward, 5'-CCA TCG GCA TGC TCC ACC GTC AAG GAG-3' and reverse 5'-CCA TCG AAG CTT GCG AGT TTA TAA CTG CAG-3'. The PCR product was subcloned into *SphI*–*HindIII* sites of pQE-30 (QIAGEN) to yield pQE-30-mLDH-X. A *BamHI* and *HindIII* fragment of pQE-30-mLDH-X was inserted into the same digestion site of a pMAL-c2x vector (New England Biolabs) to yield pMAL-c2x-mLDH-X.

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Expression of MBP-mLDH-X. pMAL-c2x plasmid encoding MBP-mLDH-X was transferred to *E. coli* JM109. The cells were grown in 1 L of TB medium at 37 °C until the optical density at 600 nm increased to 0.5–0.6, and the cells were grown further at r.t. for 4 h with isopropyl- β -D(-)-thiogalactopyranoside induction (0.5 mM). The cells were centrifuged for 10 min at 4,400 rpm, resuspended in 40 mL of lysis buffer (phosphate-buffered saline (PBS), 10% glycerol), and lysed by sonication on ice. Insoluble materials were removed by centrifugation (4,400 rpm, 10 min \times 2) to collect the soluble fraction containing the MBP-mLDH-X. The soluble fraction (40 mL) was adsorbed onto 2 mL of amylose resin in a plastic column and then washed with 40 mL PBS. The resin-bound protein was eluted from the column with PBS containing 10 mM maltose. The fraction containing the purified MBP-mLDH-X (confirmed by SDS-PAGE) was dialyzed in PBS twice to remove the excess maltose. The concentration of MBP-mLDH-X was determined using a modified Lowry protein assay kit (Thermo Scientific). The solution of MBP-mLDH-X was stored at –20 °C.

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3. Synthesis

General. The reagents and solvents were purchased from standard suppliers and used without further purification. The NMR spectra were measured using a Bruker AVANCE III spectrometer (400 MHz, 9.4 T). Tetramethylsilane (0 ppm) was used as an internal standard. Mass spectra (MS) were
5 measured using a JEOL JMS-HX110A (FAB).

Synthesis of 2-(dimethylamino)-2-oxoacetic acid (6). Oxalyl chloride (0.52 mL, 6.0 mmol) was added to dimethylamine·HCl (0.16 g, 2.0 mmol) in CCl₄ (20 mL) and the mixture was stirred for 3 days at 65 °C under N₂. The solvent was removed *in vacuo*. The resulting residue was quenched by
10 addition of water. The solvent was again evaporated to dryness and the resulting residue was purified using silica gel column chromatography (eluent: chloroform:acetic acid = 100:1), followed by washing of impurity with chloroform to give 2-(dimethylamino)-2-oxoacetic acid (110 mg, yield = 47%): ¹H NMR (CDCl₃, 400 MHz) δ = 3.11 (s, 3H), 3.48 (s, 3H); ¹³C NMR (CD₃OD, 100 MHz) δ = 34.1, 37.5, 164.9, 165.8; HRMS (FAB): *m/z* calc. for C₄H₇O₃NNa⁺ [M+Na]⁺ = 140.0324, found = 140.0325.

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4. Cellular experiments

Construction of Ac-GFP-mLDH-X expression plasmid (pAcGFP-mLDH-X). The full length cDNA clone of mLDH-X was amplified from pBluescript KS (+) encoding mLDH-X by PCR using primers designed to introduce restriction sites for *Xho*I and *Hind*III at the 5' and 3' ends respectively: forward 5'-ATC GCT CGA GGA TCC ACC GTC AAG GGA G-3', and reverse 5'-CCA TCG AAG CTT GCG AGT TTA TAA CTG CAG-3'. The PCR product was subcloned into *Xho*I and *Hind*III sites of pAcGFP1-Hyg-C1 vector (Clontech).

Expression of AcGFP-mLDH-X in HeLa cell. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and penicillin-streptomycin-amphotericin B. The cells were plated in 35 mm glass based dishes (2.2×10^5 cells, fluorescence imaging) or in 10 cm dishes (1×10^6 cells, ^{13}C NMR measurement). After 24 h of incubation, the cells were transfected with pAcGFP-mLDH-X plasmid using HilyMax. Then, the cell cultures were incubated in a humidified atmosphere of 5% CO_2 at 37 °C.

Fluorescence imaging of HeLa cells transfected with pAcGFP-mLDH-X. After 36 h of incubation, fluorescence image was acquired with a confocal laser scanning microscope (LSM510 META, Carl Zeiss) using the 488 nm line of an argon laser for excitation and a 505 nm long-pass filter for emission (GFP) (Fig. S3). The specimen was viewed using water immersion objective ($\times 40$, 1.2 numerical aperture (NA), C-Apochromat, Carl Zeiss).

^{13}C NMR measurement of Y to Y^r conversion by HeLa cells transfected with pAcGFP-mLDH-X. After 24 h of incubation, the cells were harvested by trypsinization and were centrifuged for 2 min at 800 rcf. The 1×10^5 cells were plated in 24-well plate. The cells were cultured in DMEM with 10 mM Y for 30 h in a humidified atmosphere of 5% CO_2 at 37 °C. Then supernatants were mixed with D_2O (10% v/v) and were subjected to ^{13}C NMR measurements (Fig. S4). The ^{13}C NMR spectra were obtained using a 30° flip angle pulse (32 scans) with a Bruker AVANCE III (400 MHz, 9.4 T).

5. Enzymatic Assays

General information on enzymatic assays. Absorption spectra were measured by a Shimadzu spectrophotometer (UV-1650PC) at 37 °C. One molecule of cofactor NADH is consumed for the reduction of one molecule of substrate by LDH, therefore the enzymatic activity was determined by monitoring the absorbance change at 340 nm using an extinction coefficient of 6,220 M⁻¹ cm⁻¹ (NADH, 340 nm). All assays were performed in 100 mM potassium phosphate buffer, pH 7.5 (final reaction volume 1.5 mL). All reagents except pyruvic acid derivative **6** were purchased from standard suppliers and were used without further purification.

Substrate-specificity assay. The reaction mixture contained 115 μM NADH, 200 μM pyruvic acid derivatives **1–6**, and the enzyme LDH. The concentration of each LDH was set to provide an absorbance change of 0.05–0.075 per min at 340 nm for 200 μM pyruvic acid. The enzymatic activities were measured by the absorbance change at 340 nm. In these experiments, the net absorption change was calculated by subtracting the absorption change of the substrate-free enzymatic reaction solution as a background.

Determination of apparent kinetic parameters. The concentrations of MBP-mLDH-X and NADH were fixed at 75.6 nM (for pyruvic acid derivative **2**) or 18.9 nM (for pyruvic acid) and 100 μM, respectively. The substrate concentrations varied from 0.02 to 0.16 mM, and the initial rates were plotted against the substrate concentrations. Kinetic parameters were obtained using the Michaelis–Menten equation (1)

$$V_0 = k_{\text{cat}}[E]_0[S]/([S] + K_m), \quad (1)$$

where V_0 represents the initial rate of the mLDH-X reaction; k_{cat} and K_m represent the first-order rate constant from the catalyst–substrate complex and Michaelis–Menten constant, respectively; and $[E]_0$ and $[S]$ represent the initial concentration of MBP-mLDH-X and concentration of substrate, respectively. Standard deviation was determined by three independent experiments.

6. MTT assay

MTT assay. MTT assay was performed using the commercial kit (MTT Cell Proliferation Assay Kit, Cayman Chemical) according to the manufacture's procedure as follows. HeLa cells were seeded in a 96-well plate at a concentration of 3.0×10^4 cells/well in 100 μL of Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin-streptomycin-amphotericin B and various concentrations of 4-methyl-2-oxopentanoic acid (1, 10, or 50 mM). Then, the cell cultures were incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37 $^\circ\text{C}$. After the incubation period, 10 μL of the MTT labeling reagent (final concentration: 5 mg/mL) was added to each well. After incubating the 96-well plate for additional 3 h in a humidified atmosphere of 5% CO_2 at 37 $^\circ\text{C}$, the culture medium was aspirated from each well. 100 μL of the solubilization solution was added into each well. Finally, complete solubilization of the purple formazan crystals was checked and the absorbance to each sample at 570 nm was measured by using a microplate reader (SpectraMax 340PC³⁸⁴, Molecular Devices).

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