Journal of Visualized Experiments

Protocols for non-radioactive, in vitro, cardiac myosin light chain kinase assay

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61168R1
Full Title:	Protocols for non-radioactive, in vitro, cardiac myosin light chain kinase assay
Section/Category:	JoVE Biochemistry
Keywords:	MYLK3, MLC2v, Kinase activity, ATP, ADP, Phos-tag SDS-PAGE, photoluminescence
Corresponding Author:	Osamu Tsukamoto Osaka Daigaku Daigakuin Igakukei Kenkyuka Igakubu Suita, 565-0871 JAPAN
Corresponding Author's Institution:	Osaka Daigaku Daigakuin Igakukei Kenkyuka Igakubu
Corresponding Author E-Mail:	tsuka@medbio.med.osaka-u.ac.jp
Order of Authors:	Kenta Kamikubo
	Osamu Tsukamoto
	Yuki Uyama
	Ryohei Oya
	Tomoya Tsubota
	Noboru Fujino
	Yoshihiro Asano
	Hisakazu Kato
	Ken Matsuoka
	Seiji Takashima
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Suita, Osaka, Japan

March 1st, 2020

Journal of Visualized Experiments (JoVE)

Dear Chief Editor,

We would like to submit our revised manuscript entitled “Protocols for non-radioactive, in vitro, cardiac myosin light chain kinase assay” for publication in JoVE.

We would like to thank the editor and reviewers for their excellent comments. In addition to the response to the editor’s and reviewer’s comments, according to the great suggestions from Technical Editor, we have made some changes relevant sections in our revised manuscript and Figures. We believe that the manuscript has been substantially improved by responding to all of the comments.

All of the authors contributed to the work described in the paper and take responsibility for it. Moreover, none of the work described in the paper has been published elsewhere. We certify that there are no conflicts of interest with any financial organization regarding the material discussed in the manuscript.

The enclosed manuscript consists of text, 2 figures, supplemental table including material information, and point-by-point response to the editor’s and reviewer’s comments.
Figure 2 in revised figure was an improved one with Figure 4 and 5 in our previous report[1].

1. Hodatsu, A. *et al.* Impact of cardiac myosin light chain kinase gene mutation on development of dilated cardiomyopathy. *ESC Heart Failure*. **6** (2), 406–415, doi: 10.1002/ehf2.12410 (2019).

We sincerely hope that this revised manuscript is now acceptable for publication in JoVE.

Best wishes,

Osamu Tsukamoto
Department of Medical Biochemistry,
Osaka University Graduate School of Medicine
Address: 2-2 Yamadaoka, Suita, Osaka 565-0871, JAPAN
TEL: +81-6-6879-3492, FAX: +81-6-6879-3493,
E-mail: tsuka@medbio.med.osaka-u.ac.jp

TITLE:

Non-Radioactive In Vitro Cardiac Myosin Light Chain Kinase Assays

AUTHORS AND AFFILIATIONS:

*Kenta Kamikubo[1], *Osamu Tsukamoto[1,*]Yuki Uyama-Saito[1], Ryohei Oya[1], Tomoya Tsubota[1], Noboru Fujino[3], Yoshihiro Asano[2], Hisakazu Kato[1], Ken Matsuoka[1], Seiji Takashima[1]

[1]Department of Medical Biochemistry, Osaka University Graduate School of Medicine/Frontier Biosciences, Suita, Osaka, Japan
[2]Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine/Frontier Biosciences, Suita, Osaka, Japan
[3]Department of Cardiovascular and internal medicine, Kanazawa University Graduate School of Medicine, Kanazawa, Japan

*Three authors contributed equally to this manuscript.

Corresponding Author:
Osamu Tsukamoto (tsuka@medbio.med.osaka-u.ac.jp)

Email Addresses of Co-authors:
Kenta Kamikubo (kamikubo@fbs.osaka-u.ac.jp)
Yuki Uyama-Saito (uyamaaaa@fbs.osaka-u.ac.jp)
Ryohei Oya (roya@ent.med.osaka-u.ac.jp)
Tomoya Tsubota (tomoy4n@fbs.osaka-u.ac.jp)
Noboru Fujino (nfujino@mhs.mp.kanazawa-u.ac.jp)
Yoshihiro Asano (asano@cardiology.med.osaka-u.ac.jp)
Ken Matsuoka (kmatsuoka@medbio.med.osaka-u.ac.jp)
Hisakazu Kato (katohisa@medbio.med.osaka-u.ac.jp)
Seiji Takashima (takasima@cardiology.med.osaka-u.ac.jp)

Keywords:
MYLK3, *MLC2v*, kinase activity, ATP, ADP, phosphate-affinity SDS-PAGE, photoluminescence

SUMMARY:
This study describes protocols for nonradiometric methods, a bioluminescent ADP detection assay and a phosphate-affinity SDS-PAGE, to determine the kinase activity of cardiac myosin light chain kinase (cMLCK) and the phosphorylation level of its substrate, myosin regulatory light chain (MLC2v).

ABSTRACT:
Cardiac-specific myosin regulatory light chain kinase (cMLCK) regulates cardiac sarcomere structure and contractility by phosphorylating the ventricular isoform of the myosin regulatory light chain (MLC2v). MLC2v phosphorylation levels are significantly reduced in failing hearts, indicating the clinical importance of assessing the activity of cMLCK and the phosphorylation level

of MLC2v to elucidate the pathogenesis of heart failure. This paper describes nonradioactive methods to assess both the activity of cMLCK and MLC2v phosphorylation levels. In vitro kinase reactions are performed using recombinant cMLCK with recombinant calmodulin and MLC2v in the presence of ATP and calcium at 25 °C, which are followed by either a bioluminescent ADP detection assay or a phosphate-affinity SDS-PAGE. In the representative study, the bioluminescent ADP detection assay showed a strict linear increase of the signal at cMLCK concentrations between 1.25 nM to 25 nM. Phosphate-affinity SDS-PAGE also showed a linear increase of phosphorylated MLC2v in the same cMLCK concentration range. Next, the time-dependency of the reactions was examined at the concentration of 5 nM cMLCK. A bioluminescent ADP detection assay showed a linear increase in the signal during 90 min of the reaction. Similarly, phosphate-affinity SDS-PAGE showed a time-dependent increase of phosphorylated MLC2v. The biochemical parameters of cMLCK for MLC2v were determined by a Michaelis-Menten plot using the bioluminescent ADP detection assay. The V_{max} was 1.65 ± 0.10 mol/min/mol kinase and the average K_m was around 0.5 USA μM at 25 °C. Next, the activity of wild type and the dilated cardiomyopathy-associated p.Pro639Valfs*15 mutant cMLCK were measured. The bioluminescent ADP detection assay and phosphate-affinity SDS-PAGE correctly detected defects in cMLCK activity and MLC2v phosphorylation, respectively. In conclusion, a combination of the bioluminescent ADP detection assay and the phosphate-affinity SDS-PAGE is a simple, accurate, safe, low-cost, and flexible method to measure cMLCK activity and the phosphorylation level of MLC2v.

INTRODUCTION:

The cardiac-specific myosin regulatory light chain kinase (cMLCK) encoded by the *MYLK3* gene is the kinase predominantly responsible for maintaining the phosphorylation of cardiac ventricular myosin regulatory light chain 2 (MLC2v)[1,2]. By phosphorylating MLC2v at Ser-15, cMLCK promotes sarcomere organization[1] and potentiates cardiac contractility[2,3] as a result of increasing cross-bridge formation and therefore an increase in the lever-arm stiffness of myosin II[4]. Defects in cMLCK activity or reduced levels of MLC2v phosphorylation contribute to the development of heart failure in animal models[3,5,6]. Thus, cMLCK activity plays critical roles in cardiac contractility in both physiological and pathological conditions by regulating the phosphorylation level of MLC2v.

Dilated cardiomyopathy (DCM) is characterized by systolic dysfunction and an enlarged left ventricular chamber size and is a major cause of congestive heart failure and heart transplantations. So far more than 40 genes have been identified as DCM-causing mutations[7]. Recently, a novel DCM-associated *MYLK3* mutation (p.Pro639Valfs*15) was identified that completely abolishes kinase activity due to truncation of the cMLCK protein at the middle portion of its catalytic domain[8]. Two cases of familial DCM-associated mutations in *MYLK3* showing depressed or abolished cMLCK activity have also been reported[9]. Thus, depressed or abolished cMLCK activity in familial DCM may contribute to the development of the disease by decreasing MLC2v phosphorylation levels. MLC2v phosphorylation levels are also significantly reduced in failing human hearts even without mutations in *MYLK3*[10,11]. Thus, the reduction of the MLC2v phosphorylation levels seems to be common in human heart failure, indicating that the assessment of cMLCK activity and MLC2v phosphorylation levels is clinically important. It is

necessary to explain how the reduced MLC2v phosphorylation levels contribute to depressed cardiac contractility. Accordingly, assays that measure cMLCK activity and MLC2v phosphorylation levels are extremely important for elucidating the pathogenesis of heart failure.

The classical method for measuring cMLCK activity is a radiometric-based assay that quantifies the incorporation of [γ-^{32}P] from radioactively labelled ATP into MLC2v[2]. However, due to its hazardous nature it requires special safety and environmental considerations, and the cost of waste disposal is high. In addition, the short half-life of ^{32}P restricts the flexibility of the radiometric assay. To overcome these drawbacks, alternative nonradiometric protein kinase assay techniques have been developed[12]. The bioluminescent ADP detection assay developed by Promega Corporation measures ADP generated by the protein kinase reaction without using radioisotopes[13]. It shows comparable results to the radiometric assay for protein kinases with varying levels of activity[13]. Because the bioluminescent ADP detection assay measures ADP produced by a kinase reaction, phosphate-affinity SDS-PAGE in parallel with bioluminescent ADP detection assay was used to verify whether MLC2v is actually phosphorylated. Phosphate-affinity SDS-PAGE is a phosphate-affinity electrophoresis technique that can detect changes in the mobility of phosphorylated substrate proteins compared to their nonphosphorylated counterparts[14].

This article describes protocols for measuring the activity of cMLCK and the phosphorylation level of its substrate, MLC2v, using nonradioactive methods. After performing an in vitro kinase reaction, both the bioluminescent ADP detection assay and phosphate-affinity SDS-PAGE are employed to calculate biochemical values of MLCK and the phosphorylation level of MCL2v, respectively. Overall, a protocol combining the two nonradioactive kinase assays is valuable for the study of kinases.

PROTOCOL:

1. Cloning and purification of recombinant wild type and DCM-associated mutant cMLCK

1.1. Cloning of recombinant cardiac myosin light chain kinase into plasmid

1.1.1. Design a continuous nucleotide sequence to represent the final plasmid.

1.1.2. Amplify a DNA fragment coding human *MYLK3* (NM_1829493.3) using a standard PCR method. The primer sequences are as follows:

Forward primer: 5'- CACCATGTCAGGAACCTCCAAGGAGAGTCTGGGG -3'
Reverse primer: 5'- TTAGGGAGAAGTTGGAAATTTCCTTAACCT -3'

The melting temperature is 60 °C.

NOTE: The single-stranded overhang sequence (CACC) is necessary to ligate to the TOPO cloning vector.

1.1.3. Introduce a DCM-associated mutant construct by primer-derived mutagenesis. The primer sequences are as follows:

Forward primer: 5'- GTACAAGCCTCGAGAGAAGCTGAAGGTGAAC -3'
Reverse primer: 5'- CTTGAGGTCCAGGTGCAGGATGTAGTGCTGGT -3'

The melting temperature is 60 °C.

1.1.4. Run a 0.8% agarose gel at 135 V for 20 min, and cut out the bands corresponding to the PCR products. Then extract and purify the PCR products using a gel extraction kit (see **Materials**) following the manufacturer's instructions.

1.1.5. Perform a TOPO cloning reaction following the manufacturer's instructions (see **Materials**) and transform *Escherichia coli* (pENTR/cMLCK WT, mutant vector).

1.1.6. Confirm the correct sequence by PCR using the following universal M13 primers. The primer sequences are as follows:

Forward primer: 5'- GTAAAACGACGGCCAGT -3'
Reverse primer: 5'- GTCATAGCTGTTTCCTG -3'

1.1.7. Incorporate a FLAG-tag into the pENTR/cMLCK vector and perform a LR recombination reaction between *MYLK3* and a GateWay destination vector (i.e., pEF-DEST51) following the manufacturer's instructions (pEF-DEST51/FLAG-cMLCK WT, mutant).

1.2. Cell culture and transfection into HEK293T cells

1.2.1. Seed a 100 mm dish with 7.5 x 10^6 HEK293T cells and culture with DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Incubate at 37 °C in 5% CO_2 for 24 h.

1.2.2. Prepare the Lipofection reagent/DNA mix for transfection into HEK293T cells by mixing 10 µg of plasmid DNA in 500 µL of MEM (see **Table of Materials**). At the same time, prepare the mixture by adding 20 µL of Lipofectamine 2000 in 500 µL of MEM.

1.2.3. Incubate for 5 min at room temperature (RT), then mix the Lipofectamine-DNA solution.

1.2.4. Incubate for 20 min at RT, then add 1 mL of the Lipofectamine-DNA solution to the 100 mm dish.

NOTE: To avoid detaching the cells, add the lipofection mixture gently.

1.2.5. Incubate the transfected cells for up to 48 h at 37 °C with 5% CO_2.

1.3. Purification of recombinant cMLCK protein

1.3.1. Place the transfected cells on ice and wash 3x with 5 mL of PBS.

1.3.2. Prepare lysis buffer (50 mM Tris-HCl, 0.15 M NaCl, 1% NP40, 0.5 mM EDTA, 0.5 mM EGTA, and protease inhibitor cocktail, pH = 7.5) and keep on ice.

NOTE: Add the protease inhibitor cocktail just before the assay.

1.3.3. Add 1 mL of lysis buffer to the cells, use a scraper to harvest cells into a 1.5 mL tube, and incubate on ice for 5 min.

1.3.4. Centrifuge at 20,000 x *g* for 5 min at 4 °C.

1.3.5. Collect supernatant in a new 1.5 mL tube and incubate with 5 µL of FLAG-M2 agarose for 1 h at 4 °C.

1.3.6. Centrifuge at 1,000 x *g* for 1 min at 4 °C, then remove the supernatant and wash in FLAG-M2 agarose 3x using washing buffer (50 mM Tris-HCl, 0.15 M NaCl, 1% NP40, 0.5 mM EDTA, 0.5 mM EGTA, and protease inhibitor cocktail, pH = 7.5).

1.3.7. Elute the binding proteins with elution buffer (50 mM Tris-HCl, 0.15 M NaCl, 1% NP40, 0.5 mM EDTA, 0.5 mM EGTA, and protease inhibitor cocktail, pH = 7.5) at 4 °C for 30 min.

1.3.8. Centrifuge at 1,000 x *g* at 4 °C for 3 min and use the supernatant as the recombinant FLAG-tagged proteins.

2. Cloning and purification of recombinant calmodulin

2.1. Cloning of recombinant calmodulin into the plasmid

2.1.1. Design a continuous nucleotide sequence to represent the final plasmid.

2.1.2. Amplify a DNA fragment coding human calmodulin (CALM1) (NM_006888) using a standard PCR method. The primer sequences are as follows:

Forward primer: 5'- CACCATGGCTGATCAGCTGACCGAAGAACAGATT -3'
Reverse primer: 5'- TCATTTTGCAGTCATCATCTGTACGAATTC -3'.

The melting temperature is 60 °C.

NOTE: The single-stranded overhang sequences (CACC) are necessary for ligating to the TOPO cloning vector.

2.1.3. Run a 0.8% agarose gel at 135 V for 20 min, and cut the bands corresponding to the PCR

products out of the gel. Then extract and purify the PCR products using a gel extraction kit (see **Materials**) following the manufacturer's instructions.

2.1.4. Perform the TOPO cloning reaction following the manufacturer's instructions (see **Materials**) and transform into *E. coli* (pENTR/Calmodulin).

2.1.5. Confirm correct sequence by PCR using the following universal M13 primers. The primer sequenced are as follows:

Forward primer: 5'- GTAAAACGACGGCCAGT -3'
Reverse primer: 5'- GTCATAGCTGTTTCCTG -3'

2.1.6. Perform the LR recombination reaction between the calmodulin and a GateWay destination vector (pEF-DEST17) following the manufacturer's instructions.

2.1.7. Transform into BL21 (DE3) chemically competent *E. coli*.

2.2. Cell culture and induction of expression

2.2.1. Inoculate 5 mL of LB medium containing 100 µg/mL ampicillin with a colony from the transformed *E. coli* and shake overnight at 37 °C.

2.2.2. Transfer to 200 mL of LB medium containing 100 µg/mL ampicillin and incubate at 37 °C until the culture optical density (600nm) reaches 0.5.

2.2.3. Add arabinose to a final concentration of 0.2% and culture for 3 h at 37 °C.

2.2.4. Centrifuge at 5,000 x *g* at 4 °C for 10 min and remove the supernatant.

2.2.5. Suspend in 10 mL of BugBuster Master Mix containing EDTA-free protease inhibitor cocktail and rotate at RT for 20 min.

2.2.6. Centrifuge at 16,000 x *g* at 4 °C for 10 min.

2.2.7. The resulting supernatant containing the N-terminus His-tagged calmodulin protein was loaded onto a column of TALON Affinity Resin equilibrated with immobilized metal ion chromatography binding buffer.

2.2.8. Elute the bound His-tagged calmodulin protein with elution buffer (50 mM sodium phosphate [pH = 8.0], 0.3 M NaCl, 0.1% CHAPS, and 0.15 M imidazole), then refold and concentrate by centrifugation at 5,000 x *g* at 4 °C using a centrifugal filter.

2.2.9. The protein concentration was adjusted to 10 µM and the solution kept at -80 °C until use.

3. In vitro kinase assay

NOTE: All steps are performed on ice to prevent the kinase reaction from proceeding. Additionally, the MLCK and substrate solutions are mixed separately to avoid an overly fast reaction.

3.1. Prepare the following solutions and keep on ice:

10x kinase buffer (200 mM HEPES, 10 mM $CaCl_2$, 50 mM $MgCl_2$, 0.1% Tween 20, pH = 7.5)
100 mM DTT
10 mM ATP
500 nM calmodulin
100 nM FLAG-tagged cMLCK
24 µM His-tagged MLC2v

NOTE: Recombinant proteins are diluted using 1x kinase buffer according to the indicated concentrations.

3.2. Prepare 100 µL of the cMLCK master solution on ice in 1.5 mL tubes using the following ingredients:

10 µL of 10x kinase buffer
20 µL of 100 nM cMLCK
20 µL of 5 µM calmodulin
8 µL of 100 mM DTT
42 µL of H_2O

NOTE: Each sample is diluted with 10x kinase buffer and distilled water and kept on ice.

3.3. Prepare 30 µL of substrate solution in the 8 strip PCR tube on ice at each MLC2v concentration as described in **Table 1**. The final MLC2v concentrations are 0, 0.25, 0.5, 1, 2, 4, 8, and 12 µM.

NOTE: Recombinant His-tagged MLC2v is diluted with 10x kinase buffer and distilled water. The volume of substrate solution is adjusted to 30 µL by adding water to obtain the appropriate MLC2v concentration.

3.4. Add 10 µL of the MLCK master solution to achieve a final reaction solution volume of 40 µL. The final concentration of each component in the kinase reaction is 20 mM HEPES, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.01% Tween 20, 2 mM DTT, 150 µM ATP, 5 nM cMLCK, 250 nM calmodulin, and 0, 0.25, 0.5, 1, 2, 4, 8, or 12 µM MLC2v (pH = 7.5).

3.5. Mix well and spin down.

3.6. Incubate the reaction samples at 25 °C for the indicated time.

3.7. After incubation, measure kinase activity by phosphate-affinity SDS-PAGE or bioluminescent ADP detection assay.

4. Phosphate-affinity SDS-PAGE

NOTE: Phosphate-affinity SDS-PAGE was performed according to the manufacturer's protocol (see **Table of Materials**).

4.1. Pour the gel for phosphate-affinity SDS-PAGE.

4.1.1. Mix stacking gel solutions as follows: 12% wt/vol acrylamide, 0.1% wt/vol SDS, 125 mM Tris-HCl (pH = 6.8), 0.1% wt/vol ammonium persulfate, and 0.5% vol/vol N, N, N', N'- tetramethylethylenediamine.

4.1.2. Mix resolving gel solutions as follows: 12% wt/vol acrylamide, 30 µM Phos-tag acrylamide, 60 µM $MnCl_2$, 0.1% wt/vol SDS, 375 mM Tris-HCl (pH = 8.8), 0.05% wt/vol ammonium persulfate, and 0.25% vol/vol N, N, N', N'- tetramethylethylenediamine.

4.2. Prepare sample for electrophoresis.

4.2.1. Add and mix 1 mM $MnCl_2$ to the sample from the in vitro kinase reaction.

4.3. Perform electrophoresis and Western blotting.

4.3.1. Run the gel at 150 V for 80 min in running buffer (0.1% wt/vol SDS, 25 mM Tris, and 192 mM glycine).

4.3.2. After electrophoresis, soak the gel in EDTA (+) transfer buffer (10 mM EDTA, 50 mM Tris, 380 mM glycine, 0.000375% wt/vol SDS, and 20% wt/vol ethanol) for 10 min and then in transfer buffer for 10 min.

4.3.3. Transfer proteins to PVDF membrane at 15 V for 30 min in transfer buffer.

4.3.4. Rock the membrane continuously with non-fat dry milk for 30 min in RT. After blocking, wash the membrane 3x with TTBS (50 mM Tris, 150 mM NaCl, 0.001% Tween).

4.3.5. Soak the membrane with the primary antibody (anti-MLC2v, 1:4,000; Abcam ab92721) overnight at 4 °C.

4.3.6. Wash the membrane 3x with TTBS (50 mM Tris, 150 mM NaCl, and 0.001% Tween).

4.3.7. Soak the membrane with the secondary antibody (HRP-coupled goat anti-rabbit 1:8,000;

Cappel, #55696) for 1 h at RT.

4.3.8. Wash the membrane 3x with TTBS.

4.4. Detect and analyze proteins on the membrane.

4.4.1. Add ECL (Enhanced Chemi Luminescence reagent) detection reagent to the membrane for 1 min at RT.

4.4.2. Detect the proteins on the membrane using a LAS-4000 at the optimal time.

4.4.3. Quantify the phosphorylated and nonphosphorylated MLC2v, subtracting the background densitometry using ImageQuant TL software.

5. Bioluminescent ADP detection assay

NOTE: The bioluminescent ADP detection assay was performed according to the manufacturer's protocol.

5.1. Stop the kinase reaction by adding 40 µL of ATP depletion reagent into the kinase assay reaction solution. Mix well and incubate for 30 min at RT.

5.2. Add 80 µL of ADP detection reagent. Mix well and incubate for 30 min at RT.

5.3. Measure the luminescence using a luminometer with a suggested maximum integration time of 0.5 s per well.

5.4. Convert the luminescence intensity to ADP concentration using a calibration curve.

5.5. Calculate the amount of phosphates used for MLC2v phosphorylation.

NOTE: The total ADP produced during the kinase reaction is measured as described above. Background ADP, including cMLCK autophosphorylation, is measured based on a reaction without MLC2v, and the amount of ADP used for MLC2v phosphorylation is assessed by subtracting background ADP from total ADP.

6. Data Analysis

6.1. Fit data to the Michalis-Menten equation using appropriate data analysis software. Data are expressed as mean ± standard deviation.

REPRESENTATIVE RESULTS:

The classical method for measuring kinase activity is a radiometric-based assay that quantifies the radiolabeled phosphate incorporated into the kinase substrate. For the method presented

here, a nonradioactive, in vitro cMLCK kinase assay using purified wild type cMLCK (**Figure 1A**), MLC2v, and calmodulin was developed (**Figure 1B**), and kinase activity was determined using a bioluminescent ADP detection assay. For the experiments used to establish the cMLCK assay, purified cMLCK from HEK293T cells was used. In order to determine what cMLCK concentration and reaction time guarantee signal linearity, MLC2v was first incubated with seven different concentrations of cMLCK at 25 °C. After 1 h, the kinase activity was measured by the bioluminescent ADP detection assay and the linear assay region was found between 1.25 and 20 nM cMLCK (**Figure 1C**), which was confirmed by phosphate-affinity SDS-PAGE (**Figure 1D**). Next, 25 nM cMLCK was incubated for four different durations (15, 30, 60, 90 min) and kinase activities were measured. Strict linearity of the signal during 90 min of the reaction was observed (**Figure 1E**,**1F**). Thus, a cMLCK concentration of 5 nM was used to guarantee strict linearity between the assay signal and kinase activity. Next, the values of K_m and V_{max} for MLC2v of cMLCK were determined in the presence of calmodulin, ATP, $CaCl_2$, and $MgCl_2$. The average V_{max} value was 1.65 ± 0.10 mol/min/mol kinase, and the average MLC2v K_m value was around 0.49 ± 0.10 μM at 25 °C (**Figure 1G**). Thus, a nonradioactive, in vitro cMLCK kinase test using a bioluminescent ADP detection assay and phosphate-affinity SDS-PAGE was developed.

Next, the functional consequences of the p.Pro639Valfs*15 mutation of the *MYLK3* gene, which is associated with human DCM pathogenesis, was examined. Wild type and mutant cMLCK was purified from HEK293T cells (**Figure 2A**), and the best fit was obtained using a model that describes cMLCK-MLC2v reactions based on Michaelis-Menten kinetics. Wild type cMLCK had a K_m value of 0.64 ± 0.13 μM and V_{max} value of 2.15 ± 0.10 mol/min/mol kinase. Mutant cMLCK had no kinase activity. n = 2 for each point. (**Figure 2B**,**2C**)

FIGURE AND TABLE LEGENDS:

Figure 1: Establishment of nonradioactive, in vitro cMLCK kinase assay system.

(**A**) Silver staining of recombinant FLAG-tagged wild type human cMLCK proteins from insect cells used in the in vitro kinase assay. (**B**) Coomassie staining of purified His6-tagged human MLC2v and His-tagged calmodulin from *E. coli*. (**C**, **D**) Different concentrations of cMLCK (0, 1.25, 2.5, 5, 10, 20, 40, and 80 nM) were incubated with 12 μM MLC2v, 150 μM ATP, and 250 nM calmodulin at 25 °C for 1 h, and the kinase activities or MLC2v phosphorylation levels were measured by bioluminescent ADP detection assay (**C**) or phosphate-affinity SDS-PAGE (**D**), respectively. Phosphate-affinity SDS-PAGE was followed by immunoblot analysis with an anti-MLC2v antibody. Bands corresponding to phosphorylated and nonphosphorylated MLC2v are marked with open and closed circles, respectively. (**E**, **F**) In vitro kinase reactions were performed with 5 nM cMLCK in the presence of 150 μM ATP, 250 nM calmodulin, and 12 μM MLC2v at 25 °C for the indicated times (0, 15, 30, 60, 90 min), and the kinase activity was measured by bioluminescent ADP detection assay (**E**) or phosphate-affinity SDS-PAGE (**F**). (**G**) The MLC2v dose-dependence curve of the cMLCK activity was fitted using the Michaelis-Menten equation. K_m (MLC2v) = 0.49 ± 0.10 μM. V_{max} = 1.65 ± 0.10 mol/min/mol kinase. Each point represents the mean of duplicate measurements using two different protein preparations.

Figure 2: The kinase activities of wild type and DCM-associated mutant cMLCK.

(**A**) Silver staining of recombinant FLAG-tagged wild type and p.Pro639Valfs*15 mutant human

cMLCK proteins from HEK293T cells used in the in vitro kinase assay. (**B**) Same amounts of four different concentrations of wild type and p.Pro639Valfs*15 mutant cMLCK were incubated with 12 µM MLC2v, 150 µM ATP, and 250 nM calmodulin at 25 °C for 1 h, and the phosphorylation levels of MLC2v were measured by phosphate-affinity SDS-PAGE. Upper panels show phosphate-affinity SDS-PAGE of MLC2v and subsequent immunoblot analysis with anti-MLC2v antibody. Bands corresponding to phosphorylated and nonphosphorylated MLC2v are marked with open and closed circles, respectively. Lower panels show the loading control of purified Flag-tagged cMLCK proteins used in the kinase assays. (**C**) The MLC2v dose-dependence curves of wild type and p.Pro639Valfs*15 mutant cMLCK activities were fitted using the Michaelis-Menten equation. Wild type cMLCK had a K_m value of 0.64 ± 0.13 µM and V_{max} value of 2.15 ± 0.10 mol/min/mol kinase. The p.Pro639Valfs*15 mutant had no kinase activity. Each point represents the mean of duplicate measurements using two different protein preparations. RLU, relative light unit.

DISCUSSION:

The present study was undertaken to assess whether the combination of nonradioactive methods, the bioluminescent ADP detection assay and the phosphate-affinity SDS-PAGE could successfully be used to determine the activity of cMLCK. It is essential to perform the kinase reactions under the optimal temperature and reaction time. Increasing either of these will rapidly and strongly promote the enzyme reaction. In the present study, the in vitro kinase reaction was performed with 5 nM of cMLCK at 25 °C, which ensured signal linearity for at least 90 min. The bioluminescent ADP detection assay uses a three-step process to quantitate the amount of ADP generated during the protein kinase reaction[13]. After this reaction is complete, the residual ATP is fully depleted and all ADP produced by the reaction is converted to ATP. Only the newly generated ATP is then used for the kinase activity that generates the luminescence signal. For accurate determination of the biochemical values (e.g., K_m of substrate and V_{max}), ADP-ATP standard solutions with various ADP/ATP ratios (final concentration of 150 µM) should be prepared in the same volumes as the kinase reaction solutions. In addition, it is important to measure the signal of cMLCK in the absence of MLC2v simultaneously to exclude the ADP from ATP hydrolysis that does not result in phosphorylation of MLC2v. In the present study, bioluminescent ADP detection assay determined the K_m for MLC2v value was around 0.5 µM and a V_{max} value was around 1.7 to 2.2 mol/min/mol kinase in wild type cMLCK. These values showed minor differences from the values determined by the in vitro radioactive kinase assay[2] because of the difference in the reaction temperature. However, the results appear to be within the acceptable range.

The bioluminescent ADP detection assay does not directly observe the phosphorylation level of the protein kinase substrate, while the radiometric assay can observe the direct incorporation of γ-^{32}P into the protein kinase substrate. Accordingly, the phosphate-affinity SDS-PAGE was performed in parallel using the same sample to complement the bioluminescent ADP detection assay that can visualize the phosphorylated substrate as slower migration bands with corresponding nonphosphorylated substrate[14]. Phosphate-affinity SDS-PAGE showed time- and dose-dependent increases in the amounts of phosphorylated MLC2v, which is consistent with the results of ADP-Glo assay. Furthermore, the validity of both the bioluminescent ADP detection assay and the phosphate-affinity SDS-PAGE were confirmed by the experiments using wild type

and p.Pro639Valfs*15 mutant cMLCK purified from HEK293T cells. Because p.Pro639Valfs*15 mutant cMLCK is truncated at the middle portion of its catalytic domain, its kinase activity is likely abolished[8]. Indeed, phosphate-affinity SDS-PAGE showed complete disappearance of the phosphorylated bands of MLC2v, and the ADP-Glo assay showed complete disruption of the kinase activity of the mutant cMLCK.

The bioluminescent ADP detection assay can determine the biochemical values of kinase activity accurately and will be the next gold standard assay for protein kinase activity[13]. However, it cannot directly observe substrate phosphorylation and cannot distinguish the ADP product formed during the kinase reaction from those during an ATPase reaction. On the other hand, phosphate-affinity SDS-PAGE can directly observe the phosphorylation level of the protein kinase substrate[14], although it cannot determine the biochemical values of kinase activity accurately. Taken together, combining the bioluminescent ADP detection assay and the phosphate-affinity SDS-PAGE will provide the necessary and sufficient information to determine kinase activity by covering for each other's weaknesses.

ACKNOWLEDGMENTS:

This work was supported in part by JSPS KAKENHI Grant Number JP17K09578 and JP18H04050.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Seguchi, O. et al. A cardiac myosin light chain kinase regulates sarcomere assembly in the vertebrate heart. *The Journal of Clinical Investigation*. **117** (10), 2812–2824 (2007).
2. Chan, J. Y. et al. Identification of cardiac-specific myosin light chain kinase. *Circulation Research*. **102** (5), 571–580 (2008).
3. Scruggs, S. B. et al. Ablation of ventricular myosin regulatory light chain phosphorylation in mice causes cardiac dysfunction in situ and affects neighboring myofilament protein phosphorylation. *Journal of Biological Chemistry*. **284** (8), 5097–5106 (2009).
4. Sheikh, F. et al. Mouse and computational models link Mlc2v dephosphorylation to altered myosin kinetics in early cardiac disease. *Journal of Clinical Investigation*. **122** (4), 1209–1221 (2012).
5. Ding, P. et al. Cardiac myosin light chain kinase is necessary for myosin regulatory light chain phosphorylation and cardiac performance in vivo. *Journal of Biological Chemistry*. **285** (52), 40819–40829 (2010).
6. Warren, S. A. et al. Myosin light chain phosphorylation is critical for adaptation to cardiac stress. *Circulation*. **126** (22), 2575–2588 (2012).
7. Pinto, Y. M. et al. Proposal for a revised definition of dilated cardiomyopathy, hypokinetic non-dilated cardiomyopathy, and its implications for clinical practice: a position statement of the ESC working group on myocardial and pericardial diseases. *European Heart Journal*. **37** (23), 1850–1858 (2016).
8. Hodatsu, A. et al. Impact of cardiac myosin light chain kinase gene mutation on development of dilated cardiomyopathy. *ESC Heart Failure*. **6** (2), 406–415 (2019).

9. Tobita, T. et al. Identification of MYLK3 mutations in familial dilated cardiomyopathy. *Scientific Reports*. **7** (1), 17495 (2017).
10. Van Der Velden, J. et al. Myosin light chain composition in non-failing donor and end-stage failing human ventricular myocardium. *Advances in Experimental Medicine and Biology*. **538**, 3–15 (2004).
11. Morano, I. Tuning the human heart molecular motors by myosin light chairs. *Journal of Molecular Medicine*. **77** (7), 544–555 (1999).
12. Fan, F., Wood, K. V. Bioluminescent assays for high-throughput screening. *Assay and Drug Development Technologies*. **5** (1), 127–136 (2007).
13. Sanghera, J., Li, R., Yan, J. Comparison of the Luminescent ADP-Glo Assay to a Standard Radiometric Assay for Measurement of Protein Kinase Activity. *ASSAY and Drug Development Technologies*. **7** (6), 615–622 (2009).
14. Kinoshita, E., Kinoshita-Kikuta, E., Koike, T. Separation and detection of large phosphoproteins using phos-tag sds-page. *Nature Protocols*. **4** (10), 1513–1521 (2009).

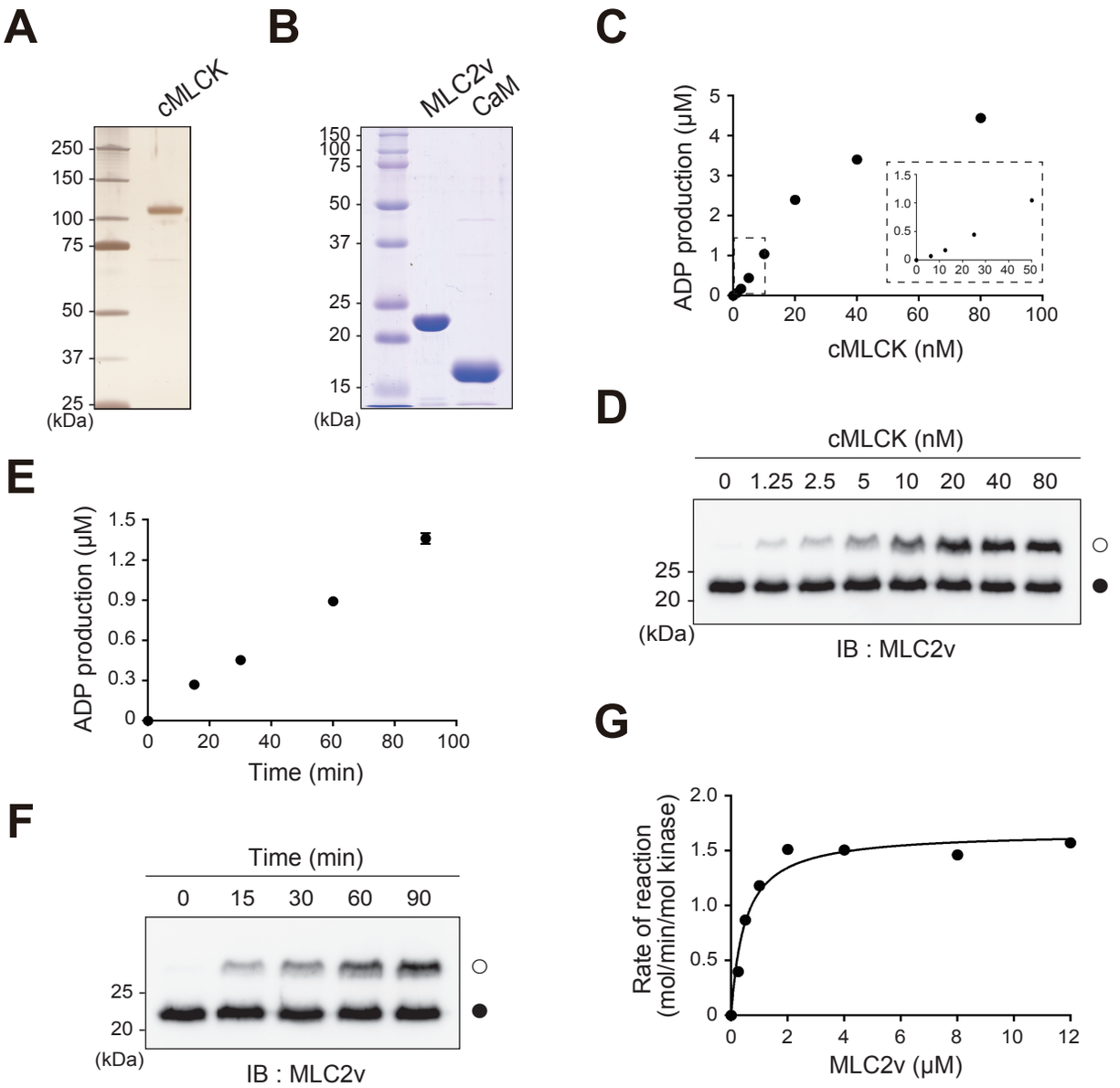

Figure 2

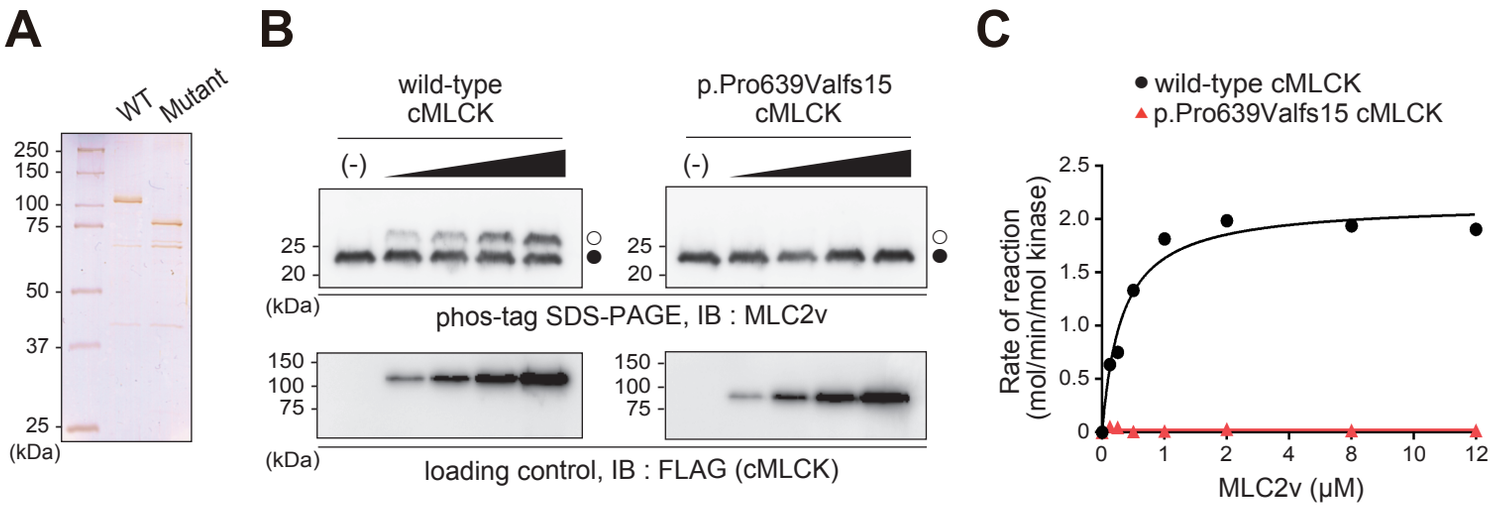

Table 1

Final MLC2v conc. (µM)	**0**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**12**
10 x kinase buffer (µL)	2	2	2	2	2	2	2	2
2 mM ATP (µL)	3	3	3	3	3	3	3	3
MLC2v (µL)	0	0.4	0.8	1.7	3.4	6.7	13.4	20
H_2O (µL)	25	24.6	24.2	23.3	21.6	18.3	11.6	5
Total (µL)	30	30	30	30	30	30	30	30

Name of Material/Equipment	Company	Catalog Number	Comments/Description
30% acrylamide/Bis solution	Bio-Rad	1610156	Store at 4℃
acrylamide	Bio-Rad	1610156	Store at 4℃
Amicon Ultra-15	Merck	UFC901008	
ammonium persulfate	Wako	019-03435	
ampicillin sodium	Wako	014-23302	Store at -20℃
BugBuster	Milipore	71456-4	Store at 4℃
CaCl2	Wako	031-00435	
CHAPS	Dojindo	349-04722	Store at 4℃
chemiluminescence imaging analyzer TriStar2	CBERTHOLD TECHNOLOGIES	LB942-A	
dithiothreitol	Wako	047-08973	Store at -20℃
ECL (Enhanced Chemi Luminescence) reagent	GE Healthcare	RPN2106	Mix reagent 1 and reagent 2 in equal
EDTA	DOJINDO	345-01865	
Ethanol	Wako	057-00456	
FBS	Sigma-Aldrich	172012-500ML	Store at -20℃
FLAG agarose	Merck	A2220	Store at -20℃
FLAG peptide	Merck	F3290-4MG	Store at 4℃
GateWay pEF-DEST51 Vector	Invitrogen	12285011	Store at -20℃
glycine	Sigma-Aldrich	12-1210-5	
HEPES	Dojindo	342-01375	
Igepal CA-630 (NP40)	Sigma-Aldrich	13021-500ML	
Imiadasole	Wako	095-00015	
L-(+)-Arabinose	Sigma-Aldrich	A3256-25G	Store at -20℃
LAS-4000	GE Healthcare	28955810	
LB	Merck	WM841485 824	
Lipofectamine 2000	Invitrogen	11668-019	
Manganase (II) Chloride Tetrahydrate	Wako	134-15302	
MgCl2	nacalai-tesque	20909-42	
N, N, N’, N’- tetramethylethylenediamin	Wako	110-18-9	
NaCl	Wako	191-01665	
OneShot BL21 AI	Invitrogen	44-0184	Store at -80℃

OptiMEM	gibco	31985-070	Store at 4℃
PBS	NISSUI PHARMACEUTICAL	5913	Store at 4℃
penicillin streptmycin	gibco	15140-122	Store at -20℃
pENTR/D-TOPO Cloning Kit	Invitrogen	K240020	Store at -20℃
Phos-tag Acrylamide	Wako	AAL-107	Store at 4℃
Promega ADP-Glo	Promega	V9104	Store at -20℃
protease inhibitor cock-tail	nacalai-tesque	25955-11	
PVDF membrane	Merck	IPVH00010	Pore size : 0.45 μm
QIAEX II Gel Extraction Kit (150)	QIAGEN	20021	
SDS	Wako	191-07145	
sodium phosphate	Wako	192-02815	
TALON affinity resin	TaKaRa	635504	Store at 4℃
Tris	Sigma-Aldrich	T1503-1KG	
Tween 20	Wako	167-11515	Store at 4℃
Ultra Pure Agarose	Invitrogen	16500-500	
Ultra Pure ATP, 100mM	Promega	V703B-C	Store at -20℃
Urea	Sigma-Aldrich	U0631-1KG	

amounts

Response to the specific comments of Editor

We thank the editor for the extensive comments and have revised the manuscript in response to these constructive comments. We think that the manuscript is now clearer and much improved. Please note that italicised sentences below are the comments of the editor.

First of all, we have to apologize for the correction of the concentration of recombinant cMLCK produced from the insect cells in Figure 1. We noticed a miscalculation of its concentration and corrected it throughout the manuscript and figures.

General Comments:

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

   Response: Thank you for the comment. We confirmed and corrected spelling and grammar to appropriate one.

2. *JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.*
   For example: Lipofectamine, OptiMEM, ADP-GloTM

   Response: Thank you for these comments. As the editor said, we removed the commercial language in revised manuscript. Thank you for giving us the opportunity to modify our manuscript.

Comments for protocols:

1. *There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.*

    Response: Thank you for these comments. We selected protocols for filming, and we highlighted relevant sections by red colour (page 6 - 8, line 255 – 306, page 9, line 361 - 379).

2. *For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.*

    Response: Thank you for these comments. According to the Editor's comments, we confirmed and corrected the statement in revised manuscript.

Comments for specific protocols:

1. *5: There are not actionable steps here; please elaborate on the data analysis or remove.*

    Response: Thank you for the comment, and we are sorry for insufficient statement. As the Editor and Reviewer #2 pointed out, we added the description of the analysis for the enzymatic reaction in this section.

Comments for figures:

1. *Please remove 'Figure 1' etc. and the titles from the Figures themselves.*

Response: Thank you for the comment. As the Reviewer pointed out, we removed the sentence in revised Figures.

Comments for discussion:

1. *Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:*

Response: Thank you for the comment. As the Reviewer pointed out, we removed the sentence in revised Figures.

Comments for References:

1. *Please ensure that the references appear as the following: [Lastname, F.I., LaseName, F.I.,] Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source.* ***Volume*** *(Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.*

Response: Thank you for the comment. According to the Editor's comments, we confirmed and corrected the reference based on the appropriate JoVE journal style.

Comments for Table of Materials:

1. *Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.*

Response: Thank you for the comment. According to the Editor's comments, we confirmed and added the materials and equipment with a missing description.

Response to the specific comments of Reviewer #1

We thank the reviewer for her/his extensive comments and have revised the manuscript in response to these constructive comments. We think that the manuscript is now clearer and much improved. Please note that italicised sentences below are the comments of the reviewer.

First of all, we have to apologize for the correction of the concentration of recombinant cMLCK produced from the insect cells in Figure 1. We noticed a miscalculation of its concentration and corrected it throughout the manuscript and figures.

Comments:

The manuscript presents reasonable approaches to quantitating rates for MLCKc ATPase activity and phosphorylation of substrate MYL2 without using a radioactive assay.

Response: Thank you for these comments. We greatly appreciate the reviewer's careful reading and correct understanding of our claims in the manuscript.

Comments:

Minor Concerns:

Absolute concentrations of products are not measured hence reaction efficiency is unknown. For instance, there may be ATP hydrolysis that does not result in phosphorylation of MYL2. Absolute concentrations are measured with the radio-assay. There are many nonradioactive ways to detect ATP hydrolysis products and phosphorylation of MYL2. Motivation for these particular methods might help users to decide the best course.

Response: Thank you for the comment. We completely agree with the comment of Reviewer #1. To exclude the ADP from ATP hydrolysis that does not result in MYL2 phosphorylation, we simultaneously measured the activity of MYLK3 in the absence of MYL2, in which ADP was

produced only by ATP hydrolysis of cMLCK. By subtracting the value in the absence of MYL2 from that in the presence of MYL2, we can detect the cMLCK activity that reflects only MYL2 phosphorylation.
We added the discussion about this point (page 12, line 478 - 485).

Response to the specific comments of Reviewer #2

We thank the reviewer for her/his extensive comments and have revised the manuscript in response to these constructive comments. We think that the manuscript is now clearer and much improved. Please note that italicised sentences below are the comments of the reviewer.

First of all, we have to apologize for the correction of the concentration of recombinant cMLCK produced from the insect cells in Figure 1. We noticed a miscalculation of its concentration and corrected it throughout the manuscript and figures.

Comments: *This methods manuscript by Kamikubo et al, describes application of commercially available kits to circumvent use of radioactivity. Novelty is lacking in that investigators in the field have already applied the use of ADP-Glo and Phostag-PAGE for this exact purpose, but a concise methods paper is not currently available. This specific manuscript is timely during a period where there is growing interest in cMLCK and cardiac myosin phosphorylation. However, there are significant problems in this manuscript that indicate it was haphazardly written. Careful correction is necessary prior to acceptance and publication. As it stands, it is very difficult for a non-expert to execute the preparations for the assay following this protocol.*

Response: Thank you for these comments. We greatly appreciate the reviewer's careful reading and correct understanding of our claims in the manuscript.

Comments:

Major Concerns:

1. The abstract states recombinant cMLCK from insect cells was used, and introduction says the kinase was purchased. However, methods describe transfection of plasmids and purification of kinase from HEK293T cells. This is a major oversight for a methods paper. The abstract must be corrected to remove focus from inconsistent purification method. The

introduction must describe in general terms, potential sources of kinases and substrates, and clarify what was used when within this manuscript.

Response: Thank you for these comments, and we would like to apologize for the inappropriate representations. As the reviewer said, we removed the name of species due to describe its generality. Thank you for giving us the opportunity to modify our manuscript.

2. *Bacterial expression and purification of the MLC2v and calmodulin is not described. At the very least, source information to a vendor or reference to a published method is needed.*

Response: Thank you for these comments. We are sorry for insufficient statement. According to the comment, we added the section of “Cloning and purification of recombinant calmodulin” in revised manuscript (page 5 - 6, line 195 - 253). We are sorry, but we declined to show the protocols to purify the MLC2v because of non-disclosure contract with other’s laboratory. Instead, we introduce the alternative recombinant MLC2v proteins which can be purchased from Abcam corporation (ab117178).

3. *Description of the expression plasmid and methods for the DCM associated mutant kinase is needed. There's no figure showing purity of the mutant kinase, and Fig 2A shows the mutant kinase is larger than the WT kinase, and catalytically inactive. This raises all sorts of questions regarding kinase purity and quality. The Vmax should be calculated and reported as mol/min/mol kinase in Figure 2. For completion, purity of mutant kinase and size should be shown on same gel as WT.*

Response: We would like to apologize for our careless mistake. We corrected Fig 2A in which the mutant kinase is smaller than the WT kinase. In addition, we added the description of the expression plasmid and methods for the mutant kinase (page 3, line 126 - 130).

Moreover, we added the picture of the silver staining of purified kinases, measured the concentrations, and expressed the Vmax of cMLCK as 2.15 ± 0.10 mol/min/mol kinase.

4. For any enzymatic reaction, analysis of the reaction curves is of utmost importance. Detailed description of the analysis or reference to specific software and built-in nonlinear regression function used is necessary.

Response: Thank you for these comments. We are sorry for the insufficient explanation, *Km* values were calculated by non-linear fit to the Michaelis–Menten equation by using Prism 6 software. We added this statement in the "Data Analysis" section (page 9, line 383 - 384).

5. Radioactive assay methods that this method paper is supposed to replace has not been cited in introduction. To show efficacy in replacing a gold-standard method, confirming similar Km and Vmax values were attained is necessary, and should be discussed.

Response: Thank you for these comments. As Reviewer pointed out, it had previously reported that cMLCK kinase activity was measured by in vitro radioactive kinase assay (Chan et al., Cir. Res. (2008)102 571-580). In this paper, in vitro kinase assay demonstrated that cMLCK had a Km value of 3.9±1.2 and a Vmax value of 16.6±2.8 mol/min/mol, which shows minor differences from our values. There could be some reasons for it. First, the reaction temperature was different. We performed the kinase reaction at 25^{o}C while they did at 37^{o}C. Second, the purity of recombinant cMLCK may be different. They used HA-tagged cMLCK expressed in 293T cell for the kinase assay, which showed Ca^{2+}/calmodulin-independent activity although cMLCK has a typical regulatory domain of Ca^{2+}/calmodulin-dependent kinase. The recombinant cMLCK used in our experiment does not show Ca^{2+}/calmodulin-independent activity. Consistent with our data, the

other lab reported as obvious Ca^{2+}/calmodulin-dependency of purified recombinant cMLCK (Kampourakis T et al. JMCC 2015; 85: 199-206). However, we think the difference of the values were acceptable range. Accordingly, we proposed non-radioactive kinase assay as an assay system that could be easily measured cMLCK kinase activity in the absence of research facility using radioisotopes. Thank you for providing with an opportunity to strengthen our results.

6. *Description of how the buffer constituents were adjusted to offset volumes added from kinase and substrate protein solution are difficult to understand or follow.*

Response: Thank you for these comments. We are sorry for insufficient description about sample preparation. We added the table that shows how the buffer constituents were adjusted in Methods section “in vitro kinase assay” (page 7 - 8, table).

Minor Concerns:

1. *Michaelis-Menten is misspelled in abstract.*

Response: Thank you for the comment. We are sorry for incorrect words, and we corrected the word to “Michalis-Menten” in the revised manuscript (page 2, line 55).

2. *Km value in abstract doesn’t match results section. In abstract, it is advised that they state “the average Km was around 0.5 μM”.*

Response: Thank you for the comment. According to this comment, in the revised manuscript, we corrected the statement to “the average Km was around 0.5 μM”.

3. *Method and figure legend, the amount of His-tagged MLC2v used does not match.*

Response: Thank you for the comment. We would like to apologize for confusing representation. In this manuscript, we used various concentrations of MLC2v in accordance with the conditions. Therefore, we changed the statement in the Method section to detail explanation in revised manuscript (page 7, line 281 - 293).

4. *ECL should be spelled out and kit needs vendor info.*

Response: Thank you for the comment. We are sorry for inappropriate words, and ECL stands for Enhanced Chemi Luminescence reagent. This reagent was purchased from GE Healthcare. In accordance with this comment, we corrected words and added the information about this reagent.

5. *"LAS-4000" needs more description.*

Response: Thank you for these comments. LAS-4000 is a lumino-image analyser for producing digital images of gels or membrane samples to detect the chemiluminescence samples. In this manuscript, LAS-4000 is not essential device, and it could be replaced with other devices.

6. *Equation for quantifying phosphorylated and non-phosphorylated MLC2v should be written out or described.*

Response: Thank you for these comments. We would like to apologize for not describing about the method to evaluate the phosphorylated MLC2v level in detail. Phosphorylated and non-phosphorylated MLC2v band were quantified by subtracting background densitometry using

using ImageQuant TL software. According to the comment, we added the method to evaluate the phosphorylated band in “Phos-tag SDS-PAGE” section (page 9, line 357 - 358).

7. *"TriStar 2 LB942" is not a required equipment but method sounds like it is. Re-wording to"...using a luminometer with a suggested maximum integration time of 0.5 s per well" is advised.*

Response: Thank you for these comments. As the Reviewer #2 pointed out, TriStar 2 LB942 was not essential equipment for this method. Since ADP-Glo assay was archived to measure the ADP-related luminescence products, other devices could be substituted. According to this comment, we corrected the statement in revised manuscript (page 9, line 369 - 370).

8. *"PDMS" membrane is likely an error. Specific information on membrane pore size and vendor is needed.*

Response: Thank you for these comments. We are sorry for our inappropriate statement. In the revised manuscript, we corrected the statement “PVDF membrane”. The description of PVDF membrane was added in Table.

9. *"Soak the membrane" suggest membrane was simply submerged in the antibody mixes. This needs to be clarified, as standard method is to continuously rock the membrane in buffer mixtures.*

Response: Thank you for these comments. We are sorry for our inappropriate statement. In the revised manuscript, we corrected the statement “Rock the membrane continuously” (page 8, line 336 - 338).