1. **Colorimetric assay (e.g. Malachite green assay)**

Very robust assay for endpoint measurements (i.e. does not allow online detection)

Cardiac myofibrils (CMF) were washed three times in ATPase assay buffer (composition in mmol L−1: 20 MOPS pH 7.0, 35 NaCl, 5 MgCl2, 1 EGTA, 1 DTT) with varying concentrations of CaCl2 (pCa 9 to pCa 4.3) and the CMF concentration adjusted to 0.5 mg ml−1. For ATPase measurements at activating Ca2+ concentrations, CMFs were partially crosslinked with 5 mmol L−1 N‐hydroxysuccinimide (NHS) and 2 mmol L−1 1‐ethyl‐3‐(3‐dimethylaminopropyl) carbodiimide hydrochloride (EDC) in myofibril buffer on ice for 90 min. The crosslinking reaction was stopped by the addition of 25 mmol L−1 glycine pH 8.0 and 10 mmol L−1 DTT for 30 min on ice and CMFs processed for experiments as mentioned above. Chemical crosslinking prevents CMFs from shortening during calcium activation.

Reactions were started by the addition of 2.5 mmol L−1 ATP and samples taken at the indicated time points (e.g., after 5, 10, 15 and 20 mins) were quenched with 0.5 volumes ice cold 25% (w/v) trichloro acetic acid (TCA) solution. Samples were kept on ice at all times, diluted with double‐deionized water (ratio between 1:10 and 1:2, depends on assay conditions and needs to be experimentally tested).

Inorganic phosphate content measured using the malachite green assay according to manufacturer's instructions (Sigma, MAK030). Briefly, standards with known Pi concentrations were prepared from 1 mmol L-1 Pi stock and 200 uL aliquoted into clear 96 well plates. 200 uL diluted sample were pipette into clear 96 well plate and 40 uL substrate mix (prepared by mixing components A and B in 1 1:1000 ratio) was added to all wells using an 8- or 12-channel pipette. Incubate in the dark for 20-30 min and measure absorbance at 630 nm using a plate reader.

Absorbance values were transformed into Pi concentrations using the standard curve and ATPase activity extracted by linear regression to the time points.

1. **NADH fluorescence-based assay**

Good assay for ‘online’ detection of ATPase activity of CMFs (i.e. allows the simultaneous measurement of >80 samples) but requires plate reader with appropriate excitation/emission filters

20 mL of enzyme mix in assay buffer containing 1 mg mL-1 CMFs, 40 U mL-1 lactate dehydrogenase and 400 U mL-1 pyruvate kinase were dispensed into a black 96-well half area plate (Greiner). Plates were incubated on a plate shaker at 30°C for 10 min at 2000 rpm. Reactions were started by adding 20 mL substrate mix in assay buffer containing 440 mmol L-1 NADH, 4 mmol L-1 2-phosphoenolpyruvate and 4 mmol L-1 ATP using a GILSON PLATEMASTER 96-channel pipette. Plates were briefly mixed by shaking at 5000 rpm on a plate shaker. NADH fluorescence intensity was measured for each well using a ClarioStar Plate Reader for 10 min every 30 sec at 30°C with the following settings: excitation at 380 nm with a 10 nm bandwidth and emission at 470 nm with a 24 nm bandwidth. ATPase activity was extracted by linear regression to changes in fluorescence intensity. The essay can be calibrated by substituting the CMFs for known concentrations of ADP to create a calibration curve.