

Notes & Tips

# NADH-coupled microplate photometric assay for kinetic studies of ATP-hydrolyzing enzymes with low and high specific activities

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Genome database searches retrieve over 10,000 sequences that either are known to use NTP or contain motifs suggestive of that. Hence, ATP hydrolysis is a convenient tool for monitoring their biological activity. Among the most popular assays are measurements of the release of  $P_i$ , ADP, or  $H^+$  [1]. For enzymes sensitive to the accumulation of ADP, an assay based on ADP recycling coupled to the oxidation of NADH represents a particular advantage [2,3]. The high molar absorption coefficient of NADH and the virtual absence of protein or DNA absorbance at this wavelength ensure reliable ATP hydrolysis measurements down to the micromolar concentration scale. A limitation of this technique is that it requires investment into relatively costly equipment with a limited capacity for parallel analysis. Microplate readers are devoid of these limitations, offering parallel analysis of many samples and great versatility. However, the lightpath of a microplate reader is variable and depends on the well fill volume, requiring proper calibration. Herein, we adapted the NADH-coupled ATPase assay for a 96-well microplate reader. A previous adaptation of this assay to the microplate format focused on P-glycoprotein, one particular ATPase [4]. Here, we elaborated general assay parameters to analyze a variety of ATPases with low and high specific activities. We show that beyond the numerous advantages of microplate readers (high throughput, low cost, ease of operation, use of disposable plasticware in place of quartz cells), the microplates ensure sufficiently high sensitivity and precision to measure ATP hydrolysis down to the submicromolar scale. We recommend practical tips for adapting the microplate-based assay for kinetic studies

(Table 1), which can be applied for developing high throughput in vitro drug screening applications for a variety of target proteins known to utilize ATP.

## Materials and methods

*Saccharomyces cerevisiae* Rad54 and Rad51 proteins were purified from the cognate host as described [5,6]. Rad51 protein produced in *Escherichia coli* was a generous gift of Dr. S. Kowalczykowski (University of California, Davis). Protein concentrations are given in moles of monomers, ssDNA concentrations are given in moles of nucleotides, and dsDNA concentrations are given in moles of basepairs. Phosphoenolpyruvate (PEP),<sup>1</sup> pyruvate kinase (PK), L-lactate dehydrogenase (LDH), and NADH were purchased from Sigma (St. Louis, MO). The regeneration system consisting of PEP and PK converts ADP back to ATP. The resulting pyruvate is subsequently converted to lactate by LDH, at the expense of oxidation of NADH. The decline of NADH absorbance at 340 nm allows monitoring the rate of steady state ATP hydrolysis in real time [7,8]. Complexes of Rad51 and Rad54 proteins with DNA were formed as described in the text and then transferred to quartz cells (100- $\mu$ l volumes, 1-cm lightpath, up to eight cells per run) or 96-well microplates (indicated volumes), blank-read, and then supplemented with NADH to give the desired optical density. Absorbance data were collected using a 8452A diode array spectrophotometer equipped with UV-visible ChemStation software (Hewlett-Packard) or a SPECTRAMax 250

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<sup>1</sup> Abbreviations used: PEP, phosphoenolpyruvate; PK, pyruvate kinase; LDH, L-lactate dehydrogenase; CS, conventional spectrophotometer; MP, Microplate reader.

Table 1  
Microplate assay guidelines and troubleshooting

Problem	Solution
Variable lightpath	Perform calibrations <ul style="list-style-type: none"> <li>• determine linearity of OD reading</li> <li>• calculate <math>K_{path}</math> over the range of NADH concentrations in the reaction buffer</li> </ul>
UV-induced NADH decomposition	Reduce UV exposure by <ul style="list-style-type: none"> <li>• increasing well fill volume (150 <math>\mu</math>l or more for 96-well plates)</li> <li>• keeping low NADH concentration (e.g., <math>A_{340}</math> 0.25–0.5 for 150-<math>\mu</math>l fill volume)</li> <li>• making longer intervals between readings</li> <li>• turning off autocalibration option (where available)</li> </ul>
Low ATPase activities	Increase precision and sensitivity of detection by <ul style="list-style-type: none"> <li>• increasing well fill volume (&gt;150 <math>\mu</math>l) or using 384-well-format reader</li> <li>• keeping NADH absorbance within the linearity range</li> <li>• running time course for longer time (where possible)</li> <li>• analyze every sample in duplicate or triplicate</li> </ul>
High ATPase activities	Increase NADH pool capacity by <ul style="list-style-type: none"> <li>• lowering well fill volume (e.g., to 100 <math>\mu</math>l)</li> <li>• increasing NADH concentration (<math>A_{340}</math> 2.0–2.5)</li> </ul>

microplate spectrophotometer equipped with SOFTmax PRO software (Molecular Devices). Readings were made in 30-s intervals.

The ATP turnover rates were calculated from the equation

$$\text{ATPase rate } [\text{ATP} \times \text{min}^{-1}] = -\frac{dA_{340}}{dt} [\text{OD}/\text{min}] \times K_{path}^{-1} \times \text{moles}^{-1} \text{ ATPase}$$

where  $K_{path}$  is the molar absorption coefficient for NADH for a given optical pathlength. For the microplate reader,  $K_{path}$  was determined experimentally for each sample fill volume. Rates were calculated per 180-s time frames (each comprising seven data points) overlapping in 30-s increments and plotted as a function of time. Where indicated, the rates were corrected for background NADH decomposition of control samples containing no ATPase. Rates were averaged over the selected time intervals during which the absorbance decline was linear.

## Results and discussion

*Determination of absorbance linearity and  $K_{path}$  for variable fill volumes.* The microplate well geometry ensures near perfect absorbance linearity with varying lightpaths between 50- and 300- $\mu$ l fill volume (Fig. 1A, diamonds). Both generic and UV-transparent polystyrene microplates have satisfactory transmittance at 340 nm, although generic ones have a slightly higher background absorbance (ca. 0.2 OD vs 0.05 OD). We calculated  $K_{path}$  by measuring absorbance of different fill volumes of an NADH solution of known concentration (Fig. 1B). As the absorbance linearity range may vary

between different models of microplate readers, we recommend an additional calibration with a series of NADH concentrations prior to establishing a new assay. Also, the lightpath may vary depending on the surface tension of the reaction buffer. Therefore, it is recommended to determine the  $K_{path}$  with several concentrations of NADH in the reaction buffer within the absorbance linearity range.

*Contribution of UV-induced NADH decomposition.* NADH in water is a relatively stable compound, and its spontaneous oxidation is usually negligible. However, we found that the UV-induced NADH decomposition rises significantly for fill volumes of less than 100  $\mu$ l (Fig. 1A, bars). Increasing the lightpath with larger sample volumes reduces the effect of UV exposure. The rate of NADH decomposition also depended on the NADH concentration (Fig. 1D). We found that the pathlength corresponding to a fill volume of 150  $\mu$ l provides a reasonable compromise between the amount of enzyme, the sensitivity of absorbance reading, and the background NADH decomposition. This volume was used in all subsequent experiments.

*Rate fluctuation during the kinetic time course.* The rate of ATP hydrolysis is usually calculated from relatively short time intervals in the beginning of the reaction. Monitoring rates over the entire time course can bring additional information about the properties of an enzyme (e.g., its overall stability and time-dependent activation/inhibition). In this case, it is important to take into account the variation of the reaction rate within a given time interval (rate fluctuation), which is a composite of the variation of enzyme activity and of instrument-borne errors. The rate fluctuation can also become a source of error when measuring low ATPase activities, where the rates of absorbance decrease due

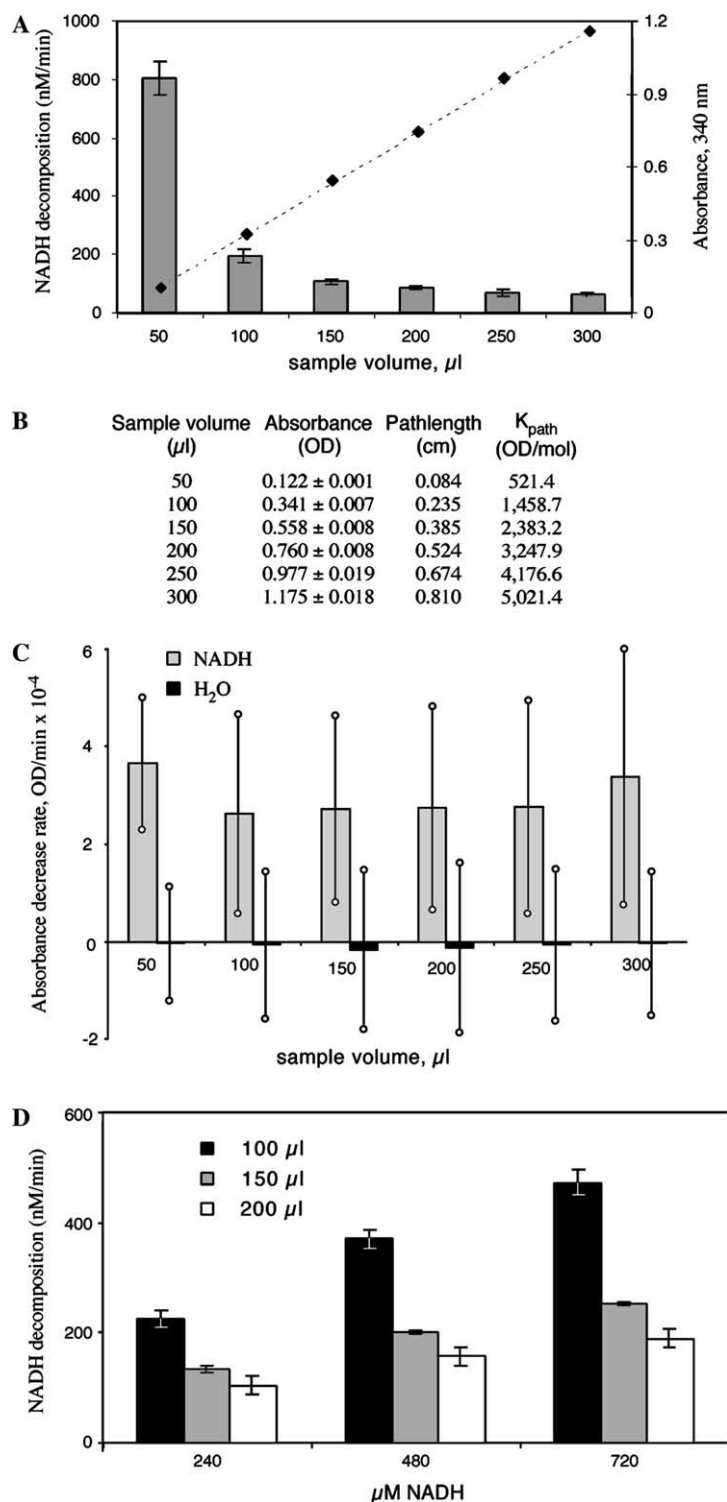


Fig. 1. Parameters of a microplate-based ATPase assay and factors contributing to the variability of kinetic data. (A) Linearity of absorbance over sample volume and UV-induced NADH decomposition as a function of pathlength. Diamonds show the single absorbance readings for each fill volume measured before the time course (OD units, plotted on the right Y axis; corresponding values are shown in B). Bars show the kinetic data expressed as the rate of NADH decomposition (nM/min; plotted on the left Y axis). Rates were calculated using the  $K_{\text{path}}$  data presented in B. NADH was at a concentration of  $234 \mu\text{M}$ . All measurements were done in triplicate. (B) Determination of pathlength for variable sample volumes. Pathlength coefficients for different fill volumes were calculated from single absorbance readings shown in A using the molar absorption coefficient for NADH in water ( $6200 \text{ OD} \times \text{mol}^{-1} \times \text{cm}^{-1}$ ) as a reference. (C) Evaluation of rate fluctuation. The kinetic data from A were replotted as averages of rates of absorbance decrease (OD/min). Circle-ended error bars represent the rate fluctuation, which is calculated as a standard deviation of the rate values within the time course. (D) Further optimization of assay parameters to reduce the background NADH decomposition. Shown is the rate of NADH decomposition (nM/min). All measurements were done in triplicate. For details see text.

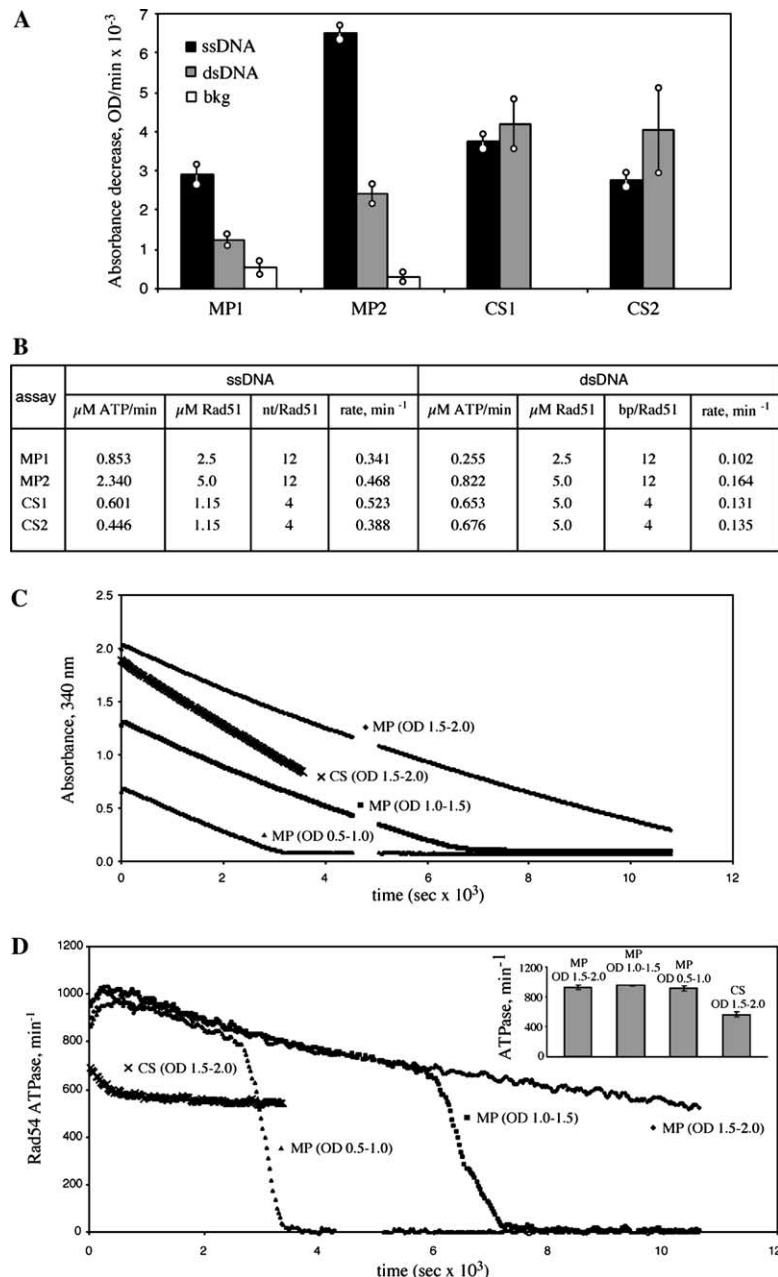


Fig. 2. Assay application for ATPases with low and high specific activities. Comparison of data obtained by conventional fixed-pathlength spectrophotometer (CS) and microplate reader (MP, 150  $\mu\text{l}$  fill volume). (A, B) ATPase with low specific activity. Complexes of Rad51 protein with ssDNA (poly dA) and dsDNA (supercoiled  $\phi\text{X174}$  DNA) were performed for 10 min at 30  $^{\circ}\text{C}$  in buffer containing 25 mM triethanolamine acetate, pH 7.5, 13 mM magnesium acetate, 1.8 mM dithiothreitol, 5 mM ATP, 100  $\mu\text{g/ml}$  bovine serum albumin, 20 U/ml LDH, and the ATP regeneration system (3 mM PEP, 20 U/ml PK). Rad51 protein concentration and DNA/protein ratio are indicated in B. The ATPase assay for Rad51 protein was performed at 30  $^{\circ}\text{C}$  as described under Materials and methods. (A) Average rates of NADH absorbance decrease (OD/min) were calculated over the 1400- to 2900-s interval of the 1-h time course. Circle-ended error bars represent rate fluctuations calculated as standard deviations of rate values within given time course intervals. MP1 and MP2, independent experiments performed on different days with the same preparation of Rad51 protein (from *S. cerevisiae* host) using the microplate reader; CS1 and CS2, independent experiments performed on different days with two different preparations of Rad51 protein using the conventional spectrophotometer: CS1, Rad51 purified from *E. coli* host; CS2, Rad51 purified from *S. cerevisiae* host; Bkg, background NADH decomposition measured from a control containing no Rad51 protein. (B) Calculated ATP turnover rates for Rad51 protein after background subtraction. (C, D) ATPase with high specific activity; 5 nM Rad54 protein was incubated with 4.6  $\mu\text{M}$  dsDNA at 24  $^{\circ}\text{C}$  in reaction buffer as described in A. Kinetics on the CS was recorded in triplicate during 1 h (initial NADH input: 1.5–2.0 OD range). Kinetics on the MP was recorded during 3 h with three initial NADH inputs (1.5–2.0, 1.0–1.5, and 0.5–1.0 OD ranges), each in triplicate. (C) Decline of NADH absorbance (averages of triplicates) as a function of time. (D) Averages of ATP turnover rates plotted as a function of time. Note that MP reactions containing lower initial NADH inputs (0.5–1.0 and 1.0–1.5 OD range) are inhibited past the 3000- and 6000-s time points, respectively, due to NADH pool depletion. (Inset) Rates of ATP turnover calculated within 600–2100 s of the time course. Note that the lower activity of Rad54 measured by CS was due to the instability of the diluted protein, as this experiment was performed later in time.

to the ATPase may not be significantly higher than the rates of the background NADH decomposition. To evaluate the precision of the microplate reader under our assay conditions, the rate fluctuation of NADH samples for different fill volumes (kinetic data for Fig. 1A) and the corresponding blank samples (water) were calculated (Fig. 1C). Assuming linearity of the rate of UV-induced NADH decomposition in the absence of the ATPase, the measured fluctuation is mainly due to spectrophotometer-borne errors. For both blank and NADH-containing samples, the well fill volume did not appreciably affect the rate fluctuation.

*Application for measurement of ATPases with low specific activity.* A significant number of ATP-hydrolyzing enzymes have turnover rates of  $<1 \text{ min}^{-1}$  or may be unavailable for analysis in large amounts. Therefore, we aimed to determine the sensitivity of the microplate-based ATPase assay, which is limited primarily by the UV-induced NADH decomposition and by the rate fluctuation due to instrument errors. Under current assay conditions, the background rate of NADH decomposition can be kept as low as  $100 \text{ nmol/min}$  (Fig. 1D) and the rate fluctuation of the blank and NADH-containing samples do not exceed 80% of the background values (Fig. 1C). The UV-induced NADH decomposition can be further reduced by increasing the fill volume (up to  $300 \mu\text{l}$  for 96-well plates), which will also increase the sensitivity of absorbance detection but requires more enzyme. Note that the geometry of 384-well microplates allows another fourfold increase in the lightpath and sensitivity of absorbance detection of the same fill volume. Background NADH decomposition can also be reduced by lowering the NADH concentration. Practically, absorbances of  $A_{340} = 0.25\text{--}0.5 \text{ OD}$  are within the range where accurate measurements can be done. Finally, increasing the intervals between readings (from 30 s to 1 or several minutes) can minimize the UV exposure. We also recommend turning off the autocalibration mode, when available, which will significantly reduce the sample exposure to UV light.

We have performed a comparison of measurements of low ATPase activities using the conventional spectrophotometer (CS) and the microplate reader (MP). Rad51 protein, a yeast RecA-like protein, hydrolyzes ATP in a DNA-dependent manner with turnover rates of  $0.6\text{--}0.7$  and  $0.05\text{--}0.1 \text{ min}^{-1}$  for ssDNA and dsDNA, respectively [9]. Figs. 2A and B represent the data of several independent experiments in which the Rad51 ATPase activity on ssDNA and dsDNA was measured by both methods (CS and MP) at various enzyme concentrations and DNA/protein ratios. The microplate-based assay allows accurate measurement of ATP hydrolysis rates as low as  $0.25 \mu\text{mol/min}$ . The data obtained by MP were consistent with those obtained by CS and with published data that had been determined by an independent technique (release of radioactive  $P_i$ , [9]).

Note that the fluctuation rates of the NADH absorbance decrease were generally lower for the MP reader than for the CS.

*Application for measurement of ATPases with high specific activity.* The microplate-based ATPase assay has a particular advantage for kinetic absorbance measurements of enzymes with high ATP turnover rates ( $>1000 \text{ min}^{-1}$ ). The low NADH pool capacity of a CS (up to  $300 \mu\text{M}$ ) imposed by its range of absorbance linearity (in general between 0.1 and 1.7 OD) may require repeated replenishment of NADH during the time course. In contrast, an MP reader ensures fair linearity up to 2.5 OD and the NADH pool capacity can be further expanded by reducing the fill volume. We performed a comparison of the kinetics of Rad54 protein, a potent dsDNA-dependent ATPase with a turnover rate of  $\sim 1000 \text{ min}^{-1}$ , measured by the CS and MP. The microplate-based assay allows continuous recording of the ATPase kinetics during at least 3 h of time course with an initial NADH input of about 2.0 OD (Figs. 2C and D). Moreover, triplicate measurements made by MP appear to have less intraexperiment variation (0.5–3.5%) than data obtained by CS (6.5%; Fig. 2D inset).

## Conclusion

We have refined the NADH-coupled ATPase assay for use with a microplate spectrophotometer. A step-by-step guideline as to how to optimize the assays is provided (Table 1). We show that the microplate-based assay can be successfully applied to kinetic studies of a broad range of ATP-hydrolyzing proteins including those with very low specific activity (e.g., bacterial RecA protein and its eukaryotic homologs). The improved microplate assay has an increased sensitivity down to the submicromolar range comparable to that using the conventional spectrophotometer. The microplate-based method exhibits advantages over the conventional spectrophotometer assay: better data reproducibility, significantly higher capacity for parallel analysis, and the flexibility of changing assay parameters to fit particular needs.

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