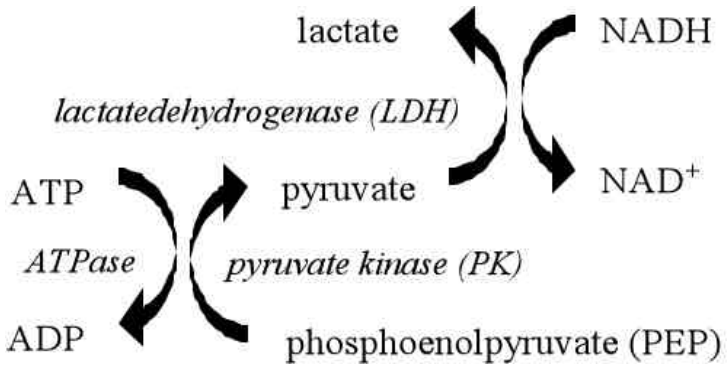
ATP/NADH coupled assay for Rad54 ATPase

The assay is based on a reaction in which the regeneration of hydrolyzed ATP is coupled to the oxidation of NADH. Following each cycle of ATP hydrolysis, the regeneration system consisting of phosphoenolpyruvate (PEP) and pyruvate kinase (PK) converts one molecule of PEP to pyruvate when the ADP is converted back to the ATP (see figure). The pyruvate is subsequently converted to lactate by L-lactate dehydrogenase (LDH) resulting in the oxidation of one NADH molecule. The assay measures the rate of NADH absorbance decrease at 340 nm, which is proportional to the rate of steady-state ATP hydrolysis. The constant regeneration of ATP allows monitoring the ATP hydrolysis rate over the entire course of the assay. A 96-well microplate format reader permits the simultaneous analysis of up to 96 samples.



The assay is typically performed at 24°C, with 5 nM Rad54 protein and indicated concentrations of DNA or preformed DNA-Rad51 complexes in 150 µl volume of the ATPase reaction buffer supplemented with a regeneration system (3 mM PEP, 20 U/ml PK), 20 U/ml LDH and NADH to give an A_{340} of 0.5-2.0. Absorbance data are collected using a SPECTRAmax 250 microplate spectrophotometer equipped with SOFTmax PRO software (Molecular Devices). The rate of ATP hydrolysis is calculated from the equation:

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

where K_{path} is the molar absorption coefficient for NADH for a given optical path length. For 150 µl well fill volume K_{path} is equal to 2,383. Rates are calculated per 180 sec time frames (each comprising 7 data points) overlapping with 30 sec increment and plotted as a function of time. The rates are normally corrected for background NADH decomposition of controls containing no ATPase.

Materials and stock solutions. ΦX174 supercoiled dsDNA (NEB, 1 mg/ml) Rad51 protein (wt 156 μM, KR ?). PEP (Sigma):150 mM stock solution. LDH (Sigma): 8.76 u/μl stock suspension in ammonium sulphate. PK (Sigma): 2.27 u/μl stock suspension in ammonium sulphate. Prepare freshly as a 50x concentrated mixture of the two enzymes as follows: pull X/2.27 μl PK and X/8.76 μl LDH stock suspensions together, spin down 2 min in Eppendorf centrifuge at max speed, discard supernatant and dissolve protein pellet in X μl of water. NADH (Sigma) lyophilized, 2 mg per vial. Prepare freshly by dissolving it in 100 ul of water (20 mg/ml stock).

The complete reaction buffer consists of

- 1x ATPase reaction buffer (25 mM TEA pH 7.5, 13 mM magnesium acetate, 1.8 mM DTT; available in 10x concentrated stock)
- 5 mM ATP (0.1 M stock)
- 100 μg/ml BSA (10 mg/ml stock)
- 3 mM PEP (150 mM stock)
- 20 u/ml PK and 20 u/ml LDH (prepared as 50x concentrated stock, see above)
- NADH is supplied just before the kinetics starts (usually 4-8 μl of a 20 mg/ml stock is added to the 150 μl of reaction mixture).

Order of assay assembly.

1. Calculate the required number of experiment and control samples and a total volume of buffer to prepare (with slight excess).
2. Prepare complete reaction buffer, dispense into tubes.
3. Make all incubations required before the ATPase assay (e.g. form DNA-Rad51 complexes).
4. Transfer 150 μl reaction samples into a 96 well microplate. Make a blank reading.
5. Add NADH, mix well (1 min on plate shaker), make a single-point reading to check the absorbance (if needed, add more NADH and verify again).
6. Add Rad54 protein, mix very well (2x1 min on the plate shaker) start kinetics promptly.