ADP/ATP Ratio Bioluminescent Assay Kit

(Catalog #BN00522; Store kit at –20°C)

I. Introduction:

The changes in ADP/ATP ratio have been used to differentiate the different modes of cell death and viability. Increased levels of ATP and decreased levels of ADP have been recognized in proliferating cells. In contrast, decreased levels of ATP and increased levels of ADP are recognized in apoptotic cells. The decrease in ATP and increase in ADP are much more pronounced in necrosis than apoptosis. ADP/ATP Ratio Assay kit utilizes bioluminescent detection of the ADP and ATP levels for a rapid screening of apoptosis, necrosis, growth arrest, and cell proliferation simultaneously in mammalian cells. The assay utilizes the enzyme luciferase to catalyze the formation of light from ATP and luciferin, and the light can be measured using a luminometer or Beta Counter. ADP level is measured by its conversion to ATP that is subsequently detected using the same reaction. The assay can be fully automatic for high throughput and is highly sensitive (can detect 100 mammalian cells/well).

II. Kit Contents:

Components	BN00522	Cap Code	Part Number
Nucleotide Releasing Buffer ATP Monitoring Enzyme ADP Converting Enzyme Enzyme Reconstitution Buffer	50 ml Lyophilized Lyophilized 2 ml	NM Green Blue Red	BN00522-1 BN00522-2 BN00522-3 BN00522-4

III. Reagent Reconstitution and General Consideration:

1. Reconstitute ATP Monitoring Enzyme with 2 ml of the Enzyme Reconstitution Buffer & mix gently by inversion.
2. Reconstitute ADP Converting Enzyme with 220 µl of the Nucleotide Releasing Buffer & mix gently by inversion.
3. The reconstituted enzymes are stable for up to 2 months at 4°C after reconstitution. For more accurate handling, the reconstituted ADP-Converting Enzyme can be diluted 10-fold with Nucleotide Releasing Buffer just before use (Section IV Step 7), then use 10 µl of the enzyme for each assay.
4. ADP Kit is significantly more sensitive than other methods used for cell viability assays. As a rule, we recommend using 1 x 10^4 - 1 x 10^6 cells per assay. Avoid contamination with ATP from exogeneous biological sources, such as bacteria or fingerprints.
5. Ensure that the Nucleotide Releasing Buffer is at room temperature before use. The optimal temperature is 22°C. Keep enzymes on ice during the assay and protect from light as much as possible.
6. The assay can be performed using either a single tube or a white walled 96-well luminometer plate (100 µl/well culture volume is recommended).

IV. ADP/ATP Ratio Assay Protocol:

1. Induce apoptosis in cells by desired method. Concurrently incubate a control culture without induction.
2. For each sample well to be measured, mix 100 µl of reaction mix consisting of:

   ATP monitoring enzyme: 10 µl
   Nucleotide Releasing Buffer: 90 µl
3. Add 100 µl of the reaction mix to the appropriate wells of a 96-well plate and read the background luminescence (Data A). For higher accuracy let the reaction mix sit at room temperature to burn off low level ATP contamination for a few hours.
4. For suspension cells, remove culture media and treat cells with Nucleotide Releasing Buffer for 5 minutes (10^5 – 10^6 cells/ml). Transfer 10 µl of the cultured cells (10^3 – 10^4 cells) into luminometer plate.
5. For adherent cells, remove culture medium and treat cells (10^3 – 10^4) with 50 µl of Nucleotide Releasing Buffer for 5 minutes at room temperature with gentle shaking. Transfer into luminometer plate.
6. Mix well. After ~2 minutes, read the sample in a luminometer or luminescence-capable plate reader (Data B).
7. To measure ADP levels in the cells, read the samples (from step 6) again (Data C), then add 1 µl of ADP Converting Enzyme. Read the samples after ~2 minutes (Data D).

   Note:

   The results can be analyzed using cuvette-based luminometers or Beta Counters. When Beta Counter is used it should be programmed in the "out of coincidence" (or Luminescence mode) for measurement. The entire assay can directly be done in a 96-well plate*. It can also be programmed automatically using instrumentation with injectors (When using injector the ATP Monitoring Enzyme and the ADP Converting Enzyme can be diluted with the Nuclear Releasing Buffer at 1:50 for injector).

   *The assay utilizes a "glow-type" luciferase which has replaced the original "flash-type" luciferase. While still sensitive to sub-picomole amounts of ATP, the glow-type reactions can still be read an hour later. This means that ATP & ADP levels are now determined by quasi-steady-state light output levels. This makes the reading of an entire 96-well (384-well) plate much more feasible.
8. **Calculation**:

   ADP/ATP Ratio is calculated as: $\frac{\text{Data D} - \text{Data C}}{\text{Data B} - \text{Data A}}$

Interpretation of Results:

Cell Fate	ADP Level	ATP Level	ADP/ATP
Proliferation	Very Low	High	Very Low
Growth Arrest	Low	Slightly Increased	Low
Apoptosis	High	Low	High
Necrosis	Much Higher	Very Low	Much Higher

The interpretation of different ratios obtained may vary significantly according to the cell types and conditions used. However, the following criteria may be used as guidelines:

a. Test gives markedly elevated ATP values with no significant increase in ADP levels in comparison to control cells = proliferation.
b. Test gives similar or slightly higher levels of ATP and with little or no change in ADP compared to control = growth arrest.
c. Test gives lower levels of ATP to control but shows an increase in ADP = apoptosis.
d. Test gives considerable lower ATP levels than control but greatly increased ADP = necrosis.

FOR RESEARCH USE ONLY! Not to be used on humans.

GENERAL TROUBLESHOOTING GUIDE:

Problems	Cause	Solution
Assay not working	• Use of a different buffer	• Refer data sheet and use buffers as indicated
	• Omission of a step in the protocol	• Refer and follow the data sheet precisely
	• Plate read at incorrect wavelength	• Check the wavelength in the data sheet and the filter settings of the instrument
	• Use of a different 96-well plate	• Fluorescence: Black plates ; Luminescence: White plates; Colorimeters: Clear plates
Samples with erratic readings	• Use of an incompatible sample type	• Refer data sheet for details about incompatible samples
	• Samples prepared in a different buffer	• Use the Nucleotide releasing buffer provided in the kit or refer data sheet for instructions
	• Samples were not deproteinized (if indicated in datasheet)	• Use the 10 kDa spin cut-off filter or PCA precipitation as indicated
	• Cell/ tissue samples were not completely homogenized	• Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope
	• Lysates used after multiple free-thaw cycles	• Aliquot and freeze sample lysates, if needed to use multiple times
	• Presence of interfering substance in the sample	• Troubleshoot if needed, deproteinize samples
	• Use of old or inappropriately stored samples	• Use fresh samples or store at correct temperatures till use
Lower/ Higher readings in Samples	• Improperly thawed components	• Thaw all components completely and mix gently before use
	• Use of expired kit or improperly stored reagents	• Always check the expiry date and store the components appropriately
	• Allowing the reagents to sit for extended times on ice	• Always thaw and prepare fresh reaction mix before use
	• Incorrect incubation times or temperatures	• Refer datasheet & verify correct incubation times and temperatures
	• Incorrect volumes used	• Use calibrated pipettes and aliquot correctly
Unanticipated results	• Measured at incorrect wavelength	• Check the equipment and the filter setting
	• Samples contain interfering substances	• Troubleshoot if it interferes with the kit
	• Use of incompatible sample type	• Refer data sheet to check if sample is compatible with the kit or optimization is needed
	• Sample readings above/below the linear range	• Concentrate/ Dilute sample so as to be in the linear range
Note: The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.		