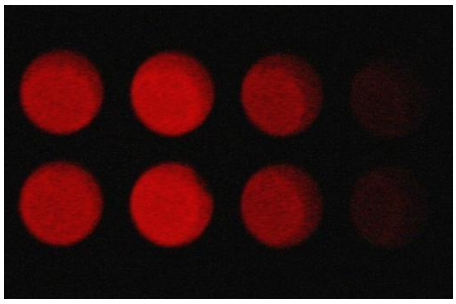
PRODUCT INFORMATION SHEET



RubyGlow™

LUMINESCENT CELL PROLIFERATION ASSAY KIT
LUMINESCENT CELL VIABILITY ASSAY KIT
LUMINESCENT CYTOTOXICITY ASSAY KIT

(Product M1574/1575/1576)

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RubyGlow™ Luminescent Cell Proliferation Assay Kit Product M1574;
RubyGlow™ Luminescent Cell Viability Assay Kit Product M1575;
RubyGlow™ Luminescent Cytotoxicity Assay Kit Product M1576

NOTE: This kit series provides easy and quick methods to quantify cell proliferation based on ATP generation in metabolically active cells. Each kit contains a genetically engineered luciferase which generates very stable luminescent signals. This luciferase emits red light and thus opens the door to multiplexing with currently available luciferase in cell-based assays. For more information about these techniques, please visit our website at www.markergene.com or contact our technical assistance department at techservice@markergene.com.

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I. OVERVIEW

Researchers often require rapid and accurate measurement of viable cell number and cell growth. They have traditionally assessed cell viability via membrane integrity (e.g., trypan blue exclusion), and cell proliferation via the incorporation of labeled nucleotides (e.g., [^{3}H]-thymidine) into newly synthesized DNA during cell division. An alternative technology used for quantifying cell proliferation is based on the generation of ATP in metabolically active cells.

Studies have shown that the level of intracellular ATP per cell is highly regulated and remains essentially constant in a cell population. The cell responds to the increased demand for ATP by increasing the production or turnover rate of ATP. Increasing levels of ATP are therefore reflections of cell proliferation and an increase in the number of viable cells per well. In addition, controlled studies have shown that ATP measurements correlate well with traditional tritiated thymidine incorporation methods. Any form of cell injury results in a rapid decrease in cytoplasmic ATP levels, and thus measuring ATP in drug treated cells can assess the viability of the cells as well as cytotoxicity of the drug.

The most successful technique for ATP measurement has proven to be the luciferin-luciferase bioluminescent assay. The use of the luciferase enzyme has become highly valuable as a genetic marker gene due to the convenience, high sensitivity and linear range of the luminescence assay. The reaction involved is described as below:

$$\text{Luciferase} + \text{ATP} + \text{Luciferin} + O_2 \longrightarrow \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{Light}$$

Thus measuring the units of light output will accurately reflect the amount of ATP in a sample or reaction.

Using genetic engineering, we have generated a new luciferase which exhibits long-wavelength light emission, as well as improved thermostability, compared to the native firefly luciferase often used in these assays. The light output is stable over several hours and thus eliminates the necessity for substrate injection. We have also adapted this kit series for use in a microtiter plate format and thus made it amenable to high-throughput applications. Our new RubyGlow™ luciferase emits red light (EM 619 nm) upon luciferin conversion, while most of the commercially available luciferases emit green light (562 nm). This unique feature enables the possibility of multiplexing.

The kit components involve no radioisotopes or toxic materials and are environmentally safe. The provided kit components are sufficient for 100 reactions in 96-well microplate format.

II. MATERIALS

1. **Buffer Solution**. Reaction buffer
2. **Buffer Solution**. Cell lysis buffer
3. **Substrate:** Luciferin, powder
4. **Enzyme:** Red Luciferase, lyophilized
5. **Control Cytotoxic Reagent:** Doxorubicin, 10mM in DMSO

Storage and Handling. Reagents, standards, and buffers should be handled with care, kept cold when not in use, and stored at -20ºC. In case of contact with skin or eyes, wash thoroughly with soap and cold water. Reagents should be stable for at least 6 months following purchase if stored properly.

<u>Equipment and materials not provided but required:</u>
Luminometer or plate reader with luminescence detector
96-well black walled assay plate (Corning #3792 round-bottom or #3915 flat bottom)
Multi-channel pipette

III. PROTOCOL

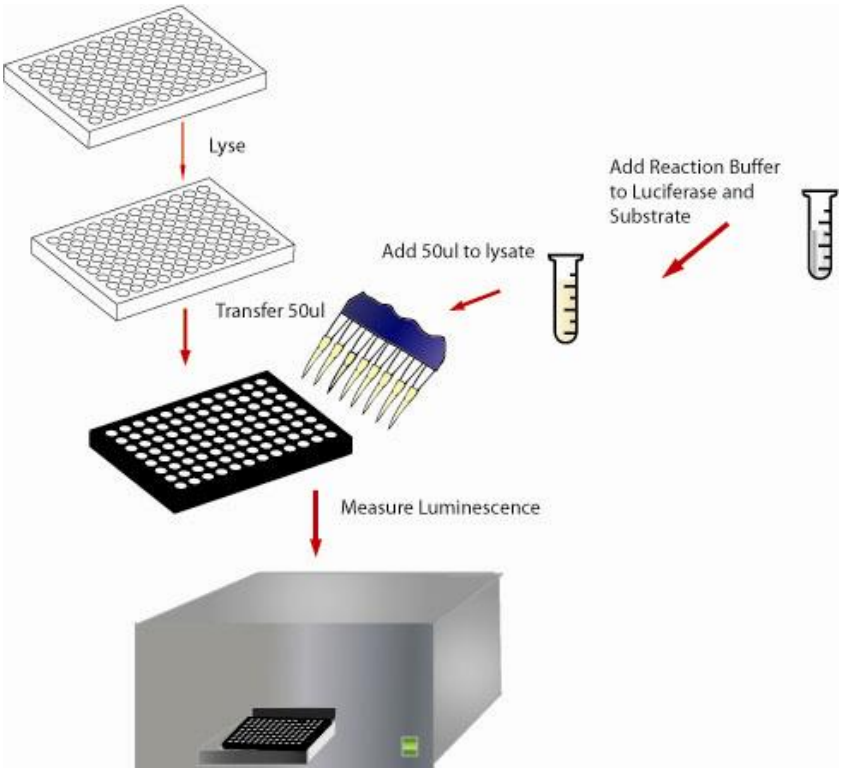


Figure 1. Diagram of assay flow.

A. Reconstitute to working solutions

1. Thaw “reaction buffer” and keep on ice once thawed.
2. Bring the lyophilized substrate and enzyme to room temperature.
3. Transfer exactly 5ml of reaction buffer to the bottle containing the lyophilized substrate, swirl or vortex gently to mix.
4. Transfer approximately 1ml of reconstituted substrate to the vial containing red luciferase; pipet up and down gently to resuspend the lyophilized enzyme. Transfer back this resuspended solution to the substrate bottle; swirl to mix.
5. The well mixed solution currently in the substrate bottle is the luciferin/luciferase working solution for the assay. This working solution should be kept on ice. Any unused solution can be stored at 4ºC and will remain active for at least 2 weeks according to our test. Alternatively, unused portions can be aliquotted and stored at -20ºC. Avoid repeated freeze/thaw cycles.

B. Protocols for proliferation, cell viability and cytotoxicity assays

Measuring cell number/proliferation:

1. Cells are seeded up to 30000 cells per well (varying the seeding as appropriate with different cell lines) in a 96-well microplate. Avoid seeding in the outer wells which include row A, H; column 1, 12. Instead, fill cell culture media in these wells. Incubate the plate at 37ºC in incubator overnight.
2. Next day, thaw out “cell lysis buffer”. Prepare luciferin/luciferase working solution as described in Section III A: 1-5.
3. For adherent cells, simply wash once with PBS and then add 60μl per well of cell lysis buffer to lyse the cells. For suspension cells, centrifuge the cells, aspirate off the medium and add cell lysis buffer at 60μl per well. Cell lysis is carried out for 30min on ice.
4. Transfer 50μl of the cell lysate to a 96-well black-walled flat or round bottom microtiter assay plate (Use the cell lysis buffer as blank). Dispense 50μl luciferin/luciferase working solution per well, mix and read in a plate reader [time for integration: 1 second] using the instructions provided by your microtiter plate reader manufacturer (see Figure 1 assay flow).
5. Plot the light output (RLU) versus cell number as shown in Example Figure 1a &1b below for cells plated with a known number of cells.
6. Use the curve generated in Step 5 to determine the number of cells present in the wells of unknown seeding. We suggest running steps 1-5 for each distinct cell line as the linear curve may vary with different cells.

Measuring cell viability:

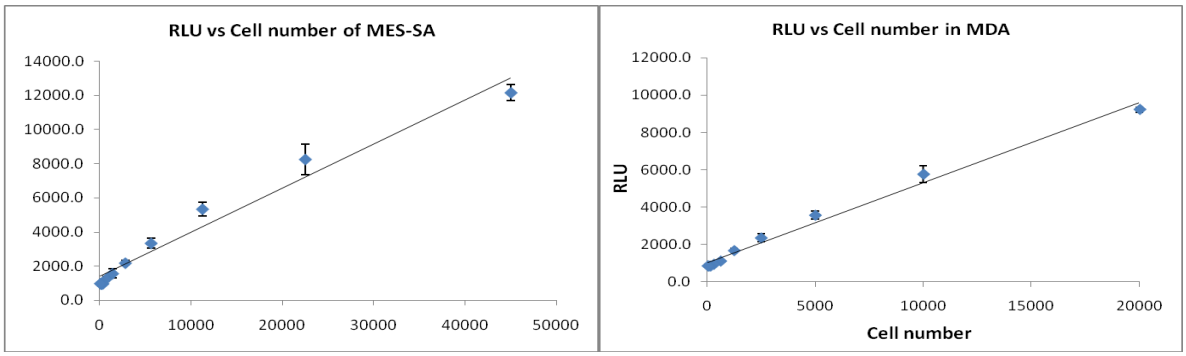
1. Cells are seeded in complete culture medium into 96-well microplate wells. Seeding should be optimized to reach 20-30% confluency following overnight incubation. Again avoid seeding into outer wells.
2. Next day, a series of dilutions of doxorubicin in complete medium are prepared and added to the cells to reach a final concentration of 0.05 to 5μM in the wells. Similarly, a series of dilutions of the test compound are prepared and added to the wells to reach a final concentration up to 30μM. The cells are incubated at 37ºC for an additional 48hrs.
3. Thaw out cell lysis buffer. Prepare the luciferin/luciferase working solution for assay as described in Section III A: 1-5.
4. For adherent cells, simply wash the cells once with PBS and then add 60μl per well of cell lysis buffer to lyse the cells. For suspension cells, centrifuge the cells, aspirate off the medium and add cell lysis buffer at 60μl per well. Cell lysis is carried out for 30min on ice.
5. Transfer 50μl cell lysate to a 96-well black walled assay plate (cell lysis buffer as blank). Dispense 50μl luciferin/luciferase working solution per well, mix and read with a microtiter plate reader [time for integration: 1 second] using the instructions provided by your microtiterplate reader manufacturer (Figure 1).
6. Plot the light output versus concentration of drug/sample as shown in Example Figure 2 below.

Measuring Cytotoxicity of a reagent:

1. Cells are seeded in complete culture medium into 96-well microplate wells. Seeding should be optimized to reach 50-60% confluency following overnight incubation. Again avoid seeding in outer wells.
2. Next day, a series of dilutions of doxorubicin in complete medium are prepared and added to the cells to reach a final concentration of 0.05 to 5μM. Similarly, a series of dilutions of the test compound are prepared and added to the wells to reach a final concentration up to 30μM. The cells are incubated with the reagents at 37ºC for an additional 24hours.
3. Thaw out cell lysis buffer. Prepare luciferin/luciferase working solution for assay as described in Section III A: 1-5.
4. For adherent cells, simply wash the cells once with PBS and then add 60μl per well of cell lysis buffer to lyse the cells. For suspension cells, the cells are centrifuged, the medium is aspirated and cell lysis buffer is added at 60μl per well. Cell lysis is carried out for 30min. on ice.

5. Transfer 50µl of cell lysate to wells in a 96-well black-walled assay plate (Use 50 µL of cell lysis buffer as blank wells). Dispense 50µL luciferin/luciferase working solution per well, mix and read with a plate reader [time for integration: 1 second] using the instructions provided by your microtiterplate reader manufacturer (Figure 1).
6. Plot the light output versus concentration of drug/sample as shown in Example Figure 3 below.

IV. Examples



2a 2b

Figure 2. Cell proliferation measured by luciferase: a. 80-45000 MES-SA cells/well were seeded in triplicate; b. 37-20000 MDA cells/well were seeded in triplicate. Cells were incubated at 37ºC overnight, then lysed with 60µL Cell lysis buffer. 50µL of the lysate was mixed with equal volume of Luciferase/Luciferin working solution. Luminescence was immediately recorded with Biotek Synergy Mx microplate reader under gain setting of 135 and integration time of 1 second.

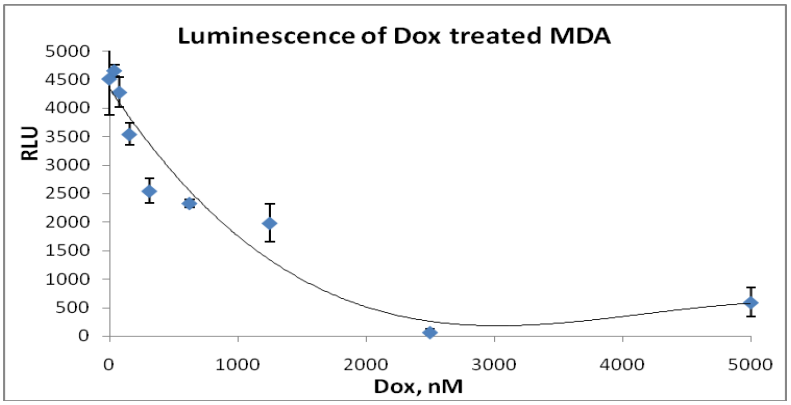


Figure 3. Cell viability measured by Luciferase: 7000 MDA cells/well were seeded and incubated at 37ºC overnight. Next day, Doxorubicin was added to the cells to reach the final concentration of 0.05-5µM and incubated for additional 48hrs. Then cells were lysed with 60µl Cell lysis buffer. 50µl of the lysate was mixed with equal volume of Luciferase/Luciferin working solution. Luminescence was immediately recorded with Biotek Synergy Mx microplate reader under gain setting of 135 and integration time of 1 second.

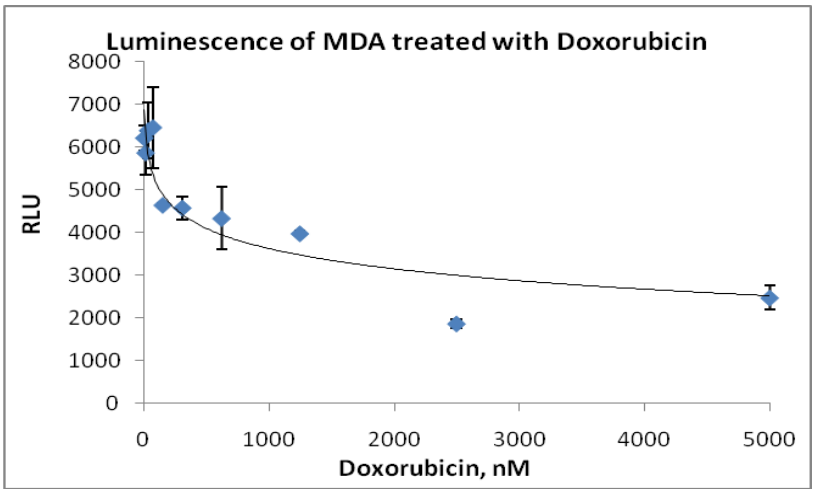


Figure 4. Cell cytotoxicity measured by Luciferase: 10000 MDA cells/well were seeded and incubated at 37ºC overnight. Next day, Doxorubicin was added to the cells to reach the final concentration of 0.05-5μM and incubated for additional 24hrs. Then cells were lysed with 60μl Cell lysis buffer. 50μl of the lysate was mixed with equal volume of Luciferase/Luciferin working solution. Luminescence was immediately recorded with Biotek Synergy Mx microplate reader under gain setting of 135 and integration time of 1 second.

M1574/ M1575/ M1576 KIT CONTENTS			
DESCRIPTION	**QUANTITY**	**PART NO.**	**STORAGE**
REAGENTS			
LUCIFERIN SUBSTRATE REAGENT	1.4 MG	1574/5/6-001	F, L
RUBYGLOW™ LUCIFERASE	0.5 MG	1574/5/6-002	F, L
DOXORUBICIN (10MM)	50 UL	1575/6-005	F
BUFFERS			
REACTION BUFFER	6 ML	1574/5/6-003	F
CELL LYSIS BUFFER	7 ML	1574/5/6-004	F
DOCUMENTATION			
MSDS SHEETS	3		
PRODUCT INFORMATION SHEET	1		

Notes: F=store at or below -20°C; C=store cold (4°C); L=lyophilized; T=avoid repeated freeze/thaw; R=read protocol/instructions carefully prior to use.

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