MGM Instruments, Inc.

Analytical Instruments for Science, Medicine and Industry

APPLICATION NOTE

USING THE PROMEGA DUAL-LUCIFERASE REPORTER ASSAY SYSTEM WITH THE OPTOCOMP™ II LUMINOMETER

Introduction

Promega Corporation's Dual-Luciferase Reporter Assay System adds the benefit of an internal control to the firefly luciferase reporter gene assay.

The internal control can be used to normalize for sample-to-sample variations, such as in cell population and condition or transfection efficiency. This benefit is made possible by the use of two reporter vectors in a single assay. Thus, the ability to individually and sequentially activate and measure each reporter enzyme is necessary.

To process a dual reporter gene assay, the Optocomp™ II Automated Luminometer has the ability to perform an inject-read-inject-read sequence on each sample processed. In processing the Promega Dual-Luciferase Reporter Assay System, the first injection adds the firefly luciferase reagent, activating luminescence of the firefly luciferase enzyme in the sample, which is then measured by the instrument's sensitive photon-counting photomultiplier detector. Once the firefly luciferase activity has been measured, the Renilla luciferase reagent is added to the sample, quenching firefly luciferase luminescence and activating luminescence of the Renilla luciferase in the sample. The Renilla luciferase luminescence is then measured. All of the reagent addition and measurement of the resulting luminescence is made automatically by the Optocomp™ II, with precisely repeated timing for every sample.

The purpose of this application note is to provide information on how to program, run and maintain your Optocomp™ II luminometer to properly process the Promega Dual-Luciferase Reporter Assay.

A Technical Manual is available from Promega (Promega part number TM040), which provides important information on using the Dual-Luciferase Reporter Assay System. ***We recommend, very strongly, that you obtain the Dual-Luciferase Reporter Assay System Technical Manual and read it thoroughly before attempting to run the assay.***

Contact us at: 800-551-1415, or at (203) 288-3523, or visit our web site at http://www.mgminstruments.com

Programming the Optocomp™ II to process the Dual-Luciferase assay

The Optocomp™ II Dual Read Protocol performs two reads on each sample, and always uses two injectors. The first read is timed from the first injection; the second read is timed from the second injection. The ratio of the first read and the second read is then calculated and printed. Dual Read Protocols are used to process assays that measure two reactions in each sample, such as the Promega Dual-Luciferase Reporter Assay.

We wish to suggest, very strongly, that you read Promega's Dual-Luciferase Reporter System Technical Manual thoroughly before programming the luminometer or attempting to run the assay.

In particular, take note of Promega's cautions regarding careful checking of the linear range of the assay/instrument.

Proper maintenance of the Optocomp™ II injector system is critical. Please read the Maintenance section of this Application Note.

In a Dual Read Protocol, the RLU for each read of each sample, the average RLU and %CV of the replicates, and ratio of the first and second reads for each replicate and the averages are reported.

The Blank Tube Subtraction feature subtracts the machine and reagent background from each sample.

Programming a Dual Read Protocol involves selecting:

- Count time for the first and second read of each tube
- Time delays between injections and reads
- Number of sample replicates
- Blank tube subtraction and number of replicates

Procedure:

At the Main Menu

1. Press **PROGRAM**, **0**, **ENTER**, to program a protocol (as opposed to the QUEUE), enter a protocol number, press **ENTER**.
2. Press **ENTER** to program or edit the protocol.
3. Press **1**, to enter a name for the protocol, or **ENTER**, to skip entering a name, or to accept the displayed name, if any.
4. Press **4** [=DUAL READ], **ENTER**.
5. Enter the delay required between the first injection and the first count (read) (**Promega recommends 2.0 seconds**).
6. Enter the first count time (**Promega recommends 10.0 seconds**).

Programming the Optocomp™ II to process the Dual-Luciferase assay

7. Enter the delay required between the first count and the second injection (**0 seconds for the Dual-Luciferase Reporter Assay**).
8. Enter the delay required between the second injection and the second count (**Promega recommends 2.0 seconds**).
9. Enter the second count time (**Promega recommends 10.0 seconds**).
10. Select the ratio to be reported;

    Press **0**, **ENTER**, to report the results of the first read for each tube divided by the results of the tube's second read. This will provide the ratio of the firefly luciferase activity to the Renilla luciferase (control) activity.

11. Select the RS-232 data output format;

    Comma delimited format is ideal for importing data into popular spreadsheet programs.

    Press **0**, **ENTER**, to output all printed text.

    Press **1**, **ENTER**, to output data in RSP format.

    Press **2**, **ENTER**, to output data in comma delimited format. This is the recommended selection for importing data into spreadsheet programs.

12. Press **0**, **ENTER**, for no Blank tube subtraction, press **1**, **ENTER** to perform Blank tube subtraction.

    Refer to Promega Technical Manual no. TM040, Dual-Luciferase Report Assay System, for information on background (Blank tube) subtraction.

    If Blank tube subtraction is selected, you are next prompted for the number of Blank tube replicates, enter from **1** to **10**, **ENTER**.

13. Enter the number of sample replicates, from **1** to **10**, **ENTER**.

Programming Notes

Blank Tube Subtraction

When this option is selected, each blank tube replicate is processed the same as the samples will be, using the same injections, injection delay timing and count times. The average of the first read made for all of the blank tube replicates is then subtracted from the first read of each sample tube, and the average of the second read made for all of the blank tube replicates is subtracted from the second read of each sample tube. The use of multiple Blank Tube replicates is encouraged, as blank tubes usually result in relatively low count values and thus are prone to significant statistical variance.

Refer to Promega Technical Manual no. TM040, Dual-Luciferase Report Assay System, for information on background (Blank tube) subtraction.

Injector parameters

Both injectors are automatically used in Dual Read protocols; therefore the injector selection menu encountered in other protocol types is not displayed when programming a Dual Read protocol. However, programming the delays between the injections and the reads, and between the first read and the second injection, correctly is critical to ensure proper assay performance. Please refer to the instructions provided with the assay you will be using to ensure these parameters are programmed correctly.

RS-232 Output Format

Please refer to the Computer Interface section of the Optocomp™ II user's manual for more information on this feature.

Listing the Dual-Luciferase Protocol

List Protocol

Before running the Promega Dual-Luciferase Report Assay, LIST (print-out) the protocol that you have programmed for the DLR assay.

LIST

Press* LIST*, then the protocol number you programmed, and* ENTER*.

Confirm that the injector timing and count times match this listing.

```
****************************************

         PROTOCOL    4 LISTING

NAME:                  DUAL READ PROTOCOL
TYPE:                          DUAL READ
INJECTORS:                       A AND B
1ST INJECT TO COUNT DELAY:         2 SEC.
1ST COUNT TIME:                   10 SEC.
1ST COUNT TO 2ND INJECT DELAY:     0 SEC.
2ND INJECT TO COUNT DELAY:         2 SEC.
2ND COUNT TIME:                   10 SEC.
PRINT RATIO OF:                1ST / 2ND
RS-232 OUTPUT FORMAT:     DELIMITED TEXT
BLANK TUBE SUBTRACTION:               NO
NO. OF BLANK TUBE REPLICATES:          0
NO. OF SAMPLE REPLICATES:              2
```

This sample Dual Read protocol has been programmed to read the Promega Dual-Luciferase Reporter Assay System. For this reagent system a two second delay is programmed between the first reagent injection and the first 10-second read (the firefly luciferase activity). The second injection begins immediately upon the conclusion of the first read. A two second delay is programmed between the second reagent injection and the second 10-second read (the Renilla luciferase activity).

Running the Dual-Luciferase Protocol

Dual Read Protocols perform two separate reads on each sample, with injector A injecting prior to the first read and injector B injecting prior to the second read. The RLU for each read and the ratio of the two reads is reported. If more than one replicate is programmed in the protocol, then the average and %CV for each read and the ratio of the two averages is reported. Count values reported are in RLU per second only if the count time programmed for the protocol is one second.

The following example is a Dual Read protocol programmed for the Promega Dual Luciferase Reporter Assay System using two replicates per sample.

1. Fill a cassette with empty tubes, and place the cassette in position #1 in the instrument (refer to figure 1, page IV-2 in the Optocomp™ II Operators Manual).
2. Place the injector inlet tubes into the containers of the appropriate reagents for the assay. ***For the Promega Dual-Luciferase Reporter Assay, the inlet tube for injector I (A) should go into the LAR II (Luciferase Assay Reagent II) container, and the inlet tube for injector II (B) should go into the Stop & Glo reagent container.*** Insure that the tips of the injector inlet lines are at the bottoms of the reagent containers, and that there is enough reagent in each container to prime the injectors and process all of the blank and sample tubes that will be run in the assay.

INITIALIZE

3. Press **INITIALIZE**, **START**. The instrument will prime the injectors, into the empty tubes in the first cassette, to insure the dispensed volumes are accurate during the assay. This should be done periodically to insure the reagents dispensed are fresh.

START

4. Place your blank tubes (if used) and samples in the cassettes, starting at the cassette at position 1. Close the lid, and rotate the handles to the locked position.
5. At the Main Menu, press **START**, **START**, **0** (**INITIALIZE** was done above), **ENTER**, **0** (=**MANUAL MODE**), **ENTER**, enter the protocol number for the desired Dual Read protocol, **ENTER**, select an Operator's name, if desired, and **ENTER**. The instrument will start the protocol and read all of the tubes automatically. When the instrument detects two consecutive empty tube positions, the protocol will be terminated, and the instrument will return to the Main Menu.

A sample printout is shown on the next page.

Running the Dual-Luciferase Protocol

This sample printout gives an example of the results of a Dual-Luciferase Report Assay System run on the Optocomp™ II.

```
**************************************

PROTOCOL  27
  FRIDAY      OCT.  11, 1996    10:07 AM
  SYSTEM SERIAL NUMBER:            121789
  SOFTWARE REVISION:                 2.11
  PROTOCOL TYPE:                DUAL READ
  1ST COUNT TIME:                   1 SEC.
  2ND COUNT TIME:                   1 SEC.
  OPERATOR:                           SCM
  *** FOR INVESTIGATIONAL USE ONLY ***

---------------------------------------
TUBE BLNK    1ST RLU    2ND RLU  RATIO
   1                9         12   0.75
   2                9         10    0.9
   3               13         10    1.3
 19% 9.1%          10         11 0.9091

TUBE SAMP    1ST RLU    2ND RLU  RATIO
   4    1      67331      55232   1.22
   5    1      66989      55656   1.20
.25% .38%       67160      55444   1.21

   6    2     124736      57888   2.15
   7    2     128901      57921   2.23
1.6% .03%      126819      57905   2.19
```

Protocol header:

Records the protocol number, name and type, the date the assay is run, the instrument's serial number and software revision, programmed count times and operator name.

Results:

- The first column is tube number within assay.
- The second column is tube type, and sample number.
- The third column is the net RLU counted for the first read (the firefly luciferase).
- The fourth column is the net RLU counted for the second read (the Renilla luciferase).
- The fifth column is the ratio of the two reads (the normalized firefly luciferase activity).

Following each sample's results (if programmed for more than one replicate) the following is reported:

- The first column is the %CV for the first read.
- The second column is the %CV for the second read.
- The third column is the mean of the first read.
- The fourth column is the mean of the second read.
- The fifth column is the ratio of the means.

Important Considerations

This section provides information that can help insure accurate results are obtained when using the Promega Dual-Luciferase Report Assay System with the Optocomp™ II luminometer.

Dynamic Range

The luminescent activity (i.e. the intensity of the light emitted by your samples) is dependant on a number of factors, including; the health and number of cells in each sample, the ratio of the two reporter vectors in the transfection mix, the transfection efficiency, and any factors that affect the level of expression of the reporter vectors.

High levels of expression of either reporter vector can result in saturation of the Optocomp™ II detector, resulting in inaccurate results.

Purified luciferase, or luciferase expressed in cell lysates, can be prepared in a serial dilution to establish the linear range of the Optocomp™ II luminometer. Please refer to section V., B. in Promega's Technical Manual for the Dual-Luciferase Reporter Assay System for details on determining the linear dynamic range of the instrument with the Dual-Luciferase Reporter Assay System.

If the ratio of the firefly luciferase vector to the Renilla luciferase vector used in the transfection mix is high (as Promega recommends, please refer to Promega's Technical Manual for the Dual-Luciferase Reporter Assay System), and the Renilla luciferase activity measured by the instrument is relatively high, this is an indication that the firefly luciferase activity in some samples may be too high for the Optocomp™ II to measure accurately.

Static Electricity

Certain test tubes can become charged with static electricity; polystyrene test tubes are particularly prone to static electricity.

Static on test tubes can discharge during the luminescent measurement, causing inaccurate assay results. Static can also cause injected reagents to splatter on the inside wall of the test tubes, also causing inaccurate results.

This effect can be minimized by carefully wiping the outside of each test tube with a clean lint-free wipe (such as a Kim-Wipe), lightly moistened with water, prior to inserting each test tube into the instrument. This removes the static charge from the exterior of the test tube.

Polypropylene test tubes are much more resistant to static electricity, and are recommended.

Maintenance

Proper maintenance of the Optocomp™ II is essential, and is very simple to perform.

The reagents used in luciferase assays will foul injectors very quickly if left in the instrument while it is not used. It is essential that the injector system be flushed out thoroughly whenever an assay is completed.

Flushing the injector system out every time the instrument is used, and keeping the instrument clean, especially inside the measurement chamber, will help insure accurate results from your assays. We have had a number of instruments returned for improper injector performance; in nearly all cases the injector bellows and valves are clogged with congealed reagents.

When you have completed an assay, flush out the injector system thoroughly by performing the following steps:

1. Remove the injector inlet line(s) from your reagent container(s)
2. Place the injector inlet line(s) into a container of filtered or DI water (up to 50% methanol may be added).
3. Place a cassette containing 10 empty tubes into the instrument, at the first cassette position (the right, front of the instrument).
4. Press **INITIALIZE**, **START**.
5. The Optocomp™ II will pump the reagents out of the injector system, and pump the water through the injector system, and into the test tubes.
6. When the Initialize sequence is completed, remove the injector inlet lines from the container of water.
7. Remove the cassette from the instrument, and discard the ten test tubes containing the reagent and water.
8. Place a cassette containing 10 empty tubes into the instrument, at the first cassette position (the right, front of the instrument).
9. Press **INITIALIZE**, **START**.
10. The Optocomp™ II will empty the water out of the injector system, into the test tubes.
11. Remove the cassette from the instrument, and discard the ten test tubes containing the water.
12. **Carefully**, using a clean, lint-free cloth or tissue, wipe the area around the injection nozzles to remove any fluid splattered there when the injectors emptied. ***Be very careful not to disturb the nozzle tips.***

Troubleshooting Problems

The following table lists possible problems that might occur with the Dual-Luciferase assay or your Optocomp™ luminometer. Possible causes and solutions for each problem are listed.

PROBLEM	POSSIBLE CAUSE	POSSIBLE SOLUTION
Poor results	Samples are outside the dynamic range of the Optocomp™ II.	See discussion under **Important Considerations**, and refer to the Promega Technical Manual for the Dual-Luciferase Reporter Assay System.
	Static electricity.	See discussion under **Important Considerations**.
	Injector system clogged with old reagents.	Flush out injector system with mixture of 50% methanol and 50% DI water. See instructions under **Maintenance.**
	Contaminated injector system.	Flush out injector system with mixture of 50% methanol and 50% DI water. See instructions under **Maintenance.**
	Contaminated or old reagents.	Replace reagents.
Reagents splattered on sides of test tubes	Static electricity.	See discussion under **Important Considerations**.
	Injector system clogged with old reagents.	Flush out injector system with mixture of 50% methanol and 50% DI water. See instructions under **Maintenance.**
Low injector volume	Injector system clogged with old reagents.	Flush out injector system with mixture of 50% methanol and 50% DI water. See instructions under **Maintenance**.