APPLICATION NOTE

Time Course for LumiGLO® HRP Chemiluminescent Substrates on Nitrocellulose Membrane

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Chemiluminescent substrates are popular among researchers because they are typically more sensitive than precipitating substrates. Despite their popularity, the properties of chemiluminescent substrates on membranes are not well described. Typically, only the sensitivity of the substrate is mentioned; however, there are other factors to consider when designing a chemiluminescent Western blot such as the length of time to expose a membrane. Deciding how long to expose Western blots, especially when using film, often involves guess work as well as wasting expensive film in the process. From a practical standpoint, determining the kinetics of chemiluminescent substrates when using film is technically difficult to perform. As a result, substrate kinetics had to be extrapolated from experiments utilizing solution-based assays and a luminometer. While these solution-based assays are analogous experiments to Western blotting there are major differences. With the advent of imagers and automatic digital processing, kinetic experiments on membranes can now be explored and compared with solution-based assays.

Chemiluminescent substrates emit light due to an enzymatic reaction. As a reporter signal for the chemical reaction, light allows for the visualization and depiction of proteins in membrane-based immunoassays such as Western blots and dot blots. Light is emitted during the reaction when a molecule, excited by the enzymatic reaction to a higher energy level, returns to the ground state. When compared to alkaline phosphatase (AP) enzymes, chemiluminescent substrates that utilize horseradish peroxidase (HRP) reach peak intensity or "flash" quickly and are, therefore, useful for rapid immunoassays where images are often acquired shortly after the application of the substrate. In addition, researchers often covet the higher peak intensity produced by HRP chemiluminescent substrates as compared with AP substrates.

To help researchers choose the right HRP chemiluminescent substrate for their experiments, KPL explored the kinetic properties of the LumiGLO® product line. Specifically, the time courses or "glow times" of LumiGLO (Catalog No. 54-61-00), LumiGLO Reserve™ (Catalog No. 54-71-00), and LumiGLO Ultra™ (Catalog No. 54-51-00) were examined in solution using a luminometer and on a nitrocellulose membrane with an imager. Nitrocellulose membrane was chosen because it is the most commonly used membrane. Comparing HRP substrate kinetic performance in solution and on a membrane will determine the similarity between the two experiments. Ultimately, understanding the light-emitting kinetics of the LumiGLO product line on a membrane is essential in determining the proper experimental conditions for a successful immunoassay.

MATERIALS AND METHODS

Luminometry Protocol

1. The substrates tested were LumiGLO, LumiGLO Reserve, and LumiGLO Ultra. Each substrate was tested independently according to the following procedure. The substrate was mixed according to the manufacturer's instructions in a glass test tube at ambient temperature (20–25°C). After mixing, the substrate was added to a 1-cm^2 quartz cuvette.

2. KPL's Anti-Rabbit IgG (H+L) HRP-labeled Antibody (Catalog No. 074-1506) at 1 mg/mL was diluted (1:100,000 for LumiGLO and LumiGLO Reserve and at 1:1,000,000 for LumiGLO Ultra) in deionized water.

3. The diluted HRP (2 µL) was added to the cuvette, mixed by inversion 3 times, and placed in a fluorometer for measurement. The time between mixing and measuring was less than 20 s.

4. Light emission, at the peak emission wavelength (LumiGLO: 428 nm, LumiGLO Reserve: 431 nm, LumiGLO Ultra: 447 nm), was measured in a Shimadzu Spectrofluorophotometer (Model No. RF-5301PC) with no excitation light source over the course of 150 min.

Direct Assay Detection Protocol

1. A twofold serial dilution of 1 ng mouse IgG antigen (8 lanes) was separated by reducing SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked overnight at 4°C in KPL's Detector Block (5X) (Catalog No. 71-83-00, diluted according to instructions).

2. The membrane was washed 3 times for 5 min on a platform shaker with KPL's Wash Solution (20X) (Catalog No. 50-63-00, diluted to 1X with deionized water).

3. KPL's Anti-Mouse IgG (H+L) Human Serum Adsorbed and Peroxidase-labeled Antibody (Catalog No. 074-1806) was diluted to 0.1 µg/mL in Detector Block minus casein powder

and used to probe the membrane for 1 h at ambient temperature (20–25°C) on a platform shaker.

4. The immunoblot was washed 1 time for 15 min and 3 times for 5 min in KPL's Wash Solution, followed by a final 5-min wash in deionized water.

5. KPL's LumiGLO, LumiGLO Reserve, or LumiGLO Ultra was prepared according to manufacturer's directions. Membranes were incubated with substrate for 1 min and blotted dry with blotting paper. Immunoblots were placed in Syngene's G:BOX® imager for a series of non-additive 5-min exposures over 150 min. One additional Western blot was imaged with 2 mL of LumiGLO substrate pooled on top (i.e., not blotted dry).

6. Densitometry data was collected using GeneTools® version 4.03. The mouse IgG light chain protein band (1-ng lane) was used for densitometry on all 3 immunoblots. The protein band was background subtracted and charted versus time.

RESULTS

A luminometer was used to monitor the light emitted from the LumiGLO substrate line after addition of a HRP-conjugated antibody. Due to the intensity of the signal, the antibody conjugate added to LumiGLO Ultra was diluted tenfold compared to LumiGLO and LumiGLO Reserve. The luminometry data, displayed in Figures 1–3, shows the kinetics of the reaction. Light emitted from all three substrates fades after about 150, 75, and 28 min for LumiGLO, LumiGLO Reserve, and LumiGLO Ultra, respectively. The time at which the intensity of the light is half of the starting value (a pseudo half-life) was 14, 11, and 25 min for LumiGLO, LumiGLO Reserve, and LumiGLO Ultra, respectively. These results are summarized in Table 1.

Figure 1. Light Emission Kinetics for LumiGLO Chemiluminescent Substrate in Solution

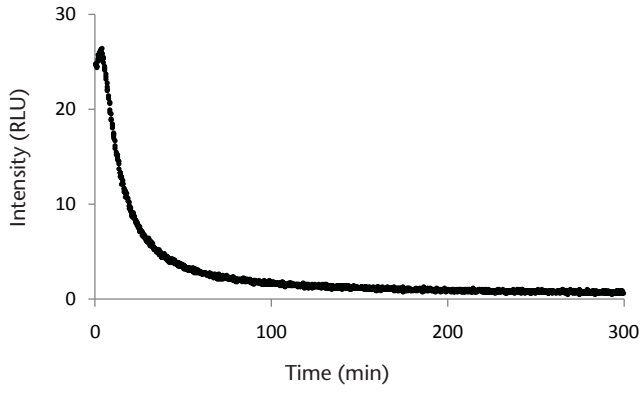


Figure 2. Light Emission Kinetics for LumiGLO Reserve Chemiluminescent Substrate in Solution

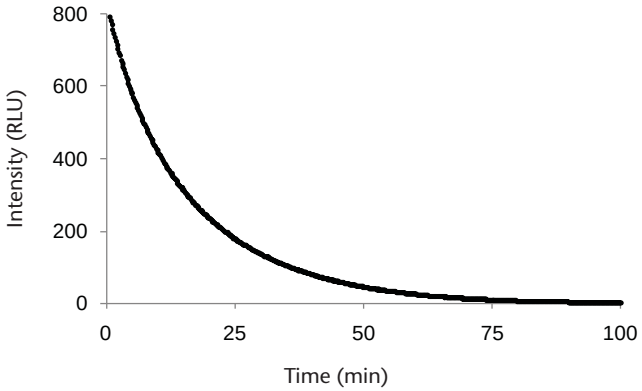
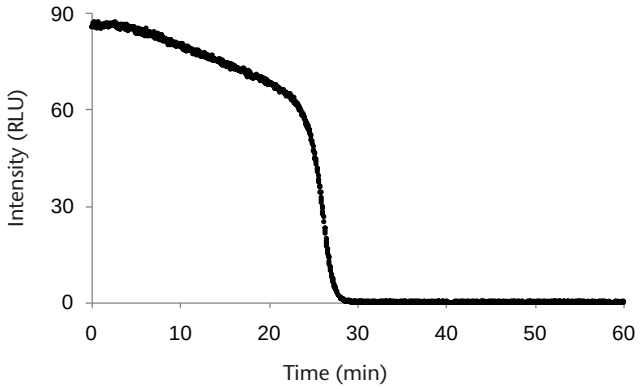


Figure 3. Light Emission Kinetics for LumiGLO Ultra Western Blotting Substrate in Solution



While the parameters of the luminometry experiments were chosen to mimic a Western blot, in reality, the experiments are very different. Notably the amount of substrate is in excess in the luminometry experiment and limited when a membrane is blotted dry. Also, the concentration of HRP-conjugated antibody is different. In one case, the HRP is suspended in solution, and when bound to a membrane, HRP is localized and concentrated.

To determine the kinetics of the LumiGLO substrates on membranes, Western blots were examined on an imager. Mouse IgG was directly detected with a HRP-labeled antibody. The light chain protein band (~50 kDa) was picked for densitometry analysis due to its initial signal intensity and distinctness (i.e., the protein band was well separated from other protein bands) Figures 4–6 show the kinetics of the membrane reaction. Note that the total "glow times" for all three substrates was similar, from 60 to 75 min. The time at which the intensity of the light is half the starting value (the pseudo half-life) varied from 10 to 30 min. These results are summarized in Table 1.

The most obvious result when comparing the luminometry-based experiments with the membrane-based experiments is that the kinetics are distinctly different. This dissimilarity is probably due to the differences between solution-based chemistry and chemistry on a solid support. Surprisingly the total "glow time" for all substrates on membranes was very similar (60–75 min). The results suggest that "glow time" estimates for Western blots cannot be extrapolated from a solution-based assay.

Note that in almost all cases the reaction rate maximum was reached with the dead time of mixing the substrate and the start of detection. Immunoblots detected with HRP-based substrates do not need lengthy immersion periods in substrate before detection (unlike AP-based substrates). The results suggest that for maximum signal intensity the HRP-based chemiluminescent substrates should be imaged immediately after blotting dry. Researchers should also note that maximum exposures were essentially complete after 1 h, and further imaging time is not needed.

Finally, one would expect that adding additional substrate would change the rate of the reaction on the membrane. Pooling chemiluminescent substrates on top of membranes in an imager is one technique to extend the "glow time" of Western blots. Indeed, when we pooled LumiGLO over a Western blot, the signal and lifetime was greatly improved (Figure 4). However, while pooling LumiGLO improved the lifetime of the light signal, pooling LumiGLO Reserve and LumiGLO Ultra on membranes produced swirls of activated substrate and made Western blot analysis difficult.

CONCLUSION

Assay developers should note that the kinetics of the three KPL HRP-based chemiluminescent substrates are distinctly different in solution than on a membrane. Western blots should be imaged immediately after the substrate has been blotted dry, and the light-emitting reaction will stop after 1 h.

Table 1.

Substrate	Time at 1/2 Intensity in Solution (min)	Signal Duration in Solution (min)	Time at 1/2 Intensity of Western Blots (min)	Signal Duration on Western Blots (min)
LumiGLO	1	150	10	60
LumiGLO Reserve	11	75	30	60
LumiGLO Ultra	25	28	25	75

Figure 4. Light Emission Kinetics for LumiGLO Chemiluminescent Substrate on Nitrocellulose

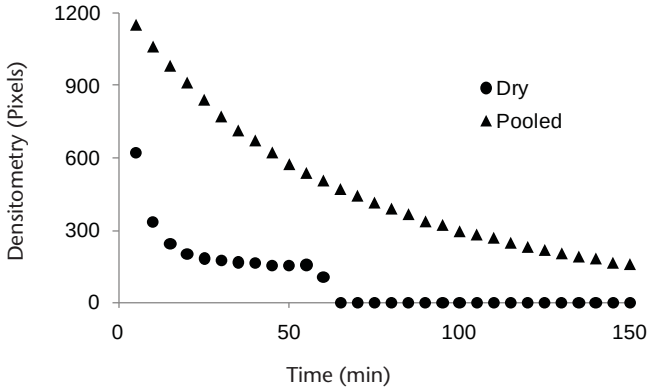


Figure 5. Light Emission Kinetics for LumiGLO Reserve Chemiluminescent Substrate on Nitrocellulose

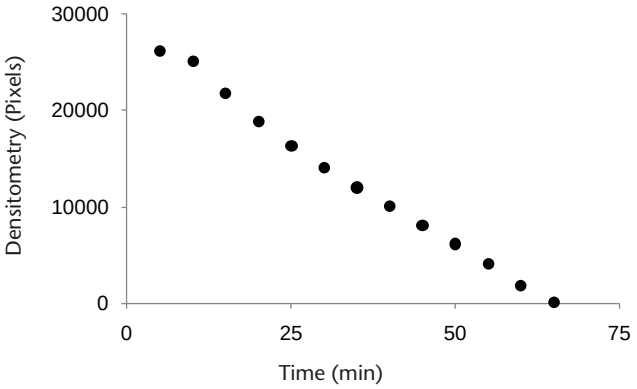
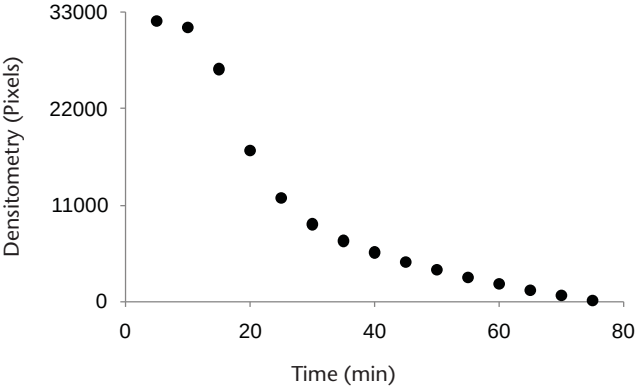


Figure 6. Light Emission Kinetics for LumiGLO Ultra Western Blotting Substrate on Nitrocellulose



PRODUCTS

Description	*Size*		*Catalog Number*
LumiGLO Chemiluminescent Substrate	240	mL	54-61-00
LumiGLO Reserve Chemiluminescent Substrate	1000	cm^2	54-71-02
LumiGLO Ultra Western Blotting Substrate	50	mL	54-51-00
5X Detector Block	240	mL	71-83-00
Wash Solution (20X)	800	mL	50-63-00
Anti-Mouse IgG (H+L) HSA and HRP-Labeled Antibody	1.0	mg	074-1806
Anti-Mouse IgG (H+L) HSA and HRP-Labeled Antibody	1.0	mg	074-1506

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