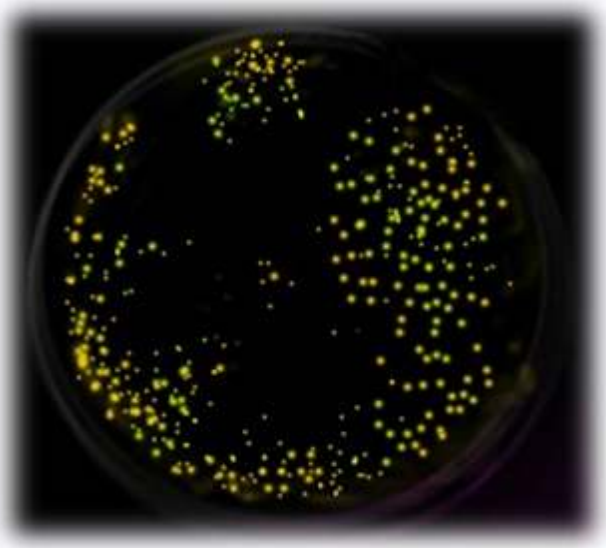
GOLDBIO®

Luciferin *In Vitro* Handbook



D-Luciferin *in vitro* Protocol

Procedure for use with Gold Biotechnology D-Luciferin;
Catalog #: LUCK® (Luciferin, Potassium Salt) and LUCNA (Luciferin, Sodium Salt)

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Editor - C. Menne (7/9/2021)

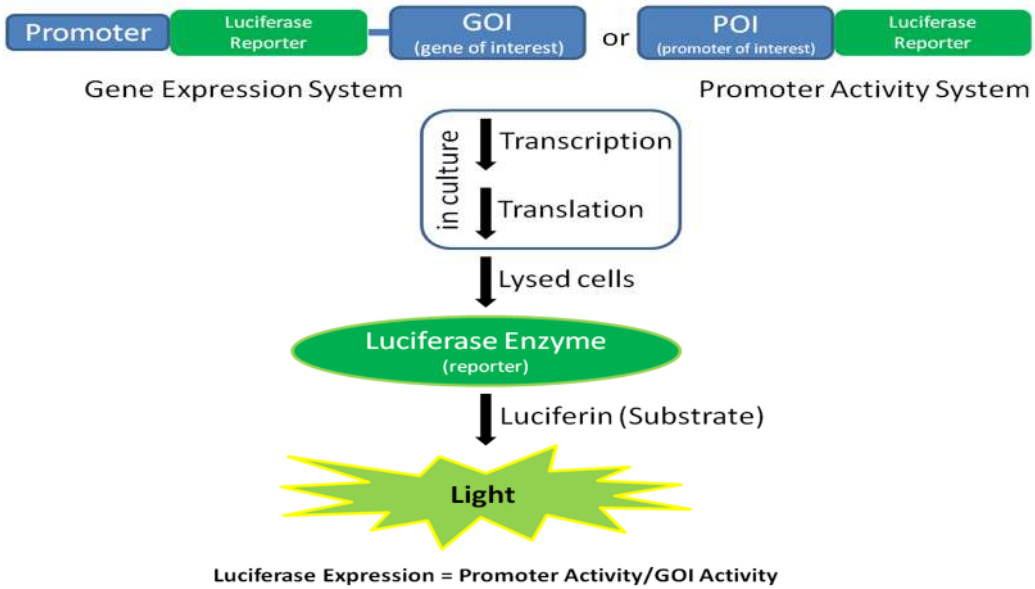
Gold Biotechnology
D-Luciferin *in vitro* Protocol

Introduction

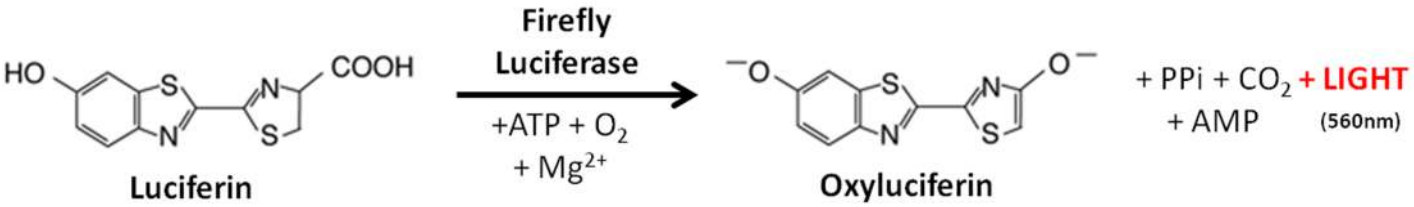
Luciferin is a common bioluminescent reporter used for *in vitro* imaging of the expression of luciferase. This water soluble substrate for the Firefly luciferase enzyme (typically from *Photinus pyralis*) utilizes ATP and Mg^{2+} as co-factors to emit a characteristic yellow-green emission in the presence of oxygen. Through the utilization of ATP, the reaction can be further used to indicate the presence of energy or life in order to function as a life-death stain.

Firefly luciferase is one of the most commonly used reporter genes for the study of gene expression due to its extreme sensitivity, and rapid, easy to use system. Luciferase is used to monitor promoter response activity in bacteria, cultured cells, as well as transgenic plants or animals. It is a ~61 kDa protein which is active as a monomer and does not require processing for its activity. By catalyzing the oxidative carboxylation of Luciferin, firefly luciferase produces a reaction with one of the highest efficiencies of any known bioluminescent. The luciferase activity can be used to characterize gene regulation by correlation with promoter/enhancer elements. When optimized, there is a direct relationship between the light produced by the chemiluminescent reaction and the transcriptional activity of the regulatory elements.

This handbook is intended to present a simple, affordable and reliable method for the detection of luciferase activity in your* in vitro *reporting system.

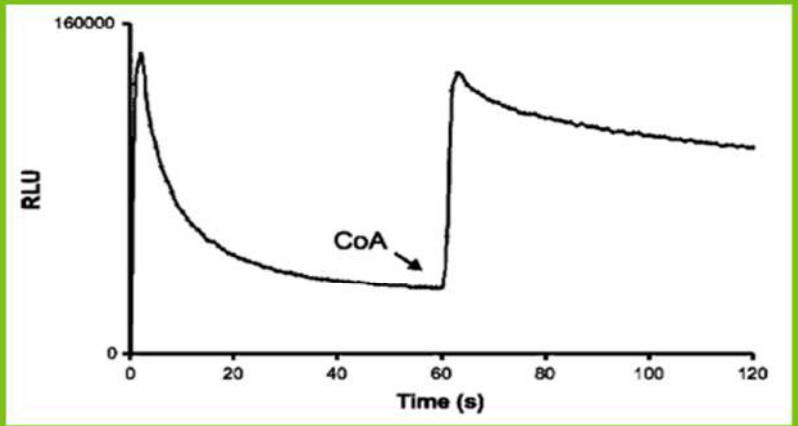


Bioluminescent Reaction



Several factors may affect the sensitivity of the reaction, including temperature, pH or substrate concentration. We recommend using buffers with a pH 7.8 and that all reagents are pre-warmed to room temperature before use for best results. We also recommend adding an excess of ATP and Mg^{2+} in the assay buffer for better reactions.

When Luciferin is added to the luciferase sample, there is an immediate flash of light that reaches peak intensity within 0.3-0.5 seconds. The light then begins to decay rapidly with a half life around 0.5-1.0 min. But when Coenzyme A has been initially added to the assay buffer, it is able to prevent the fast reaction decay extending the half-life of the reaction from 2-5 minutes. If added later to the reaction mixture, CoA also promotes additional, secondary flashes (Fraga, 2008).



The kinetic profile of Luciferase with high substrate concentrations (150µM ATP, 60µM Luciferin) and the effect of injecting 50µM CoA after 1 minute.

Fraga, H. (2008). Firefly luminescence: A historical perspective and recent developments. *Photochemical & Photobiological Sciences*, *7*(2), 146-158.

At Gold Bio, we recommend a luciferase assay buffer based on Yuichi Oba, *et al.* (2003):

- 100mM Tris-HCl (pH 7.8)
- 5mM $MgCl_2$
- 250µM CoA
- 150µM ATP (Fraga, 2008)
- 150 µg/mL d-Luciferin (GoldBio)

Some researchers have additionally added components such as dithiothreitol (DTT) or EDTA (Steghans, 1998) in the assay buffer. You may adjust your particular assay buffer to best fit your needs. We recommend conducting a pilot test of the kinetic curve on your specific cells for best results.

Product Specifications

D-Luciferin, Potassium Salt
4,5-Dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazolecarboxylic acid potassium salt
$KC_{11}H_7N_2O_3S_2$
MW: 318.42 g/mol

D-Luciferin, Sodium Salt
4,5-Dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazolecarboxylic acid sodium salt
$NaC_{11}H_7N_2O_3S_2 \cdot H_2O$
MW: 320.32 g/mol

Storage/Handling: Store at -20°C. Protect from light.

Materials

- D-Luciferin salt for GoldBio Luciferin Stock Solution (**GLSS**)
  - Luciferin, Potassium Salt (GoldBio Catalog # LUCK®), Proven and Published®
  - Luciferin, Sodium Salt (GoldBio Catalog # LUCNA), Proven and Published®
- GoldBio Luciferase Assay Buffer (**TMCA**)
  - Tris-HCl, Ph 7.8
  - $MgCl_2$
  - Coenzyme A (CoA) (hydrate)
  - ATP (disodium salt hydrate)
  - **GLSS** (GoldBio Luciferin Stock Solution)
- PBS (without Ca^{2+} or Mg^{2+})
- Cell Lysis Buffer
- Luciferase (for control)

GoldBio Luciferin Stock Solution (GLSS) Preparation

1. Prepare 15 mg/mL (100X) **GLSS** in molecular biology grade H_2O.
   a. For best results, the **GLSS** should be used immediately, but it may be separated into small aliquots and stored at -80°C for up to 1 month.

2. The final concentration of Luciferin substrate, **GLSS**, in the Luciferase Luminescence Assay should be: **150 µg/ml** (471µM for Luciferin-K or 468µM for Luciferin-Na).

GoldBio Luciferase Assay Buffer (TMCA) Preparation

Prepare all reagents as stock solutions in molecular grade H_2O prior to combining into **TMCA**.

1. Prepare a **500mM $MgCl_2$** (100x) solution.
    a. Weigh 476.05 mg (95.21 g/mol) of $MgCl_2$ and dissolve in 10 ml H_2O.
    b. Solution may be stored at RT.

2. Prepare a **25mM CoA** (100x) solution.
    a. Weigh 191.88 mg (767.53 g/mol) of CoA and dissolve in 10 ml H_2O.
    b. Prepare a 0.2 µm filter by drawing 5-10 ml of H_2O through the filter.
    c. Sterilize 100x CoA through pre-wet filter and store at -20°C.

3. Prepare a **15mM ATP** (100x) solution.
    a. Weigh 82.67 mg (551.14 g/mol) of ATP and dissolve in 10 ml H_2O.
    b. Store at -20°C.

4. Prepare **400mM Tris-HCl, pH 7.8** (4x) buffer.
    a. Weigh 4.85 g (121.14 g/mol) of Tris Base and dissolve in **85 ml H_2O**.
    b. Adjust pH with 1M HCl until pH 7.8 and bring volume to 100 ml with H_2O.

5. Prepare fresh 2x **TMCA** working stock at room temperature.
    a. Calculate the required **TMCA** for the experiment; combine all components to 2x working concentration (in H_2O) (subsequently reduced to 1x in luciferase assay).

        *Example: for 1 ml **TMCA** (2x):*
        500 µl Tris-HCl (4x stock)
        20 µl $MgCl_2$ (100x stock)
        20 µl CoA (100x stock)
        20 µl ATP (100x stock)
        Bring to 1 ml volume with H_2O.

    b. You may include additional ingredients (*i.e.* DTT, EDTA) at this time, if necessary.

GoldBio Cell Lysis Buffers

Gold Biotechnology currently offers a variety of Lysis Buffers for your particular needs. If you have questions about which one will work best for you, please contact us for more information!

- Bacterial Cell Lysis Buffer (GB-176/177)
- Mammalian Cell Lysis Buffer (GB-180)
- Tissue Culture Lysis Buffer (GB-181)
- Yeast Lysis Buffer (GB-178/GB-179)

Preparation of Cell Lysate for Luciferase Extraction

Adherent (Monolayer) vs. Nonadherent (Suspension) Cultures

There are two basic systems for growing cell cultures; as a monolayer culture on a substrate (adherent) or as a suspension (nonadherent) culture. Most animal-derived cells are anchorage-dependent (with the exception of **hematopoietic** or similar cell lines) and require a suitable substrate on which to be cultured to allow proper cell adhesion (often referred to as "tissue-culture treated"). But many lines can also be adapted for suspension cultures as well. **Suspension cultures** are not required to be "tissue-culture treated", but the medium should be agitated to facilitate adequate gas exchange and prevent cell death. Suspension cultures should also be monitored with daily cell counts to determine cell growth and density. Suspension cultures can always be diluted to stimulate additional growth.

Adherent cell cultures should be monitored daily with an inverted microscope to determine confluence. Most adherent cell cultures should be subcultured when they reach 80-90% confluence to avoid nutrient depletion and cell death. **Adherent cells <u>always</u> require tissue-culture treated dishes for proper adherence and growth.** It is possible to use glassware instead of disposable plastic plates for cell culture purposes, but it is then essential that all residual cleaning detergents are removed and that proper sterilization is carried out prior to use. The degree of adhesion will vary from cell line to cell line, but in most cases, trypsin (or some other protease) can be used to release the cells. Proteases might not be appropriate for some cell lines (e.g., when they may be harmful to the cells or when there are membrane markers or receptors of interest). Cells may also be brought into suspension using a cell scraper along with a small volume of media in order to physically detach them from the surface of the media plate or dish.

To Detach an Adherent Cell Culture:
(Based on - Methods in Molecular Biology, vol. 290: Basic Cell Culture Protocols)

1. Aspirate the culture medium from the dishes that have achieved the desired level of confluence and wash the monolayer of cells with 2–3 ml of RT PBS (no Ca^{2+} or Mg^{2+}) to remove any residual growth medium.

2. Aspirate the PBS and add 3–4 ml of RT trypsin–EDTA (T/E). Incubate the dishes at 37°C for 3–5 minutes. Progress should be monitored by examining the cultures using an inverted phase-contrast microscope.

3. Once the cells have begun to detach, transfer them to a centrifugation tube containing 6–7 ml culture medium (which contains sufficient **serum** to inhibit the trypsin activity) for centrifugation. To quantitatively recover all cells from the tissue culture dish, rinse the dish once or twice with 5 ml of the cell/medium mixture and add this cell suspension to the initial trypsinized cells.

4. Count cells using a hemacytometer and continue to lysis step.
   Note: The cells will be centrifuged and washed prior to lysis (see below).

Eukaryotic Cell Lysis (Mammalian or Yeast)

(Adapted from GoldBio's Mammalian Cell Lysis Buffer Protocol *or* Yeast/Fungi Cell Lysis Buffer Protocol)

Preparation before Use: Depending on applications, DTT and EDTA may be added. Prepare an appropriate volume of the specific Cell Lysis Buffer (Mammalian or Yeast) by adding DTT and EDTA both to a final concentration of 5mM. If the presence of a divalent metal ion is necessary for any application, do not add EDTA; instead, add an appropriate divalent salt to a final concentration of 5mM.

Protease Inhibition – If the inhibition of protease activity is required, add a cocktail of protease inhibitors to prevent protease activities during the extraction procedure (See ProBlock™ Gold Mammalian Protease Inhibitor, GoldBio #GB-331, *or* ProBlock™ Gold Yeast/Fungi Protease Inhibitor, GoldBio #GB-333).

Mammalian

1. Pellet the cells by centrifugation at 200-500 x g for 5 minutes. Remove and discard the supernatant. For adherent cells, scrape or detach cells from the culture plate (See above: "To Detach Adherent Cell Culture"), centrifuge and discard the supernatant.

2. Wash the cell pellet once with 5-10 ml PBS (without Ca^{2+} or Mg^{2+}). After removal of the supernatant, gently tap the side of the centrifuge tube to loosen the cell pellet. Resuspend the cells in 5-10 ml PBS by gently pipetting up and down. Pellet the cells again by centrifugation.

3. Remove and discard the PBS wash.

4. Resuspend the pellet in the remaining volume of PBS wash by gently pipetting up and down. Add Mammalian Cell Lysis Buffer and suspend the cell pellet. For each 10 ml of fully-grown suspension culture, add approximately 1 ml Mammalian Cell Lysis Buffer.

Alternatively: Add 1 ml Mammalian Cell Lysis Buffer for each 0.05 g of wet cell pellet. For making an even more concentrated cell extract, the volume of Mammalian Cell Lysis Buffer added to the pellet may be reduced. In such cases, one freeze and thaw cycle will ensure complete lysis of the cells.

5. Use a pipette to suspend the cells until you have a homogeneous suspension. Incubate the suspension on ice for 15-30 minutes. Periodically invert the suspension to aid in the lysis of the cells.

   Note: Freeze and thaw step is not necessary for lysis; however, one or two freeze and thaw cycle is not detrimental to the cell extract, and often ensures complete lysis.

6. Centrifuge the suspension at 20,000 x g for 30 minutes in a refrigerated centrifuge. Collect the clear suspension for analysis.

   Note: The cellular debris may contain some nuclear and membrane bound proteins, which may be further extracted with a variety of detergents.

Yeast

1. Pellet Yeast cells (culture OD_{600} 1.5-2.0) by centrifugation at 5000 x g for 5-10 minutes. Suspend the cell pellet in an equal volume of the Yeast Suspension Buffer. Add 1 µl of β-mercaptoethanol per 100 µl Yeast suspension.

2. Gently pipet up and down until the cell suspension is homogeneous. Incubate the suspension for 5 minutes at 4°C. Pipet gently again to suspend the cells.

3. Flick the vial containing Zymolyase® to mix the solution. Add 10 µl Zymolyase® for each 100 µl cell suspension. Gently mix the content.

4. Incubate the suspension at 37°C for 30-60 minutes. Lysis can be monitored by taking a 25 µl suspension, mixing with 1 ml Yeast Lysis Buffer and reading optical density at 800nm.

5. At the end of incubation, centrifuge the suspension at 1,500 x g for 5 minutes. Remove and discard the supernatant carefully, leaving the spheroplast pellet in the tube.

   OPTIONAL: *Add 5-10 volumes of the Yeast Suspension Buffer to the spheroplast pellet. Resuspend the spheroplast by gently tapping the tube. Centrifuge again as above and discard the supernatant.*

6. **Lysis:** Suspend the yeast pellet (now spheroplasts) in an appropriate volume of the Yeast Lysis Buffer (2-3 times the volume of cell pellet). Gently pipet the suspension up and down a few times. Invert the tube periodically and incubate on ice for 30 minutes.

Incubating the cells for 1-3 minutes at 37°C or a brief sonication step may further facilitate the lysis. Sonication is necessary for shearing genomic DNA. **Please note**: the higher Yeast Lysis Buffer to yeast pellet ratio, the better the cell lysis.

7. Centrifuge at 20,000 x g for 30 minutes at 4°C. Collect clear lysate. The lysate is now ready for analysis.

   NOTE: *Additional volume of Yeast Lysis Buffer can be purchased separately for downstream applications e.g. chromatography and dialysis, etc.*

- Zymolyase® is a registered trademark of Kirin Brewery Co. Ltd.

Bacterial Cells

(Adapted from GoldBio's Bacterial Cell Lysis Buffer Protocol)

Preparation before Use: Depending on applications, DTT and EDTA may be added. Prepare an appropriate volume of the Bacterial Cell Lysis Buffer by adding DTT and EDTA both to a final concentration of 5mM. If the presence of a divalent metal ion is necessary for any application, do not add EDTA; instead, add an appropriate divalent salt to a final concentration of 5mM.

Protease Inhibition – If the inhibition of protease activity is required, add a cocktail of protease inhibitors to prevent protease activities during the extraction procedure (See ProBlock™ Gold Bacterial Protease Inhibitor, GoldBio #GB-330).

Protein extraction with concurrent removal of nucleic acids

1. Pellet bacterial cells (Bacterial Culture OD_{600} 1.5-3.0) by centrifugation at 5000 x g for 10 minutes. Suspend the cell pellet in 5-10x volume of the Bacterial Lysis Buffer (e.g. for a cell pellet size of 25 µL, use 125-250 µL Bacterial Lysis Buffer).

2. Gently pipet up and down until the cell suspension is homogeneous. Incubate the suspension for 5 minutes in ice or at 4°C. Pipet gently again to suspend the cells.

3. Vortex the tube containing Lysozyme to mix the frozen suspension. Add 5 µL Lysozyme for each 100µL cell suspension in Bacterial Lysis Buffer. Gently mix the content.

4. Incubate the suspension at 37°C for 30-60 minutes.

   OPTIONAL - Lysis can be monitored by taking 25 µL of suspension and mixing with 1 ml Bacterial Cell Lysis Buffer and reading the optical density at OD_{590}.

5. At the end of incubation period, invert the content of the tube several times to complete the lysis. Lysis may be further assisted by pipetting the suspension up and down a few times with a narrow bore pipet tip or or a 20-gauge syringe needle.

Note - Additional volumes of the Bacterial Lysis Buffer may be purchased separately for downstream applications such as chromatography, dialysis, etc.

6. Removing DNA - During lysis, cellular DNA and RNA are cleaved which reduces the viscosity of the lysate. Some DNA fragments may survive, which would not interfere with downstream processing. However, for complete removal of nucleic acids, do not add EDTA in to the Bacterial Lysis Buffer. After lysis is complete, EDTA may be added to a final concentration of 2.5mM.

7. Centrifuge the lysate at 20,000 x g for 30 minutes at 4°C and collect the clear lysate. Lysate is now ready for analysis.

Protein extraction with spheroplast formation
Suitable when Lysozyme contamination is not acceptable. Use the above bacterial protein extraction method steps 1-4, then:

5. At the end of incubation, centrifuge the suspension at 200-500x g for 10 minutes. Remove and discard the supernatant carefully, leaving the spheroplast pellet in the tube.
    a. **OPTIONAL** - Re-suspend the spheroplast pellet in 5-10 volumes of the Bacterial Suspension Buffer. Centrifuge again as above and discard the supernatant.

6. **Lysis:** Suspend the spheroplast pellet in an appropriate volume of the Bacterial Lysis Buffer (2-3 times the volume of the spheroplast pellet). Pipet the suspension up and down a few times. Invert periodically and incubate on ice for 30 minutes. The lysis may be further facilitated by incubating the cells for 1-3 minutes at 37°C or a brief sonication step. Please note, the higher Bacterial Lysis Buffer to spheroplast pellet ratio the better the cells will lyse.

7. Centrifuge the lysate at 20,000 x g for 30 minutes at 4°C and collect the clear lysate. Lysate is now ready for analysis.

Isolation of Inclusion Bodies: For inclusion bodies isolation, after the lysis step centrifuge the bacterial lysate at 30,000x g for 30 minutes at 4°C. Collect the inclusion bodies pellet and wash twice with 10 fold diluted Bacterial Lysis Buffer (e.g. suspend in buffer and centrifuge to pellet the inclusion bodies). Collect the inclusion bodies for solubilization and refolding.

Luciferase Calibration Curve

It is always important to determine the linear range of light detection for your specific luminometer in order to calculate the absolute amount of luciferase in samples (if required). Luminometers can also experience signal saturation at high light intensities. To determine the linear range of your luminometer:

1. Make a series of dilutions of luciferase (using either your cell culture lysate or purified luciferase) in 1x lysis buffer (or in PBS buffer for purified luciferase) with 1 mg/ml BSA (BSA will help to stabilize the diluted luciferase enzyme).
    a. Also include a sample without luciferase (as a negative control) in order to measure the background luminescence.

    ***Purified Luciferase Stock**: Dissolve 1 mg luciferase in 100mM Tris-HCl pH 7.8/5mM $MgCl_2$. The luciferase may be aliquoted and frozen at -20°C or mixed with an equal volume of 100% glycerol and stored at -20°C (The glycerol stock will not freeze). Both the frozen luciferase stock and luciferase glycerol stock solutions remain active for at least 2 years when stored at -20°C.*

2. Add 100 µl of 2x **TMCA** (1x final concentration) to a cuvette or into the wells of a 96 well plate.

3. Add 2 µl of 100x **GLSS** (1x final concentration) to each sample.

4. Add 98 µl diluted luciferase stock solution or lysate to the cuvette or wells. Incubate at room temperature for 5-10 minutes, protected from light (this will allow for a more stable luminescence that will be more accurate for a calibration curve).
    a. For best results, incubate each sample for the same amount of time before reading.
    b. **Note**: This volume is calculated to account for addition of 100x **GLSS** for a final 1x **TMCA** concentration. For different total volumes, recalculate reaction mix accordingly.

5. Record light intensity with a luminometer.

6. Generate your luciferase calibration curve and calculate the light production vs. cell number (or vs. µg luciferase).
    Note: If your assay conditions do not yield a suitable linear profile, adjust the Luciferin concentration (usually lower).

Luciferase Luminescence Assay

The luciferase luminescence assay will largely depend on the luminometer (whether it is a manual, single-tube with injection or plate reading luminometer). Refer to your specific luminometer concerning its injection capability and programming directions (Note: **GLSS** concentrations may need to be adjusted to accommodate for the injection volume requirements for your specific luminometer).

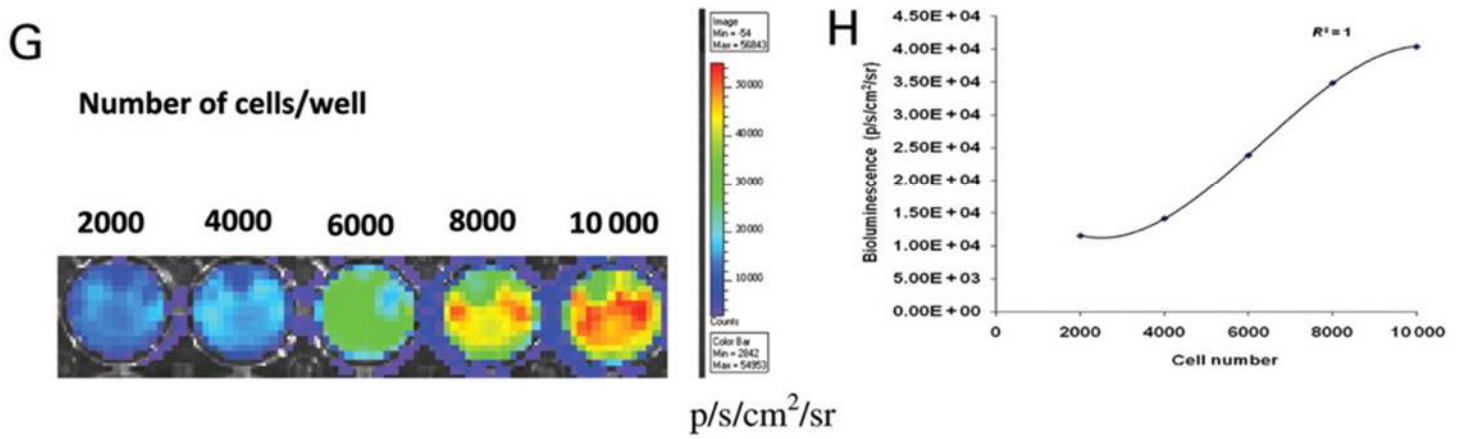
In the assay reaction mix, the final concentration of **TMCA** should each be 1x. The volume of lysate and **GLSS** should be adjusted accordingly to equal the final **TMCA** volume:

*Example 1: **Manual Luminometer** (200 µl final reaction volume)*

1. *Pipet 100 µl **TMCA** to a clean luminometer cuvette.*
2. *Pipet 50-98 µl lysate to cuvette.*
3. *Pipet additional H_2O, if necessary, to bring total volume up to 198 µl.*
4. *Pipet 2 µl **GLSS** (1x final concentration) and immediately read on the luminometer.*

*Example 2: **Injectible Luminometer** (single sample or plate) (200 µl final reaction volume)*

1. *Pipet 100 µl of **TMCA** into the luminometer cuvette or each well of a 96-well plate (solid white, flat bottom plates work best).*
2. *Pipet 50-98 µl of your lysate to cuvette or well.*
3. *Pipet additional H_2O, if necessary, to bring total volume up to 198 µl.*
4. *Program luminometer for injection of **GLSS**, with a 2-5 second delay, followed by 10 second measurement.*



(*G*) Bioluminescence imaging of increasing numbers of human adipose tissue-derived stem cells *in vitro*. Colour scale bar represents range of signals. (*H*) Bioluminescence imaging is proportional to cell number, with an R^2 value of 1.
Credit: Bai, X., Yan, Y., Song, Y. H., Seidensticker, M., Rabinovich, B., Metzele, R., ... & Alt, E. (2010). Both cultured and freshly isolated adipose tissue-derived stem cells enhance cardiac function after acute myocardial infarction. *European heart journal*, *31*(4), 489-501.

References

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