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A step by step protocol for absolute quantification of protein using *Arabidopsis thaliana* transgenic lines carrying NanoLUC-tagged genes

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Uriel Urquiza-Garcia^{1,2,3}, Nacho Molina^{4,3}, Karen Halliday², Andrew Millar³

¹Institute for Synthetic Biology, Faculty of Mathematics and Natural Sciences, CEPLAS-Cluster of Excellence on Plant Sciences, University of Düsseldorf, Universitätsstr. 1 Building 26.12.U1.23, 40225 Düsseldorf, Germany;

²Institute for Molecular Plant Sciences, D. Rutherford Building, School of Biological Sciences, University of Edinburgh, King's Buildings, Edinburgh EH9 3BF, United Kingdom;

³Centre for Engineering Biology and School of Biological Sciences, University of Edinburgh, King's Buildings, Edinburgh EH9 3BF, United Kingdom;

⁴Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) CNRS UMR 7104, INSERM U964, Université de Strasbourg, 1 Rue Laurent Fries, 67404 Illkirch, France

Uriel Urquiza-Garcia: ORCID 0000-0002-7975-5013;

Nacho Molina: ORCID 0000-0003-0233-3055;

Karen Halliday: ORCID 0000-0003-0467-104X;

Andrew Millar: ORCID 0000-0003-1756-3654; Corresponding author



Uriel Urquiza-Garcia

Heinrich-Heine-University Düsseldorf

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Abstract

Quantification of protein levels in absolute units will allow testing quantitative models of gene regulatory networks. Better quantitative models have application in fundamental plant biology but also in synthetic biology. Models in absolute units can, for example, help guide the design of promoter modifications using CRISPR genome editing technologies, such as base editing. Absolute protein quantification can also be applied to assessing the quality and biological meaning of affinity-constant measurements of transcription factors for DNA- or Protein-Protein interactions. Some absolute quantification methods using targeted proteomics have been described for mammalian cells and plant cells. However, gathering data using such methods is expensive, making it prohibitive for experimental set ups in which dynamic data is required (e.g.: inferring model parameters from time series in chronobiology applications). To overcome these shortcomings, we developed a Nano Luciferase based toolkit and methodology for performing absolute quantifications using recombinant NanoLUC and tagged lines. The steps presented in this chapter are extensively exemplified in the Doctoral thesis “A Mathematical Model in Absolute Units For the Arabidopsis Circadian Clock” (Urquiza Garcia 2018) and preprint (Urquiza Garcia et al. 2024; to be published in Molecular Systems Biology, 2025). Here we described steps to follow and tips for using NanoLUC for absolute quantification of a protein of interest.

Introduction

In systems and synthetic biology, quantitative data is important for model calibration and testing. In plant biology the largest source of semi-quantitative transcript data generated by RT-qPCR. Which has provided valuable data for quantitative model building and testing. This can be exemplified by many mathematical models of gene regulatory networks (circadian clock, flowering). In contrast, the generation of quantitative protein data has lagged significantly behind. However, having quantitative abundance data of proteins will aid in model refinement by providing the material for challenging model dynamics and parameters for plant systems (Andrew J. Millar, 2016; Andrew J. Millar et al., 2019).

The gathering of reliable, dynamic and quantitative protein data remains very challenging. Quantitative Western blots can be used to extract information in absolute units. However, quantitative Western blots can only be performed if the reagents are available (native antibodies, epitope tagged stable lines) and, although possible, there are practical limits with regards to the dynamic resolution that can be achieved with this technique. This leaves vast room for improvement in terms of sensitivity, reproducibility and ease in experimental design. Mass-spectrometry (MS) has been applied for absolute quantification of low abundance protein-like transcription factors in mammalian systems (Simicevic et al., 2013). Further work providing better control on the generation of multiplexed absolute quantification has been reported for animal clock proteins using MS-based Quantification by isotope-labeled cell-free products (MS-QBiC) (Narumi et al., 2016), in which multiplexed production of internal standards for quantification is achieved through Cell-free synthesis using the PURE system (Shimizu et al., 2001). The advantage of this approach is that it does not require the generation of time consuming stable transgenic lines. However, this approach still requires access to expensive



equipment and highly trained personnel. So extended time series over different experimental conditions that would be informative for model building and parameter inference can become prohibitively expensive. Therefore a methodology that provides quantitative data of enough quality for easy to access equipment and minimal training would be of high value.

Reporter based approaches are important tools in the elucidation of molecular mechanisms. In chronobiology and photobiology the use of Firefly luciferase (FLUC) has served as an invaluable tool. Data generated with this automatable reporter assay boosted the elucidation of key molecular mechanisms governing the emergence of circadian and photobiological responses in plants (A. J. Millar et al., 1995; Ulm et al., 2004). The instability of firefly provides a suitable reporter for tracking changes in transcriptional activity in a relatively straightforward and inexpensive way. However, the instability of FLUC has its drawbacks when studying molecular events that present half-lives significantly larger than the FLUC half-life in vivo. Therefore the use of a more stable reporter was desirable, for which the half-life is mainly determined by the process it is reporting. In particular, we noticed that the NanoLUC technology offers several advantages for tracking protein dynamics in plants (Hall et al., 2012; Urquiza-García & Millar, 2019). Here we describe in detail the process for performing absolute protein quantification, which is publicly available in the Doctoral thesis "A Mathematical Model in Absolute Units For the Arabidopsis Circadian Clock" (Urquiza García, 2018).

Guidelines

Notes

1. If the salts are mixed and the pH is measured at this point it will be lower than 5.5. However, the Agar type can impact the final pH therefore care has to be taken to ensure a final pH of 5.5.
2. We have observed significant lower MBP-NL30F10H expression in BL21 Rosetta 2 pLyS at temperatures below 35 °C.
3. Pelleting the resuspension before freezing facilitates handling of samples because Rosetta 2 pLyS tends to burst and release genomic DNA which results in a significant viscosity increase. This can then be reduced by passing the crude lysate through a narrow-gauge blunt-ended syringe needle.
4. We have observed that storing 35S:NL3F10H at -80°C results in a small decrease in activity (Urquiza-García & Millar, 2019). Therefore, we recommend maintaining the tissue in liquid nitrogen throughout the duration of the sampling and processing, or treating the calibration curve plants in exactly the same way (to control for changes in NanoLUC activity due to storage).
5. All photosynthetic organisms present a phenomenon called Delayed Fluorescence. This is luminescence that results after illumination due to processes related to the Photosystems II (PSII) and Cytochrome P680, which results in the emission of a photon. In seedlings, the signal is negligible after 60 seconds in darkness (Gould et al., 2009)
6. In order to facilitate interpretation of results, create a calibration curve that already represents the number of molecules per cell. It has been estimated that Col-0 fully expanded leaves contain on average 25 million cells per gram of fresh tissue (Flis et al., 2015). The user can create a standard curve with units of copies per cell by using this information plus the quantification of the NanoLUC standard, the molecular size of the NanoLUC standard, and the weight of the tissue collected.
7. Proteins related to the circadian clock and photobiology pathways can be highly unstable with high half-lives in the order of minutes (Jo et al., 2018). Therefore, working on and with protease and proteasome inhibitors is absolutely required while processing the samples.
8. Some plates can become autofluorescent upon exposure to light. Therefore, in order to minimize background noise, use plates that have been designed for bioluminescent measurements (like Greiner Lumitrac plates). NanoLUC activity is so high that using white plates might result in contamination of neighbouring wells by high emitting samples. Therefore, black plates provide a much better option given the strong signal emission of NanoLUC.
9. The NanoGlo® reagent has an emulsifier that is prone to generating foam. This might increase the experimental error during the assay.

Materials

Equipment

1. 250 ml Erlenmeyer flasks and shaker (e.g. Innova 44 New Brunswick)
2. High speed centrifuge up to 20,000xg for 2 ml polypropylene tubes
3. 37 °C orbital incubator shaker
4. Tristar2 LB 942 microplate reader (Berthold)
5. Liquid nitrogen
6. Spectrophotometer (600 nm)
7. Vibra-cell sonicator (Sonics & Materials Inc., Newton, Connecticut, US)
8. 1.5 ml polypropylene microtubes
9. 2 ml polypropylene Safelock (Eppendorf)
10. 50 ml conical polypropylene centrifuge tubes
11. 2 mm grinding balls
12. 96-well sterile plates (LUMITRAC, Greiner Cat. #655075)
13. Light source (e.g. Light DNA8 Valoya, cool white lamps, LED panels)
14. TopSeal-A plus plate seal (Perkin Elmer Cat. #6050185)
15. Tissue Lyser (Qiagen)
16. Analytical scale

2 Reagents

2.1 ROBUST media

1. Weight Agar (Sigma Cat. #A1296) ½ MS salts including vitamins (Duchefa Cat. #M0222.0001). MES Sigma. For 400 ml of Media add 4.8 g of Agar and 0.86 g of ½ MS salts with vitamins. Add 300 ml of deionised water and measure the pH. Adjust to 5.5 using either NaOH or HCl (see Note 1). Transfer the pH adjusted solution into a measuring cylinder and fill to 400 ml. Then transfer to a 500 ml borosilicate bottle and sterilize.

2.3 NanoLUC expression

1. 1M IPTG, dissolve 2.383 g of IPTG (Sigma Cat. #I6758) in 5 ml ddH₂O then fill to 10 ml. Prepare 250 µl aliquots in 1.5 ml polypropylene tubes and store at -20 °C.
2. Rossetta 2(DE3)pLysS Competent Cells - Novagen (Sigma Cat. #71403-3). Follow manufacturer instructions for handling and plasmid transformation.
3. Plasmids: pET28a(+) and pET28a(+):MBP-NanoLUC-3xFLAG-10xHis (Addgene ID141291) both Kan^R Cm^R have been described before (Urquiza-Garcia U. and Millar A. J. 2019).
4. Ni-NTA Agarose 25 ml (Qiagen Cat. #30210).
5. Lysis Buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM Imidazole, pH 8.0 NaOH adjusted).
6. Washing Buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM Imidazole, pH 8.0 NaOH adjusted)

7. BSIII buffer preparation buffer: 100 mm sodium phosphate, pH 8.0, 150 mm NaCl, 5 mm EDTA, 5 mm EGTA, 0.1% Triton X-100, 1 mM PMSF, Protease inhibitor Cocktail (Sigma Cat. #P9599), and 5 μ M MG132 (S)-MG132 (STEMCELL Technologies Cat. #73264).
8. 2ml Safe Lock tubes (Eppendorf) with 2 $\times$ 2 mm stainless steel grinding balls. Weigh each individual tube to control for the experimental error associated with weight variations due to the manufacturing process. Label each tube for the specific time point on the side of the tube.
9. NanoGlo[®] Luciferase Assay (Promega Cat. #N1110)

2.4 Plant strains, growth and transformation material

1. Col-0 (NASC id. 30210) and Col-0 pGWB601::35S:NanoLUC-3xFLAG-10xHis described in (Urquiza-Garcia U and Millar A. J. 2019)
2. Binary vectors pGWB401NL3F10H (KmR, Addgene ID 141285), pGWB501NL3F10H (HygR, Addgene ID 141286), pGWB601NL3F10H (BASTAR, Addgene ID 141287), pGWB701NL3F10H (TunR, Addgene ID 141288).
3. Agrobacterium strains ABI (KanR, CmR), AGL-1 (RifR, AmpR), GV3101 (pMP90) (RifR, GentR, KmR),
4. 300 mM Acetosyringone stock solution (Sigma Cat. #D134406). Weigh 0.588 g dissolve in 5 ml and fill to 10 ml with DMSO (Sigma Cat. #D8418). Can be stored at -20 °C, however it should be preferably prepared fresh.
5. Infiltration media (½ MS media containing 5% Sucrose, 3mM MES, pH 5.5 KOH adjusted, 200 μ l/L Silwet L-77, 150 μ M Acetosyringone) (all Sigma, except MS). For 1 L: 2.165 g ½ MS, 50 g sucrose, 0.59 g 3mM MES, 200 μ l Silwet L-77 and 500 μ l of 300 mM acetosyringone stock solution.
6. 2.5 mg/L glufosinate-ammonium (Sigma Cat. #45520), 0.01% Triton X-100 (Sigma Cat. #X100).
7. Hand-pumped sprayer bottle.
8. Levington Advance Seed & Modular F2S Compost mix or equivalent
9. Pots, Growth Chambers (e.g. Binder KBWF720)

Before start

Exploratory in planta time series (optional)

Before performing a discrete time series (manual sampling), we recommend performing a pilot time series experiment using a luminometer to perform whole plant assays. This will provide valuable information about the overall dynamics of the NanoLUC tagged protein. With this information the user can then select the most appropriate time points for absolute quantification assays. In particular the user is interested in determining the dynamic range of the signal. This will allow the user to select time points of lowest and highest signal level (see Urquiza Garcia 2018, chapter 5 or 6). Then this time points can be taken to optimize the protocol to avoid saturation of plate reader readings. Also, these in planta preliminary experiments need only 50 μ l of 1:10 Furimazine:0.01% Triton X-100 for each replicate and provide substantial amounts of quantitative data in arbitrary units (see Urquiza-Garcia U. and Millar A. J. 2019).

1. Melt ROBUST media and then pipette 100 μ l into each well that will contain a plant.
2. Once it has cooled down, place 1-3 seeds with a pipette in each well, keeping the number of seeds constant in all wells.
3. Seal the plate with a TopSeal-A plate cover and wrap them with aluminium foil. Stratify at 4°C for 3-4 days.
4. Germination synchronization can be optimized by giving a 2 hours white light pulse (100 μ mol m⁻² s⁻¹) followed by 22 hours of darkness at 21 °C.
5. After 22 hours transfer the plate to the growth conditions of interest.
6. Add 50 μ l of 1:5 Furimazine:0.1% Triton X-100 on the top of the seedlings of each well.
7. Place the plate in the luminometer and set your desired sampling conditions. For example, for the Tristar LB 942, we recommend measurements every 30 min - 1 hr with 1.5 s of integration time and 1 min delay in darkness prior to the measurement (see Note 5). Under these conditions we have been able to collect data for up to 9 days in a combination of photoperiodic and constant white light conditions.

Recombinant expression and purification of MBP-Nanoluc-3xFLAG-10xHis (MBP-NL3F10H)

- 1 Usually this step will be performed just before the absolute quantification will take place, to provide a fresh batch of pure NanoLUC. This step should be started two days before preparing the calibration curve to determine the absolute amount of NanoLUC tagged protein of interest
- 2 Perform transformation of chemical competent RosettaTM 2 (DE3) pLysS cells using pET28a(+) as (Control) and pET28a(+):MBP-NanoLUC-3F10H (or using other transformation method of choice), select on LB Cm34 Kan50 plate and incubate at 37 °C overnight.
- 3 Inoculate 50 ml of LB Cm34 Kan50 with two large colonies and track O.D.600. When cell culture reaches an O.D.600=of 0.1, add IPTG to a final concentration of 1 mM, and incubate at 37 °C 200 rpm overnight (see Note 2).
- 4 Collect the cells by centrifugation at 2,400×g for 15 min and resuspend in Lysis Buffer (2-5 ml / g of wet weight) (see Note 3). Cells can be flash frozen at this point and kept at -80 °C (see Note 4) or further processed.
- 5 Lyse cells by sonication using a Vibracell Sonicator or any other accessible sonicator (the conditions need to be optimised for each lab, a curve of protein release as a function of cycles can be created to follow the cell lysis). Transfer 2 ml of lysate to 2 mL Safe lock tubes (2x). Then remove insoluble debris and large particles by centrifugation at 20,000×g for 20 min at 4 °C.
- 6 Transfer carefully 1.8 ml each cleared lysates (supernatant) to a new 2 ml Safe Lock tube.
- 7 Add 200 µl of Ni-NTA agarose beads (Qiagen) and incubate at 4 °C for one hour with gentle and continuous inversion. This can be done in a 4 °C room on a rotatory shaker set at 200 rpm.
- 8 Wash 3 times the Ni-NTA agarose beads with 1 ml Washing Buffer making sure that the agarose beads are completely resuspended.

- 9 Verify the purity and homogeneity of the purification by SDS-PAGE.
- 10 Perform protein quantification by a method of choice, in our case we have used a linearized version of the Bradford assay.

Tissue preparation of calibration curve for determining number of molecules per cell

- 11 Two weeks before performing the absolute quantification measurements, sterilise Col-0 seeds and plate them in ROBUST media petri dishes. Stratify the seeds for 3-4 days at 4 °C and darkness.
- 12 Synchronise germination by treating the plates with a 2-hour pulse of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ white light at 22 °C, followed by a 22 h period of darkness at 22 °C. Then transfer to the growth conditions under which the transgenic plants tagged with NanoLUC will be analysed.
- 13 Prepare 12 of 2 ml safe-lock Eppendorf tubes by adding 2 stainless steel 2 mm grinding balls on each. Label the tubes. Afterwards, weigh the tubes containing the grinding balls and record the weights on an Excel sheet.
- 14 Collect 2-weeks old plants (total tissue of 100 mg) in a 2 ml SafeLock Eppendorf tube with 2 stainless steel 2 mm grinding milling balls.
- 15 Create a standard curve by adding purified MBP-NanoLUC-3xFLAG-10xHis (see Note 6). MBP-NL3F10 has a molecular weight of 66.833 kDa. Assuming 25 million cells/gFW the final concentration of tissue in the BSIII buffer should be 0.4 gFW/ml resulting in a total of 10 million cells/ml. Therefore standard curves with 0, 1×10^2 , 1×10^3 , 1×10^4 , 1×10^5 and 1×10^6 molecules/cell can be prepared by adding the corresponding volumes from pure and quantified MBP-NanoLUC-3xFLAG-10xHis. The pure enzyme preparation is added before flash freezing the tissue and performing tissuelyser disruption in order to simulate the possible impact of sample preparation on NanoLUC activity.
- 16 Flash-freeze the tissue in liquid nitrogen at the beginning of the time-series sampling. Maintain these aliquotes in liquid nitrogen throughout the duration of the time-series sampling (next section).

Plant growth for time series

- 17 Sterilise seeds of NanoLUC tagged lines and plate them in ROBUST media petri dishes.

Stratify the seeds for 3-4 days at 4 °C and darkness.

- 18 Synchronise germination by treating the plates with a 2-hour pulse of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ white light at 22 °C, followed by a 22 h period of darkness at 22 °C. Then transfer to the growth conditions under which the transgenic plants tagged with NanoLUC will be analysed.
- 19 After one week in these conditions transfer healthy plants to compost mix and continue under the experimental conditions of interest until sampling will take place (section 3.5).
If plants are going to be analyzed for a longer period of time in-vitro growth needs to be optimised by the user.

Time series sampling

- 20 Before starting sampling, prepare the required amount of 2 ml safelock polypropylene tubes. Add two 3 mm stainless steel grinding balls. Label the tubes, measure the weight and record this in an excel table for determining the amount of gFW collected.
- 21 Pool five 21-days-old plants into a single 2 ml safelock polypropylene tube that contains two stainless steel 3 mm grinding balls previously weighed. Flash freeze the sample in liquid nitrogen. Keep the sample stored in liquid nitrogen for the full duration of the sampling and immediately that contains grinding balls. Flash freeze them in liquid nitrogen.
Keep them stored in liquid nitrogen throughout the duration of the sampling (see Note 4).
- 22 After sampling has been completed, perform two rounds of grinding using the Tissue Lyser (Qiagen) with the following settings: 30 hz for 1 min. Perform the same procedure to the calibration curve aliquots.
- 23 Take one sample at time and record weight in the excel sheet and add 150 μl of ice-cold BSIII buffer for blocking protein degradation (see note 7). Vortex the tubes vigorously and place them on ice.
- 24 Using the excel sheet determine the tissue mass by subtracting the originally recorded empty tube weight.
- 25 Calculate the amount of additional ice-cooled BSIII buffer required for a final "concentration" of 0.4 g FW/ml. After adding the additional BSIII vortex thoroughly.
- 26 Centrifuge the samples at 20,000 x g for 5 min and transfer the cleared crude lysate to ice-cooled 1.5 ml polypropylene tubes. Keep the tubes in ice for reducing protein degradation and proceed quickly to determine NanoLUC activity.

Plate reading measurements of NanoLUC activity

27 We recommend the plate layout set up presented in Figure 1

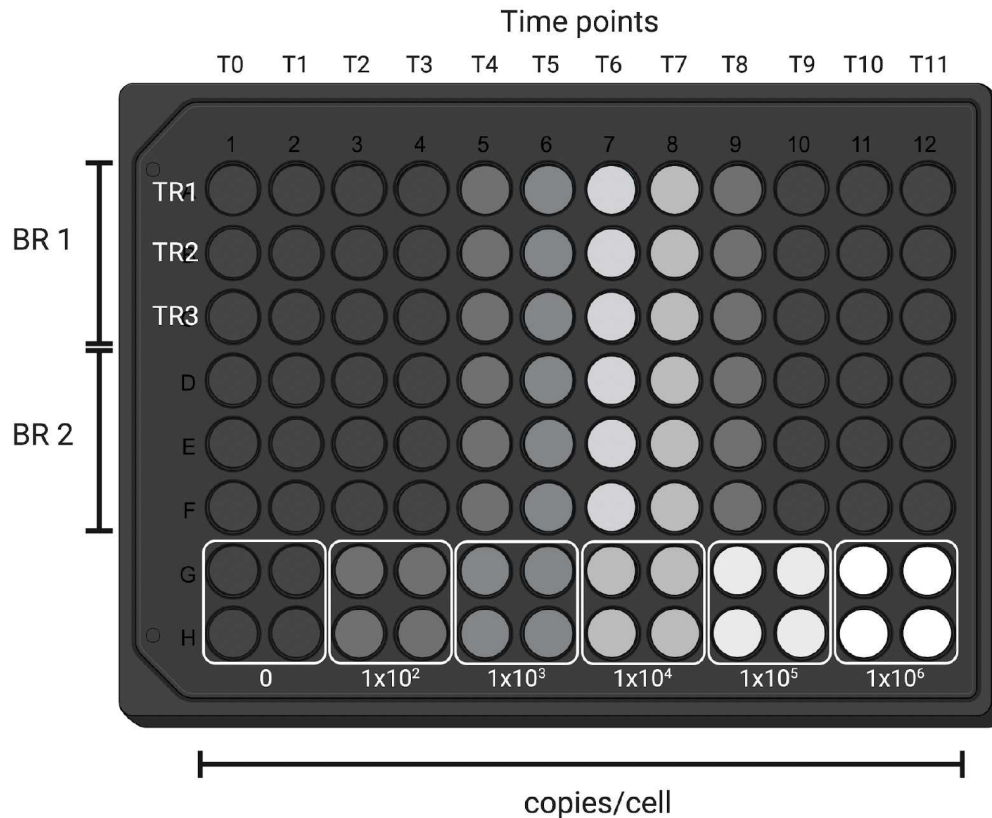


Figure 1. 96-well black plate layout for time series absolute quantification. Time points of series (T0 ... T11). 2 Biological Replicates (BR). 3 Technical replicates (TR1-TR3). Two biological replicates of time series with two technical replicates each forming a group of four wells in the last two. Each group has an order of magnitude increase in the number protein of copies/cell.

- 28 Calculate the amount of reconstituted NanoGlo® buffer aiming to deliver 100 µl/well. Choose a conical polypropylene tube adequate for the required number of samples.
- 29 Reconstitute the NanoGlo® buffer by mixing 1:50 Furimazine:NanoGlo®, then pour the substrate in a plate for loading using a multi-channel pipette.



- 30 Load 80 μ l of ice cold BSIII in a 96-well flat bottom black Lumitrac Greiner plate (see Note 8). Then proceed to load 20 μ l of cleared crude lysate. Keep the plate on ice to reduce protein degradation. This will also reduce sample evaporation.
- 31 Take 100 μ l of reconstituted substrate using a multi-channel pipette and add it to each well. Mix carefully, avoiding the formation of bubbles or foam (see Note 9).
- 32 Allow the plate to equilibrate to room temperature for 5 min (25 °C) and then proceed to perform the measurements in a plate reader (e.g. Tristar (Berthold) using 1 second integration time).

Data analysis

- 33 Using Excel or other similar calculating spreadsheet software. The dominant experimental error can be considered log-normal. Therefore, take the natural logarithm of the data and perform a linear regression against the calibration curve for determining copies/cell from the plate reader measurements.

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