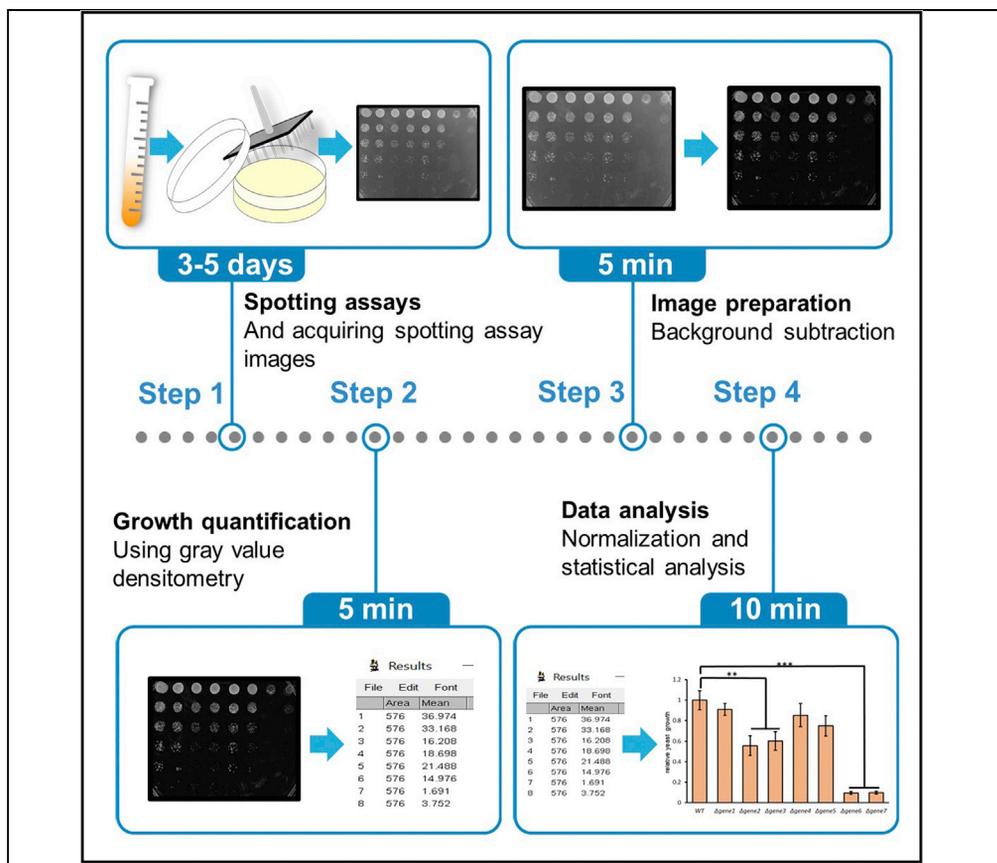# STAR Protocols

**Protocol**

# A Quantitative Imaging-Based Protocol for Yeast Growth and Survival on Agar Plates

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**HIGHLIGHTS**
Experimental
considerations for
enabling spotting
assay quantification

A step-by-step
procedure for
spotting yeast
cultures on agar
plates

Quantification and
statistical analysis of
spotting assay data

We present a detailed protocol that describes evaluation of the growth and survival of yeast cells by quantitatively analyzing spotting assays. This simple method reproducibly detects and quantifies subtle differences in growth by measuring the density of cells within a single spot of defined size on an image of a spotting assay. Our protocol is tailored specifically for low-throughput applications, can be easily adapted for specific experimental conditions, and is accessible to yeast experts and non-experts alike.

## Protocol

# A Quantitative Imaging-Based Protocol for Yeast Growth and Survival on Agar Plates

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## SUMMARY

We present a detailed protocol that describes the evaluation of the growth and survival of yeast cells by quantitatively analyzing spotting assays. This simple method reproducibly detects and quantifies subtle differences in growth by measuring the density of cells within a single spot of defined size on an image of a spotting assay. Our protocol is tailored specifically for low-throughput applications, can be easily adapted for specific experimental conditions, and is accessible to yeast experts and non-experts alike.
For an example of the execution of this protocol, please refer to DiGregorio et al. (Di Gregorio et al., 2020).

## BEFORE YOU BEGIN

There are several experimental factors that should be considered before beginning each section of this protocol. Below, we describe these experimental considerations in greater detail.

### Spotting Assays Experimental Design

1. Spots should be equal in diameter. This can be achieved by using a pronged spotter (frogger) to transfer the diluted yeast cultures onto the plate; however, careful application of defined volumes of cell suspensions using a pipette also produces satisfactory results.
2. Biological replicates should be obtained from different single yeast colonies, after the yeast cells have been transformed or mutated (e.g., gene deletion) or treated by other means (e.g., small molecules).

   ⚠ CRITICAL: Since spotting assay quantification and subsequent statistical analysis are performed relative to a control (e.g., a yeast strains bearing a vector control, WT strain etc.), it is important to include at least three biological replicates for controls and include a control on each plate (i.e. a different biological replicate of a control for each set of biological replicates).

3. To minimize the chance that results for a biological replicate become unquantifiable due to an error in spotting technique, for each biological replicate include several technical replicates (i.e., yeast cells from the same set of cultures spotted on a different plate)

**Obtaining Photographs of Spotting Assays**

Acquiring images of spotting assays with uniform background brightness aids with the subsequent steps in this protocol.

4. Images can be acquired using any digital camera (e.g., a cell phone camera), or a scanner. However, we found that taking spotting assay images with a gel imaging system, such as Bio-Rad ChemiDoc XRS, and using the "white-light" setting provides the most even background and helps to avoid glare.
5. Images should be acquired after distinct colonies can be seen, but before the colonies become too large to notice any difference between the samples (overgrown). It might be helpful to photograph the plates throughout the entire growth period once per day and then decide which pictures should be used for subsequent analysis.

**Image Preparation**

In this protocol background adjustment relies on the use of the Rolling Ball algorithm (Sternberg, 1983), which measure the average local background value for every pixel over the area of a "ball" around this pixel and subtracts this background value from the image.

**Data Analysis**

The obtained gray values are normalized against the wild-type (or other depdending on experimental design) control on the same plate. As mentioned above, for proper analysis and to enable comparison between plates it is important to include at least one control on each agar plate that is quantified. Although in this protocol we present the procedure for a statistical analysis using ANOVA followed by Tukey's test, an appropriate statistical test should be chosen depending on the specific experiment. For example, if only two means are compared, a Student's t test may be used. In some cases, when experimental data exper does not fit a standard distribution, a non-parametric test might be more appropriate.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Software and Algorithms | | |
| ImageJ | Schneider et al., 2012 | https://imagej.net/ImageJ2 |
| Microsoft Excel | Microsoft Inc. | https://www.microsoft.com/en-us/microsoft-365/excel |
| RStudio | RStudio team, 2020 | https://rstudio.com/ |
| Other | | |
| Cuvettes | VWR Inc. | Cat. #: 97000-586 |
| Dual position cap culture tubes | VWR Inc. | Cat. #: 60818-576 |
| Round bottom 96-well plates | Thermo Fisher Scientific | Cat. #: 268200 |
| SmartSpec 3000 spectrophotometer | Bio-Rad | Cat. #: 170-2525 |
| 48-pin spotter (frogger) | V&P Scientific Inc. | Cat. #: VP 407AH |
| Chemidoc XRS gel imager | Bio-Rad | Cat. #: 170-8265 |

## MATERIALS AND EQUIPMENT

Yeast strains, yeast media, and agar plates should be prepared depending on the specific experimental conditions. This protocol can be adapted for a wide variety of growth media and chemical treatments, including ones that result in slow yeast growth (Di Gregorio et al., 2020).

*Alternatives:*

- Cuvettes: Any suitable cuvette for measuring $OD_{600}$ of yeast cultures can be used.
- Culture tubes: Any sterile culture tube can be used.
- Round-bottom 96-well plates: Although round-bottom plates work better for picking up yeast cultures with a spotter, flat-bottom plates can also be used.
- Spectrophotometer: Any spectrophotometer capable of measuring $OD_{600}$ can be used.
- 48-pin spotter: We recommend using a spotter to make the assay less labor-intensive and ensure that the spots are equally sized and spaced. Yeast cultures can also be transferred onto an agar plate using a regular micropipette while making sure that the spots are round. A larger spotter (i.e., 96-pins) should be used with a larger diameter or a square plate, since a standard 100 mm diameter round petri dish fits a maximum of 8 rows of 3 μL yeast spots across.
- Gel imager: If a gel imager is unavailable, a digital camera can be used instead.

## STEP-BY-STEP METHOD DETAILS
### Performing Spotting Assays

⏱ Timing: 3–5 days

This step describes how to perform spotting assays on agar plates, starting from liquid yeast cultures. Obtaining spotting assays with spots of approximately equal size and correct dilutions is critical for subsequent steps of this protocol.

1. Inoculate transformed yeast cells in 3 mL liquid media and grow overnight in a shaker-incubator at 30°C and 200 rpm.
2. Dilute cultures after growth for 12-16 hrs in a 1:10 ratio in 1.5 mL microcentrifuge tubes and measure their $OD_{600}$ using a spectrophotometer to determine cell density.
3. Equalize the number of cells in each culture by diluting them in sterile $ddH_2O$ to $OD_{600}$=1.0, or medium, or an appropriate buffer and to a total volume of 200 μL in row A of a 96-well plate. Fill the wells in the rows B-F with 120 μL of $ddH_2O$ using a multichannel pipette.
4. Perform a 1:5 serial dilution by pipetting 30 μL of culture from each row above to the row immediately below.
5. Immediately transfer equal volumes of diluted culture from each well using a 48-prong spotter onto dry agar plates, containing selective media. Sterilize the spotter with a submersion in 70% ethanol and brief flaming after each transfer. Ensure that the spotter has sufficiently cooled down after flaming.

⚠ CRITICAL: After picking up the liquid from the 96-well plate, check the tips of the prongs to ensure that each prong contains a droplet of liquid and these droplets are of equal size.

⚠ CRITICAL: Taking the spotter out of the liquid with a relatively high speed, while keeping the hand motion vertical, helps to ensure the uniformity of the droplet size on each prong.

6. After all spotted liquid has been absorbed, incubate the plates for 2–5 days at the required temperature (typically 30°C), depending on the desired experimental conditions. Troubleshooting: Problems 1, 2, 3, and 4.

### Image Acquisition

⏱ Timing: 2–3 min/plate

Acquiring high-quality images of spotting assays, with uniform background brightness and no glare, significantly simplifies the next step of background subtraction. Images of plates containing different biological replicates or experimental groups should be taken at the same time.

7.  Turn the agar plates lid up and take the lid off. Taking the lid off helps to prevent glare, however, if contamination of the colonies on the plate is an issue, the lid can be left on as long as lighting is even.
8.  Place the plate under the camera or on the tray of the gel imager.
9.  For the best possible resolution of an image, zoom in on the plate such that most of the image frame is taken up by the spotting assay.
10. If using a camera, do not use the flash to reduce glare.
11. To avoid excessive glare from the agar, ensure uniform lighting of the plate. If taking an image with a digital camera, this can be achieved by taking images away from natural light and using a shield (e.g., cardboard) to prevent light from directly falling on the surface of the agar.

   ⏸ Pause Point: Save the spotting assay images for further analysis


**Image Preparation**

   ⏱ Timing: 5 min/plate

Image preparation is an important step in the process of spotting assay quantification as it allows to ensure the uniformity of background on a single plate and eliminate the excessive glare that might affect the consistency of the mean gray value measurements across multiple spots.

12. Import the image into ImageJ by first opening ImageJ and then clicking on "File" then "New" then "Image."
13. When the window with the image opens, click on the "image" then "type" and select "8-bit" option.
14. Click on the straight-line tool and extend the line over the diameter of the largest spot in the spotting assay. Ensure that the units of length are set to pixels. Click "analyze" then "measure." The "length" measurement in the table will be equal to the diameter of the spot. Troubleshooting: Problem 5
15. Click on "process" then "subtract background." In the newly opened window, set the rolling ball diameter to the diameter measured in step 14 + 20 pixels, select "sliding paraboloid" option and click "OK." The image with the adjusted background will appear in a new window (Figure 1A).

   ⚠ CRITICAL: The rolling ball diameter cannot be smaller than the diameter of the largest spot on the spotting assay. Troubleshooting: Problem 6

16. Using the "Image" then "adjust" then "brightness/contrast" function adjust the image parameters such that the individual spots are easier to see (Figure 1B).
17. To check for the uniformity of the background use the circular selection tool and adjust its size to the size of the smallest spot on the spotting assay. Using this selection, perform several measurements of the background mean gray value on different places on the plate by clicking "analyze" then "measure." Ensure that the background readings from different places on the plate are no more than 3 mean gray value units apart (Figure 2).
18. If the background readings fall far apart, run the Rolling Ball background subtraction algorithm again. Several iterations of background adjustment, until the background readings from different places on the plate are no more than 3 gray value units apart, can help ensuring its uniformity. Troubleshooting: Problem 7
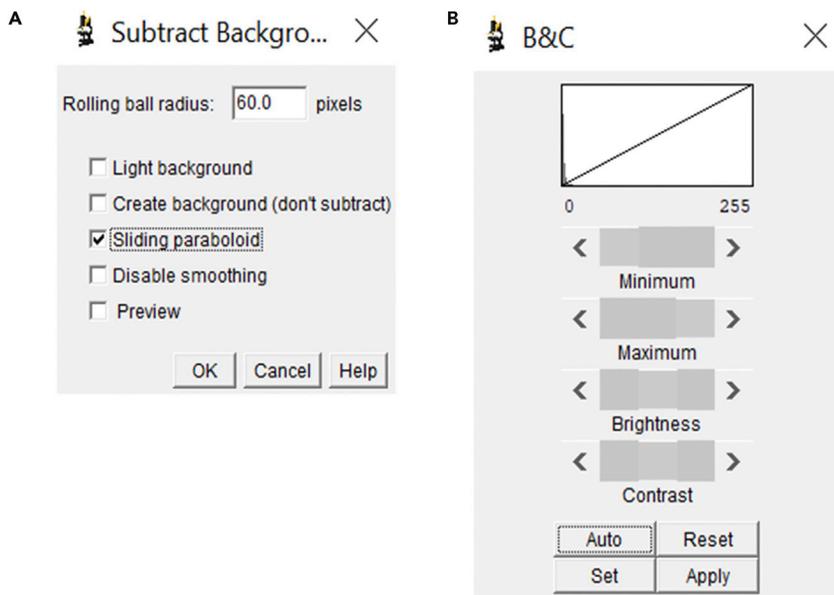
**Figure 1. ImageJ Tools for Equalizing Background on the Images of Spotting Assays**
(A) Settings for the image subtraction Rolling Ball algorithm.
(B) "Brightness and Contrast" adjustment tool that allows the individual spots on the image to be made more visible after the background has been subtracted.

19. Without closing the obtained image in ImageJ and exiting the program, proceed directly to the growth quantification step.

⚠ CRITICAL: Perform your quantification directly on the processed image. If the image is saved and then re-opened, the adjustments to the brightness/contrast might affect the results and increase variability between replicates.

## Growth Quantification

⏱ Timing: 5 min/plate

Yeast growth is quantified by measuring the gray value of the spots across different samples and for the same dilution on the processed spotting assay image. For this quantification, it is crucial to select an appropriate dilution (discussed further below), maintain the same area of the circular selection for all measurements on the same plate, and to use images with the properly adjusted background.

20. On the processed image of a spotting assay, choose a dilution that is appropriate for quantification. This dilution should fit the following requirements:
    • To be the lowest dilution (i.e., the least diluted culture) that is representative of the differences in growth between all samples (Figure 3A).
    • Display enough growth to enable quantification.

*Note:* From the spotting assays that we have quantified using this protocol, the third dilution (25×) is usually appropriate, however, this might change depending on the experiment. If a growth phenotype is too toxic, the first or second dilutions might show more appropriate differences in growth between samples, and they should be used for quantification. If the difference is not seen in the third dilution, the fourth dilution can be used for quantification.
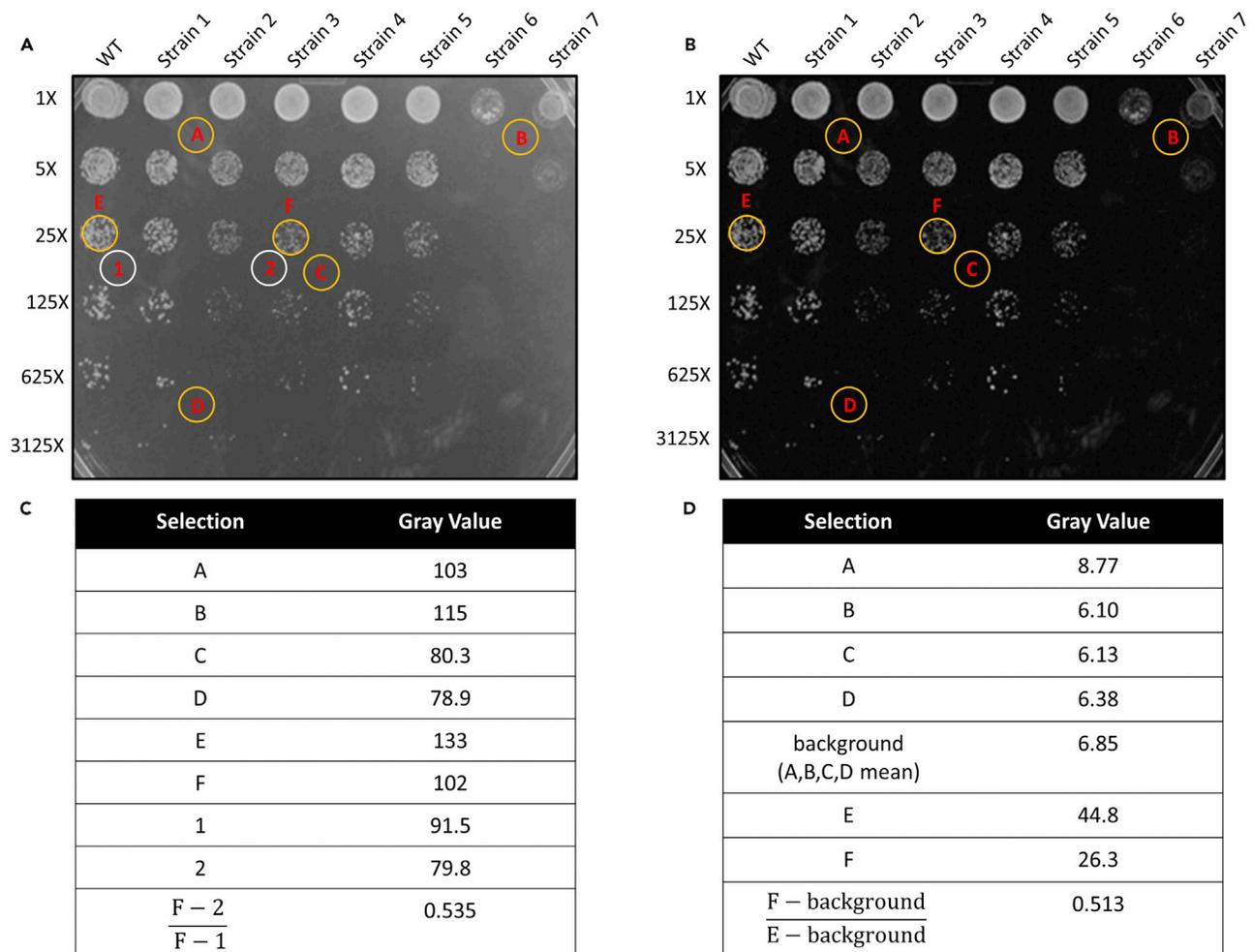
**Figure 2. The Results of Background Subtraction Using the In-Built Rolling Ball Algorithm in ImageJ**

(A) The unprocessed image that was used as the input file for background subtraction.

(B) Resulting image after background was subtracted using the Rolling Ball algorithm with settings indicated in Figure 1. Background subtraction was iterated four times, and the brightness and contrast were adjusted to make the individual spots more visible. On both images, the colored circles represent the places for measurements that are presented in the tables in (C) and (D).

(C) Gray value measurements for the input image. Background gray value measurements on the starting image are highly variable and in the case of selection B, this measurement is even higher than the value of the spot F. Selections 1 and 2 were used to estimate the background values for the spots E and F, respectively.

(D) Gray value measurements from the processed image. The background values are now grouped much closer to each other. The proximity between the F-2/F-1 ratios and F-background/E-background ratios indicates that image processing did not affect the relative gray values of the spots.

21. Click on the circular selection tool and extend the selection around the smallest spot in the dilution such that the entire spot but not the background is within its bounds.

> ⚠ CRITICAL: It is important to use the same selection tool to perform all gray value measurements on the same plate, as the area of the selection can affect the gray value readings. If throughout the measurements the area of the selection was changed, it can be restored by clicking "edit" then "selection" then "restore selection." If the selection is not restored, the measurements, including the ones for the background, should be repeated.

22. By dragging the same circular selection over five different places on the plate and clicking "analyze" then "measure," measure the gray value of the spotting assay image background.
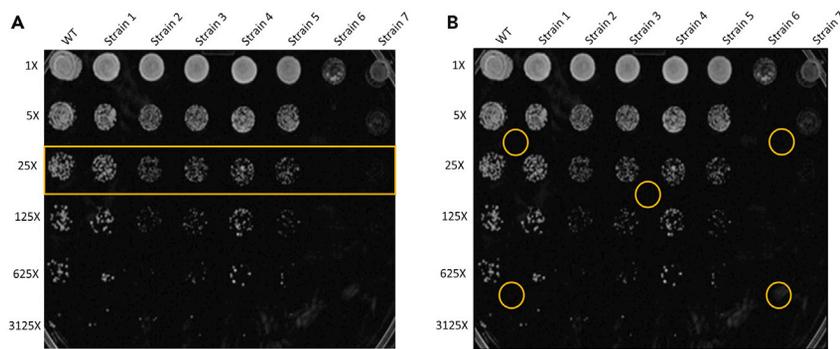
**Figure 3. Choosing an Appropriate Dilution and Measuring Background Gray Value for Spotting Assay Quantification**

(A) In this case, the third dilution (boxed) is chosen because it is the lowest most informative dilution. The second dilution was not chosen because the difference in growth between gene 2 and gene 3 samples is clearer in the third dilution. The fourth dilution was not chosen because the third dilution is sufficiently informative.

(B) Five spots on the plate that should be used to measure the gray value of the background. Those spots were chosen in different regions of the plate, because they give the optimal background value.

It is best if the background measurements are performed on different places on the plate, such as in each corner of the plate an in the middle of it (Figure 3B). Calculate the mean of the five background measurements.

Before proceeding further, check that the background measurements are within three units of each other, otherwise repeat the background subtraction algorithm again.

23. Using the same selection as in the previous steps, measure the gray value of each spot in the chosen dilution and record those values in an Excel spreadsheet. If possible, measure the gray values of different biological replicates on different technical replicates (i.e., different plates) of a spotting assay. This will help to decrease the chance that the difference in growth between samples is due to technical variability.
24. Subtract the gray value of the background from the gray values of the spots measured on the same plate. Repeat this step for all the plate images. The obtained values will represent the growth of each biological replicate, including the control. Troubleshooting: Problems 8 and 9
25. Repeat steps 12–25 for all other spotting assay images (i.e., biological replicates).

### Data Analysis

⏱ Timing: 10 min

This data analysis step relies on normalizing the growth data to a control, which should be present on each plate. This normalization or comparison between experimental groups spotted on different plates cannot be performed if the growth of the controls on these plates is considerably different from each other (See Limitations).

26. Using the gray values obtained in step 24, calculate the mean gray value for the control group.
27. Divide each of the gray values obtained in step 24 by the gray value of the corresponding control on the same plate.

⚠ CRITICAL: Within the control group, divide each of the individual control gray values by the mean value of all controls, calculated in step 26. This step allows conversion of the raw control growth values to relative growth values.

28. Calculate the mean and standard deviation for three or more relative values calculated in step 27
    Troubleshooting: Problem 10
29. Plot the obtained mean relative growth values +/- standard deviation on bar graph using Excel,
    GraphPad Prism, or any other data analysis software.

If the measurements were performed for at least three biological replicates that are distributed normally, and several means are compared at once, perform a one-way ANOVA, followed by a post-hoc Tukey's test. Statistical analysis can be performed using any available software, however, for a brief protocol of how to perform this analysis using RStudio, please refer to step 30

30.

   *Optional:* Statistical analysis of spotting assay data in R.Studio.
   a) Open a new Excel spreadsheet and in the first column type in the categories (sample names)
      of the samples to be compared, with one per each replicate. Category names should be the
      same for the replicates of the same experimental group, otherwise the program will assume
      they are different experiments.
   b) Into the next column, input the relative growth values obtained in step 3.5.2.
   c) Save the spreadsheet in the CSV format.
   d) Open R.Studio. Under the "environment" tab on the right click "import dataset" then "from
      text (base)" and select the .CSV spreadsheet containing your data.
   e) The table with your data will appear in a new window. Leave the settings in the right-hand
      side of the window as they appear and click on "import." Your data will now appear in the
      window in the top left corner.
   f) In the console window (bottom left) type the command attach (file name) and press enter. After that all commands that you type in the console will refer to your data.
   g) In the console type the code:
        myaov<-aov(V2~V1)
        summary(myaov)
        TukeyHSD(myaov, conf.level = 0.95)
   h) Interpret the output of the statistical test.

## EXPECTED OUTCOMES

Polyglutamine-repeat containing proteins are prone to misfolding and formation of insoluble aggregates (Scherzinger et al., 1999). Toxicity of these proteins is generally dependent on the number of glutamine repeats (Scherzinger et al., 1999). Using the protocol described above, we have quantified the growth of yeast strains expressing huntingtin exon I constructs with a different number of glutamine repeats under the control of a Met promoter. Yeast strains and expression plasmids used here to determine the toxicity of polyQ (polyglutamine) have been described before and are available through Addgene (Duennwald et al., 2006). Expression of fragments of the human huntingtin protein with increasing length of its polyQ region produces increasing toxicity in yeast with 25Q showing no toxicity, 46Q showing mild toxicity, and 72Q and 103Q showing strong toxicity (Figure 4A). Quantification of three independent biological replicates indicates statistically significant difference in growth between all strains, including the subtle difference between 25Q and 46Q (Figure 4B). Images of three independent biological replicates were used for quantification (Figure S1, raw data from quantification in Tables S1 and S2).

## LIMITATIONS

Unlike most existing methods for quantifying yeast growth on agar plates, our protocol does not measure the size of the individual yeast colonies (Bischof et al., 2016). It instead relies on measuring the overall growth coverage within a given spot. Thus, abnormally large colonies
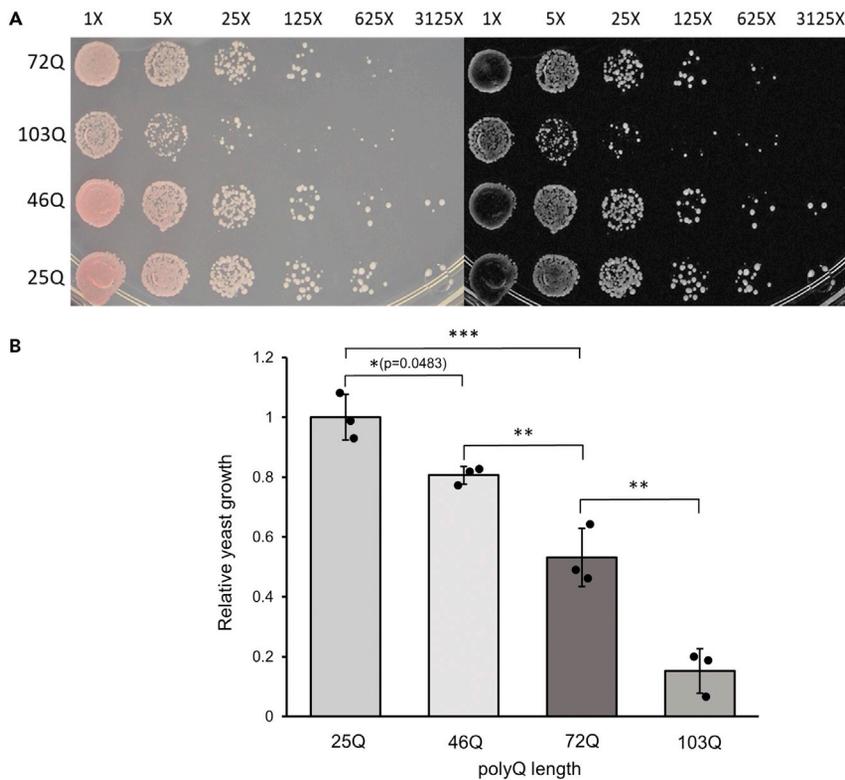
**Figure 4. Growth of Yeast Strains Expressing Huntingtin Exon I Construct with a Different Number of Glutamine Repeats**

(A) Images of the spotting assay.

(B) Results of the spotting assay quantification. Spotting assays were quantified as described in the protocol, using the third dilution. Statistical analysis was performed using a one-way ANOVA, followed by Tukey's test with a 95% confidence interval. The raw mean gray values and relative growth values for each spot are provided in Tables S1 and S2, respectively. Images used for quantification are provided in Figure S1.

may confound the results. For example, a few very large colonies that are present within a spot might produce a higher growth value than a lot of small colonies. Researchers employing our protocol should be aware of this limitation and double-check whether the obtained growth values make sense in the context of what yeast growth they see on the image of a spotting assay.

The growth of yeast colonies on agar plates is not linear and eventually the number of cells per colony reaches stationary phase (Rivas et al., 2014). This implies that the difference in growth between the control and experimental strains will vary depending on the growth time. Thus, a limitation of this protocol is that it cannot be used to directly compare growth between plates that were imaged at different times or if the growth of controls is considerably different from each other. We suggest that to enable normalization of data to the control (as described in step 27) and comparison between different plates, images of plates should be taken at the same time after spotting.

Another limitation of our protocol is that it, as other spotting assay-based techniques, assesses growth and survival of yeast cells simultaneously. While growth defects arise from reduced cell division rates, survival defects are caused by cell death or complete arrest of cell division (Mirisola et al., 2014).Thus, spotting assays do not deliver insights into the precise cellular mechanism of toxicity caused by specific growth conditions or genetic manipulations. However, many other well-established approaches, such as cell death staining or colony-forming unit counting, can help to overcome this shortcoming (Chadwick et al., 2016; Wloch-Salamon and Bem, 2013).

Finally, it is important to note that spotting assay are not always quantitative, and their quantification may not produce a result that refelcts difference in toxicity caused by specific experimental conditions. The observed difference in growth between experimental groups is sensitive to the time at which the image was taken and the initial dilution of the culture. Thus, changing these parameters may affect the quantitative difference in growth. Our protocol provides a way to make spotting assays more reproducible and enables their statistical comparison, but it does not to make them a fully quantitative technique.

## TROUBLESHOOTING

### Problem 1
Yeast spots look oval or too large (steps 1–6).

### Potential Solution
The plates were wet, which caused the yeast cultures to not be absorbed and "run" when spotted. Re-do the spotting assays and ensure that the plates are dried sufficiently

### Problem 2
Some yeast spots are too small while other spots are large (steps 1–6).

### Potential Solution
The volumes of culture on each prong of the spotter was not equal. Re-do the spotting assays and ensure that droplets on the prongs are of approximately equal size before placing the spotter on the plate.

### Problem 3
All yeast spots look too small (steps 1–6).

### Potential Solution
The volume of culture on each prong was insufficient. Re-do the spotting assays and ensure that each prong contains a clearly visible droplet of liquid. Sometimes it may be difficult to have a sufficient volume of liquid on the prongs if too many plates are spotted from the same well plate. You may need to use more than one well plate if more than 12 plates need to be spotted.

### Problem 4
Yeast spots are oval (steps 1–6).

### Potential Solution
The spotter was shifted laterally after it was placed on the agar plate. Re-do the spotting assay and lift the spotter vertically, when picking it up from the agar plate.

### Problem 5
After measuring the diameter of the largest spot on the spotting assay, the "length" measurement in displayed in decimals (step 14).

### Potential Solution
Inches are selected as the units of length measurement. Pixels are being converted to inches using a pre-set factor. To reset the units, go to "Analyze" then "Set scale" then "Click to remove scale."

### Problem 6
Running the rolling ball algorithm produced no change in background brightness and the processed image looks identical to the starting image (step 15).

**Potential Solution**

The set diameter of the ball was too small. Check that the diameter of the ball is not smaller than the largest spot in your assay. If the issue continues to persist, you might have to set the rolling ball diameter to the diameter of your largest spot +50 or more pixels.

**Problem 7**

After several iterations of the Rolling Ball algorithm, the background gray values from different parts of the plate are still far apart (step 18).

**Potential Solution**

Some areas of the plate are too bright due to excess glare. If possible, take new photographs of your spotting assays and ensure that the brightness of all parts of the plate is uniform. If new photographs cannot be taken, you might have to exclude the overly bright areas from your quantification by cropping the image.

**Problem 8**

The obtained gray values of the spots do not seem representative of the actual yeast growth seen on the plate, and for some dilutions yeast colonies see abnormally large (e.g., the gray value of the 25× dilution is smaller than the value of the 125× dilution within the same sample, and the 125× dilution contains several large colonies) (step 24).

**Potential Solution**

The yeast colonies formed papillae. The large papillae colonies will confound the results of your quantification, as these colonies are not representative of the actual yeast growth. If possible, re-do the spotting assay or exclude the dilution containing these colonies from your experiment.

**Problem 9**

The obtained gray values of the spots do not seem representative of the actual yeast growth seen on the plate, but no large colonies are seen (e.g., the gray value of the 25× diluted spot for one of the samples is close to the background value, even though yeast growth can be clearly seen) (step 24).

**Potential Solution**

The circular selection might be too large, so for some of the measurements, too much of the background without growth is included. Change the diameter of the circular selection and ensure that it is not larger than the diameter of the smallest spot. Re-do your quantifications.

**Problem 10**

The standard deviation within one or multiple experimental groups is very large, and no such large variability can be observed on the spotting assays (step 28).

**Potential Solution**

One of the measurements might have been performed inconsistently. Check which gray value deviates the most from other replicates and re-quantify that entire replicate, including all samples and the background.

## RESOURCE AVAILABILITY

### Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Martin L. Duennwald (martin.duennwald@schulich.uwo.ca).

**Materials Availability**

PolyQ plasmids used in the Expected Outcomes section of this protocol have been previously deposited to Addgene: #15589 (46Q), #15590 (72Q), #15591 (103Q). The 25Q vector is available from the lead contact, Martin L. Duennwald, upon request.

**Data and Code Availability**

Raw images used for quantifications shown in Figure 4 are provided in Figure S1. No new code was generated as a part of this study.

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100182.

## AUTHOR CONTRIBUTIONS

Conceptualization, A.A.P., M.G.T., P.L., and M.L.D.; Methodology, A.A.P., M.G.T., and M.L.D.; Validation, A.A.P. and M.G.T.; Formal Analysis, A.A.P.; Investigation, A.A.P. and M.L.D.; Resources, M.L.D. and P.L.; Writing – Original Draft, A.A.P. and M.L.D.; Writing – Review & Editing, A.A.P. and M.L.D.; Visualization, A.A.P.; Supervision, M.L.D. and P.L.; Project Administration, M.L.D.; Funding Acquisition, M.L.D.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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