Journal of Visualized Experiments

Use of microtiter dish radiolabeling for multiple in vivo measurements of Escherichia coli (p)ppGpp followed by TLC.

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59595R1
Full Title:	Use of microtiter dish radiolabeling for multiple in vivo measurements of Escherichia coli (p)ppGpp followed by TLC.
Keywords:	(p)ppGpp; thin-layer chromatography; 32P; Escherichia coli; high through-put
Corresponding Author:	Llorenç Fernández-Coll National Institutes of Health Bethesda, MD UNITED STATES
Corresponding Author's Institution:	National Institutes of Health
Corresponding Author E-Mail:	llorenc.fernandezcoll@nih.gov
Order of Authors:	Llorenç Fernández-Coll
	Michael Cashel
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Bethesda, MD, Unite States

TITLE:

Using Microtiter Dish Radiolabeling for Multiple In Vivo Measurements Of *Escherichia coli* (p)ppGpp Followed by Thin Layer Chromatography

AUTHORS AND AFFILIATIONS:

Llorenç Fernández-Coll[1] and Michael Cashel[1]

[1]Intramural Research Program, Eunice Kennedy Shriver NICHD, NIH, Bethesda, Maryland, USA.

Corresponding Author:
Llorenç Fernández-Coll (llorenc.fernandezcoll@nih.gov)
Tel: (301) 451-7206

Email Addresses of Co-Authors:
Michael Cashel (cashelm@mail.nih.gov)

KEYWORDS:
(p)ppGpp, thin-layer chromatography, ^{32}P, *Escherichia coli*, high throughput, second messenger

SUMMARY:
The growth of radiolabeled bacterial cultures in microtiter dishes facilitates high throughput sampling that allows multiple technical and biological replicate assays of nucleotide pool abundance, including that of (p)ppGpp. The effects of growth transitions provoked by sources of physiological stress as well as recovery from stress can be monitored.

ABSTRACT:
The (p)ppGpp nucleotide functions as a global regulator in bacteria in response to a variety of physical and nutritional stress. It has a rapid onset, in seconds, which leads to accumulation of levels that approach or exceed those of GTP pools. Stress reversal occasions a rapid disappearance of (p)ppGpp, often with a half-life of less than a minute. The presence of (p)ppGpp results in alterations of cellular gene expression and metabolism that counter the damaging effects of stress. Gram-negative and Gram-positive bacteria have different response mechanisms, but both depend on (p)ppGpp concentration. In any event, there is a need to simultaneously monitor many radiolabeled bacterial cultures at time intervals that may vary from 10 seconds to hours during critical stress transition periods. This protocol addresses this technical challenge. The method takes advantage of temperature- and shaker-controlled microtiter dish incubators that allow parallel monitoring of growth (absorbance) and rapid sampling of uniformly phosphate-radiolabeled cultures to resolve and quantitate nucleotide pools by thin-layer chromatography on PEI-cellulose. Small amounts of sample are needed for multiple technical and biological replicates of analyses. Complex growth transitions, such as diauxic growth and rapid (p)ppGpp turnover rates can be quantitatively assessed by this method.

INTRODUCTION:

The (p)ppGpp second messenger is a global regulator that modulates the expression of a large number of genes, including genes for synthesizing ribosomes and amino acids[1,2]. Although initially discovered in *Escherichia coli*[3], (p)ppGpp can be found in both Gram positive and Gram negative bacteria as well as in plant chloroplasts[4,5]. For *E. coli* and other Gram-negative bacteria, (p)ppGpp interacts directly with RNA polymerase at two different sites[6–8]. In Gram positives, (p)ppGpp inhibits GTP abundance, which is sensed by CodY, a GTP-binding protein with gene-specific DNA recognition motifs that lead to regulation[9,10]. (p)ppGpp accumulates in response to starvation for different nutrients and stress conditions, resulting in slow growth and adjustments of gene expression to allow adaptation to stress[11,12].

The net amount of ppGpp accumulated reflects a balance between synthetase and hydrolase activities. In *E. coli* RelA is a strong synthetase and SpoT is bifunctional, with a strong hydrolase and a weak synthetase, each of which might be regulated differently in a stress dependent manner. The strong RelA synthetase is activated when the availability of codon-specified charged tRNA bound to the ribosomal A site when it fails to keep up with the demands of protein synthesis[13–15]. The weak SpoT (p)ppGpp synthetase is activated while the strong (p)ppGpp hydrolase is inhibited in response to other stress conditions and through other mechanisms. Under some conditions, proteins such as ACP or Rsd can bind to SpoT, which also change the balance between hydrolysis and synthesis[16,17]. In Gram positives, synthesis and hydrolysis reflect a more complex balance between a single RelA SpoT homolog (RSH) protein with strong synthesis and hydrolysis activities as well as smaller hydrolases and/or synthetases[12].

The (p)ppGpp nucleotides were first discovered as unusual ^{32}P labeled spots that appeared on autoradiograms of thin-layer chromatograms (TLC) during a stringent response induced by amino acid starvation[3]. More detailed labeling protocols have been reviewed [18]. The protocol described here (**Figure 1**) is a modification of these protocols that allows monitoring growth of multiple samples on microtiter plates. This facilitates multiple biological and technical estimates of (p)ppGpp abundance changes and was initially developed for studies of diauxic shifts[19]. Labelling of (p)ppGpp with ^{32}P and detection by TLC also allows measurements of (p)ppGpp degradation rates. Alternative methods have been developed to determine (p)ppGpp levels such as mass spectrometry, HPLC[20], fluorescent chemosensors[21,22], and GFP gene fusions to promoters affected by ppGpp[23,24]. Fluorescent chemosensors currently have a limited use due small spectral shifts after binding ppGpp as well as problems distinguishing between ppGpp and pppGpp[21]. This method is efficient to detect (p)ppGpp in vitro, but not in cellular extracts. Methods involving HPLC have been improved[20] but require expensive equipment and are not well adapted to high through-put. Finally, GFP fusions can give an estimate of ppGpp dependent activation or inhibition, but do not measure ppGpp itself. While each of these alternative methods are valuable, they require expensive equipment or substantial hands-on time, or they are otherwise not amenable to multiple kinetic sampling and subsequent processing. With the method described here, 96 samples can be applied to six TLC plates in about 20 minutes (18 samples per plate), resolved by TLC development in less than a couple of hours, with quantitative data obtained after several hours or overnight, depending on labeling intensity.

PROTOCOL:

1. Media preparation

1.1. For MOPS (3-(N-morpholino)propanesulfonic acid) media[25], use 1/10 volume of 10x MOPS salts, 1/100 volume of 100x micronutrients solution, 3 mM sodium phosphate for overnight cultures or 0.2 mM sodium phosphate for uniform labeling with ^{32}P, 0.2% glucose and 1 µg/mL thiamin (vitamin B1). Add amino acids at 40 µg/mL if required.

1.1.1. For MOPS salts, use 400 mM MOPS, 40 mM Tricine, 0.1 mM $FeSO_4$, 95 mM NH_4Cl, 2.6 mM K_2SO_4, 5 µM $CaCl_2$, 10.56 mM $MgCl_2$, and 500 mM NaCl. Add the different components in the order written to avoid precipitation. Adjust to pH 7.4 with KOH and sterilize by filtration.

1.1.2. For 100x micronutrients solution, use 3 µM $(NH_4)_6(MO_7)_{24}$, 0.4 mM H_3BO_4, 30 µM $CoCl_2$, 10 µM $CuSO_4$, 80 µM $MnCl_2$, and 10 µM $ZnSO_4$. Sterilize by autoclaving.

1.1.3. For glucose (20%) and sodium phosphate (300 mM) solutions, sterilize as separate 100x stocks by autoclaving. Sterilize 100x stocks of thiamine and amino acid mixtures by filtration. Prepare fresh media from sterile stock solutions for each experiment.

2. Labelling with ^{32}P

2.1. Grow overnight cultures in MOPS media with 3 mM sodium phosphate.

2.2. In a 24 well plate, add 550 µL of MOPS media containing 0.2 mM sodium phosphate carrier and 30 µL of each bacterial inoculum (dilution 1/20). This will produce an initial inoculum of OD_{600nm} 0.1. Use one well with fresh media as a sterility control.

2.3. Place the plate on a shaking incubator at 37 °C shaking at 900 rpm for 30 min. Cultures are heated from below and shaking provides aeration. Attach the plate firmly with tape to avoid tilting or spills. Growth can be monitored with a replicate plate in parallel in media lacking ^{32}P using a plate reader and incubating under the same conditions.

2.4. Add 100 µCi to each well of ^{32}P orthophosphate (20 µL from a 5 mCi/mL stock).

NOTE: The amount of radioactivity can also be adjusted to meet experimental needs. For low basal levels 100 µCi with 0.2 mM carrier phosphate works well, but for measuring (p)ppGpp levels that are expected to become equivalent to GTP pools, label can be dropped to 25 or 50 µCi. Lowering carrier phosphate below 0.2 mM is not recommended to avoid phosphate levels becoming limiting for growth.

CAUTION: ^{32}P is a β emitting isotope so use proper shielding specified for safety. All radioactive material must be properly disposed taking all the possible precautions.

2.5. Grow in shaking incubator for 1 to 2 doublings (an hour or more) to allow external label to largely equilibrate with intracellular nucleotide pools before inducing stress.

3. Induction of stress or starvation

3.1. Induce stress using a method of your choosing, depending on the sort of stress studied, e.g. adding metabolic pathway inhibitors, changing temperature, exhausting required nutrients, shifting osmolarity shifts, inducing oxidative stress, adding toxins, etc.

3.1.1. For example, add 100 µg/mL L-valine to induce isoleucine starvation in *E. coli* K-12 strains. Increased levels of ppGpp will be observed after 5 minutes of induction.

4. Sampling and ppGpp extraction

4.1. Add 20 µL of each labelled cell sample to a 0.2 mL PCR tube containing 20 µL of ice cold 6 M Formic acid.

4.2. Immediately place the samples in dry ice.

4.3. Enhance cellular extraction efficiency by 3 cycles of freezing and thawing.

4.4. Just before spotting PEI cellulose thin layer chromatograms, centrifuge samples for 1 min at maximum speed to pellet cell debris to avoid spotting it on chromatograms.

5. Thin-layer chromatography

5.1. With a soft pencil, mark an origin line 1 cm from the edge of the 20 cm x 20 cm PEI-Cellulose TLC plate that has had 5 cm removed from the top with scissors. Apply 5 µL as a droplet in the PEI surface for each sample. Begin ascending development without allowing this spot to dry, which improve resolution by minimizing streaking.

5.2. Do ascending development in a tank with a layer of 1.5 M KH_2PO_4 (pH 3.4) solution shallow enough so that liquid does not touch the origin sample spot. Cover the tank with an air-tight seal and allow liquid ascent to the top of the trimmed sheet, 15 cm. To achieve pH 3.4 for a 1.5 M KH_2PO_4 solution, it is necessary adjust the pH by addition of H_3PO_4.

5.3. Remove the fully developed chromatogram and air dry at room temperature.

5.4. Cut and discard the top (pH front) portion of the chromatogram containing the free ^{32}P into radioactive waste. This portion is represented in yellow color in **Figure 2** and easily visualized under UV light. If measuring only (p)ppGpp nucleotides that migrate slower than GTP, it is recommended to run a UV-visible GTP standard and then cut and discard a larger upper portion (anything above GTP, as shown in **Figure 2**).

5.5. Expose autoradiographic films overnight with a phosphor screen.

5.6. Develop the film. Capture and quantitate the phosphor screen signal with a phosphoimager.

6. Quantitation of (p)ppGpp

6.1. Quantitate radioactive spots with Image J[26,27].

NOTE: The amount of (p)ppGpp can be normalized to total amount of G nucleotides observed in each sample (**Figure 2**). If the amount of background is homogeneous, a single blank (box 4 from **Figure 2**) can be subtracted to correct for background. Total G is the sum of GTP + ppGpp + pppGpp detected. This normalization assumes that GMP and GDP levels are negligible, which is true for *E. coli*. The ratio of a given nucleotide to total G provides an important way to correct for variations of applied sample volume.

7. Measurement of the rate of ppGpp decay

NOTE: A modification of this protocol allows measurements of ppGpp decay rates.

7.1. After provoking stress or starvation for a time sufficient to allow ppGpp accumulation (step 3), reverse the stress in order to block continued ppGpp synthesis, which allows detection of hydrolytic rates. The procedure for reversal will depend on the stress. For example, add 200 µg/mL of chloramphenicol to reverse any amino acid starvation.

7.2. Decay rates are usually rapid but may vary, so take samples every 20-30 seconds up to 2 minutes. Process samples as in step 4.

7.3. Once the TLC is developed (as in step 5) and the levels of ppGpp obtained during the time course, plot residual ppGpp content on a semi-logarithmic plot vs time to allow visualization of zero order rates of decay, as a straight line, and estimation of the ppGpp half-life.

REPRESENTATIVE RESULTS:

In *E. coli* K-12 strains, the addition of valine provokes an endogenous starvation of isoleucine, which results an increase of ppGpp levels after 5 minutes [3]. Cells grown in MOPS containing all amino acids except for ILV were labeled with ^{32}P as indicated in **Figure 1**. Once labelled, 6 µL of 10 mg/mL L-valine (100 µg/mL final concentration) was added to produce isoleucine starvation. Samples were taken 0 and 5 minutes after the addition of valine. After 5 minutes (**Figure 3A**), a 2 and 2.5-fold increase in the levels of ppGpp and pppGpp occured. As a negative control, a cell-free labelled sample was used to detect possible compounds that were not orthophosphate in the ^{32}P source. Also, including a ppGpp deficient strain (Δ*relA* Δ*spoT*) as a negative control could help a better identification of the spots.

Reversal of isoleucine starvation can be achieved by chloramphenicol, an inhibitor of protein

synthesis, which reduces consumption of charged ile-tRNA and in turn, restores high ratios of charged to uncharged tRNA. Activation of the strong RelA-mediated ppGpp synthetase is abolished, which allows a measure of ppGpp degradation unperturbed by residual synthesis. Therefore, 200 µg/mL of chloramphenicol was added to the starved cultures and samples were taken at 20 second intervals thereafter. In this case, ppGpp decayed with a half-life of about 64 seconds (**Figure 3B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Measurement of ppGpp by TLC workflow. Schematic representation of the protocol to extract ppGpp form bacterial cultures and its posterior detection by TLC.

Figure 2: Representative TLC analysis. Schematic representation of the results obtained after autoradiography and phosphor screen overnight with the TLC. The spots to be measured are shown in red. The formula to calculate the fractional amount of ppGpp is also shown. Total G refers to the total amount of GTP + ppGpp + pppGpp detected in the sample. The yellow band represent the pH front visible under UV light. Scissors indicate where we recommend cutting the chromatogram for discarding most of the radioactivity. The arrow indicates the direction of the flow during ascending development with 1.5 M phosphate buffer.

Figure 3: measurement of synthesis and decay of ppGpp. (**A**) Detection of ppGpp by TLC for samples 0 and 5 minutes after addition of valine. Spots corresponding to GTP, ppGpp and pppGpp are marked. A cell-free culture was included as negative control (C-). (**B**) Decay of ppGpp after addition of chloramphenicol to reverse amino acid starvation. The ppGpp half-life is determined from the exponential decay rate represented by dotted lines. Error bars reflect SD from duplicates. Y-axis is represented in a semilogaritmic (log_2) scale.

DISCUSSION:

Achieving near uniform labeling of the cells is a critical step for this protocol. Therefore, the use of defined media, such as MOPS or Tris media, is crucial to allow variation of carrier phosphate concentrations and specific activity. Phosphate buffered media, such as M9 or media A, cannot be used. Most undefined media contain variable amounts of phosphate, such as LB, tryptone and casamino acids. The phosphate isotope ^{33}P is a weaker emitter that can be substituted for ^{32}P. Advantages of the substitution are that it is safer and has a half-life of 25 days instead of 14 days for ^{32}P. However, the more energetic emissions of ^{32}P considerably enhance detection sensitivity. It is important to stress the dangers of working with isotopes and the importance of using proper shielding protections and disposal. The recent discovery of a riboswitch able to detect specifically ppGpp[28] may someday lead to a safer way to detect ppGpp in vitro and in vivo. As mentioned in the introduction, other methods exist, although the use of phosphate labelling remains a sensitive, direct and rapid approach for through-put measurements of bacterial (p)ppGpp as well as other phosphate labeled compounds, such as ribo- or deoxyribo-NTP pools, PRPP, PPi or sugar phosphates.

Another critical step is to ensure that the growth of the parallel cultures (shaking incubator and plate reader) is similar. When studying nutrient exhaustion, such as diauxic shift[19], the synchrony between cultures is crucial. The comparability of growth on labeled and unlabeled plates can be assessed by OD_{600} measurements of an unlabeled well on the otherwise radioactive plate. To increase the number of samples tested, a 96 well plate can be used instead [19], but the amount of media required to minimize evaporation will reduce culture aeration. Basal levels of (p)ppGpp will be slightly higher during growth in a 96 well plate compared to a 24 well plate due to the reduced aeration. Therefore, for measuring basal levels, a 24 well plate growth is recommended. To measure variations of more strongly induced (p)ppGpp levels, a 96 well plate is preferred. The choice between 24 and 96 well microtiter plates depends on the experimental goal.

Variations of this protocol have many potential applications for different conditions of stress. Recently we have applied it to measure accumulation and decay of ppGpp during classical diauxic shifts from glucose to lactose and to other alternative sugars. These studies revealed involvement of both glucose starvation and amino acid starvation[19]. Here we describe amino acid starvation induced by valine in *E. coli* K12 strains as a simpler and well-studied stress condition[3]. This example might be used as a control before performing more complicated starvation situations. Amino acid starvation can be provoked by adding limited amounts of a required amino acid that is exhausted during growth; adding an inhibitor of tRNA aminoacylation or synthesis (serine hydroxamate, mupirocin, or 3-aminotriazole) or for *E. coli* K12 adding valine in the absence of isoleucine). Serine hydroxamate is often used to inhibit $tRNA^{Ser}$ aminoacylation but requires high concentrations, which may cause problems due to the hydroxamate reactivity with other compounds. Reversal of serine hydroxamate effects by adding a large amounts of serine can have non-desired effects[1]. Although SHX has been proven to be effective as a diagnostic of ppGpp production, we do not recommend its use for physiological studies to produce amino acid starvation, because normal (p)ppGpp feedback mechanisms are abolished, such as physiological consequences of lowering superfluous activities but allowing activation of stress survival regulatory circuits.

Modifications of the TLC procedures described here are necessary in order to properly separate noncanonical regulatory nucleotides, such as (p)ppApp[29], from mixed samples with (p)ppGpp. It has been shown that pppApp has an antagonistic role to ppGpp in vitro[30,31]; therefore, better separation of both compounds is required for future studies.

ACKNOWLEDGMENTS:

This research was supported the Intramural Research Program, Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Cashel, M., Gentry, D., Hernandez, V., Vinella, D. The stringent Response. *Escherichia coli and Salmonella: Cellular and Molecular Biology*. 1458–1489 (1996).
2. Magnusson, L.U., Farewell, A., Nyström, T. ppGpp: a global regulator in *Escherichia coli*. *Trends in Microbiology*. **13** (5), 236–42, doi: 10.1016/j.tim.2005.03.008 (2005).
3. Cashel, M., Gallant, J. Two Compounds implicated in the Function of the RC Gene of *Escherichia coli*. *Nature*. **221** (5183), 838–841, doi: 10.1038/221838a0 (1969).
4. Braeken, K., Moris, M., Daniels, R., Vanderleyden, J., Michiels, J. New horizons for (p)ppGpp in bacterial and plant physiology. *Trends in Microbiology*. **14** (1), 45–54, doi: 10.1016/j.tim.2005.11.006 (2006).
5. Atkinson, G.C., Tenson, T., Hauryliuk, V. The RelA/SpoT homolog (RSH) superfamily: distribution and functional evolution of ppGpp synthetases and hydrolases across the tree of life. *PloS One*. **6** (8), e23479, doi: 10.1371/journal.pone.0023479 (2011).
6. Mechold, U., Potrykus, K., Murphy, H., Murakami, K.S., Cashel, M. Differential regulation by ppGpp versus pppGpp in *Escherichia coli*. *Nucleic Acids Research*. **41** (12), 6175–89, doi: 10.1093/nar/gkt302 (2013).
7. Molodtsov, V. et al. Allosteric Effector ppGpp Potentiates the Inhibition of Transcript Initiation by DksA. *Molecular Cell*. **69** (5), 828–839.e5, doi: 10.1016/J.MOLCEL.2018.01.035 (2018).
8. Ross, W., Sanchez-Vazquez, P., Chen, A.Y.Y., Lee, J.-H.H., Burgos, H.L.L., Gourse, R.L.L. ppGpp Binding to a Site at the RNAP-DksA Interface Accounts for Its Dramatic Effects on Transcription Initiation during the Stringent Response. *Molecular Cell*. **62** (6), 811–823, doi: 10.1016/j.molcel.2016.04.029 (2016).
9. Kriel, A. et al. Direct Regulation of GTP Homeostasis by (p)ppGpp: A Critical Component of Viability and Stress Resistance. *Molecular Cell*. **48** (2), 231–241, doi: 10.1016/J.MOLCEL.2012.08.009 (2012).
10. Kriel, A. et al. GTP dysregulation in *Bacillus subtilis* cells lacking (p)ppGpp results in phenotypic amino acid auxotrophy and failure to adapt to nutrient downshift and regulate biosynthesis genes. *Journal of Bacteriology*. **196** (1), 189–201, doi: 10.1128/JB.00918-13 (2014).
11. Potrykus, K., Cashel, M. (p)ppGpp: still magical? *Annual Review of Microbiology*. **62**, 35–51, doi: 10.1146/annurev.micro.62.081307.162903 (2008).
12. Hauryliuk, V., Atkinson, G.C., Murakami, K.S., Tenson, T., Gerdes, K. Recent functional insights into the role of (p)ppGpp in bacterial physiology. *Nature Reviews Microbiology*. **13** (5), 298–309, doi: 10.1038/nrmicro3448 (2015).
13. Arenz, S. et al. The stringent factor RelA adopts an open conformation on the ribosome to stimulate ppGpp synthesis. *Nucleic Acids Research*. **44** (13), 6471–6481, doi: 10.1093/nar/gkw470 (2016).
14. Loveland, A.B. et al. Ribosome•RelA structures reveal the mechanism of stringent response activation. *eLife*. **5**, e17029, doi: 10.7554/eLife.17029 (2016).
15. Brown, A., Fernández, I.S., Gordiyenko, Y., Ramakrishnan, V. Ribosome-dependent activation of stringent control. *Nature*. **534** (7606), 277–280, doi: 10.1038/nature17675 (2016).
16. Battesti, A., Bouveret, E. Acyl carrier protein/SpoT interaction, the switch linking SpoT-

dependent stress response to fatty acid metabolism. *Molecular Microbiology*. **62** (4), 1048–63, doi: 10.1111/j.1365-2958.2006.05442.x (2006).
17. Lee, J.-W., Park, Y.-H., Seok, Y.-J. Rsd balances (p)ppGpp level by stimulating the hydrolase activity of SpoT during carbon source downshift in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America*. **115** (29), E6845–E6854, doi: 10.1073/pnas.1722514115 (2018).
18. Cashel, M. Detection of (p)ppGpp Accumulation Patterns in *Escherichia coli* mutants. *Molecular Microbiology Techniques (volume 3, part A)*. 341–356 (1994).
19. Fernández-Coll, L., Cashel, M. Contributions of SpoT Hydrolase, SpoT Synthetase, and RelA Synthetase to Carbon Source Diauxic Growth Transitions in *Escherichia coli*. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2018.01802 (2018).
20. Varik, V., Oliveira, S.R.A., Hauryliuk, V., Tenson, T. HPLC-based quantification of bacterial housekeeping nucleotides and alarmone messengers ppGpp and pppGpp. *Scientific Reports*. **7** (1), 11022, doi: 10.1038/s41598-017-10988-6 (2017).
21. Rhee, H.-W. et al. Selective Fluorescent Chemosensor for the Bacterial Alarmone (p)ppGpp. **130** (3), 784–785 (2008).
22. Wang, J., Chen, W., Liu, X., Wesdemiotis, C., Pang, Y. A Mononuclear Zinc Complex for Selective Detection of Diphosphate via Fluorescence ESIPT Turn-On. *Journal of Materials Chemistry B*. **2** (21), 3349–3354, doi: 10.1039/C4TB00020J (2014).
23. Pokhilko, A. Monitoring of nutrient limitation in growing *E. coli*: a mathematical model of a ppGpp-based biosensor. *BMC Systems Biology*. **11** (1), 106, doi: 10.1186/s12918-017-0490-5 (2017).
24. Funabashi, H., Mie, M., Yanagida, Y., Kobatake, E., Aizawa, M. Fluorescent monitoring of cellular physiological status depending on the accumulation of ppGpp. *Biotechnology Letters*. **24** (4), 269–273, doi: 10.1023/A:1014032711683 (2002).
25. Neidhardt, F.C., Bloch, P.L., Smith, D.F. Culture medium for enterobacteria. *Journal of Bacteriology*. **119** (3), 736–47 (1974).
26. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. *Nature Methods*. **9** (7), 676–682, doi: 10.1038/nmeth.2019 (2012).
27. Schneider, C.A., Rasband, W.S., Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*. **9** (7), 671–5 (2012).
28. Peselis, A., Serganov, A. ykkC riboswitches employ an add-on helix to adjust specificity for polyanionic ligands. *Nature Chemical Biology*. **14** (9), 887–894, doi: 10.1038/s41589-018-0114-4 (2018).
29. Oki, T., Yoshimoto, A., Sato, S., Takamatsu, A. Purine nucleotide pyrophosphotransferase from *Streptomyces morookaensis*, capable of synthesizing pppApp and pppGpp. *Biochimica et Biophysica Acta (BBA) - Enzymology*. **410** (2), 262–272, doi: 10.1016/0005-2744(75)90228-4 (1975).
30. Travers, A.A. ppApp alters transcriptional selectivity of *Escherichia coli* RNA polymerase. *FEBS Letters*. **94** (2), 345–8 (1978).
31. Bruhn-Olszewska, B. et al. Structure-function comparisons of (p)ppApp vs (p)ppGpp for *Escherichia coli* RNA polymerase binding sites and for rrnB P1 promoter regulatory responses in vitro. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*. **1861** (8), 731–742, doi: 10.1016/J.BBAGRM.2018.07.005 (2018).

Figure

Click here to access/download;Figure;figure 1.eps

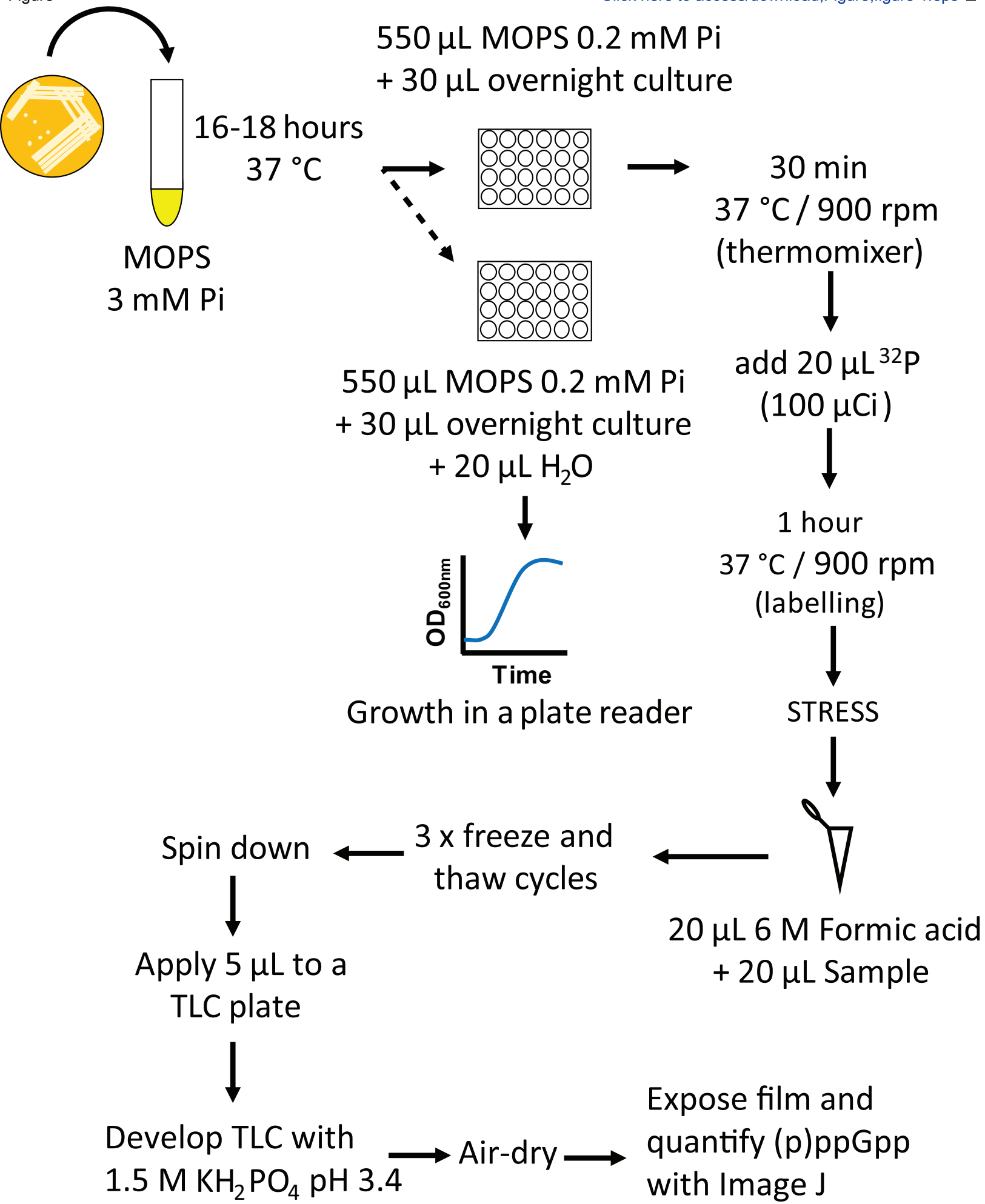
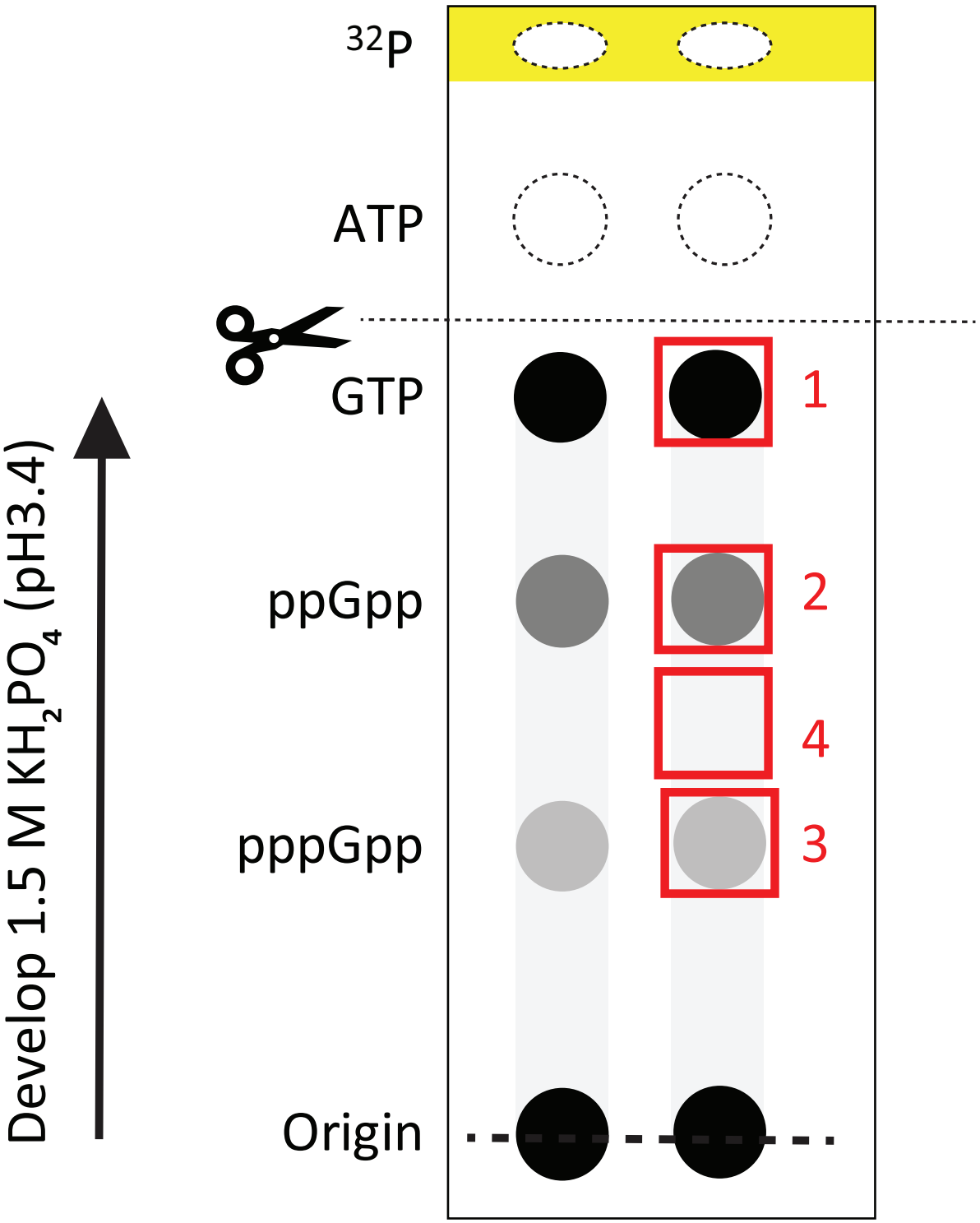
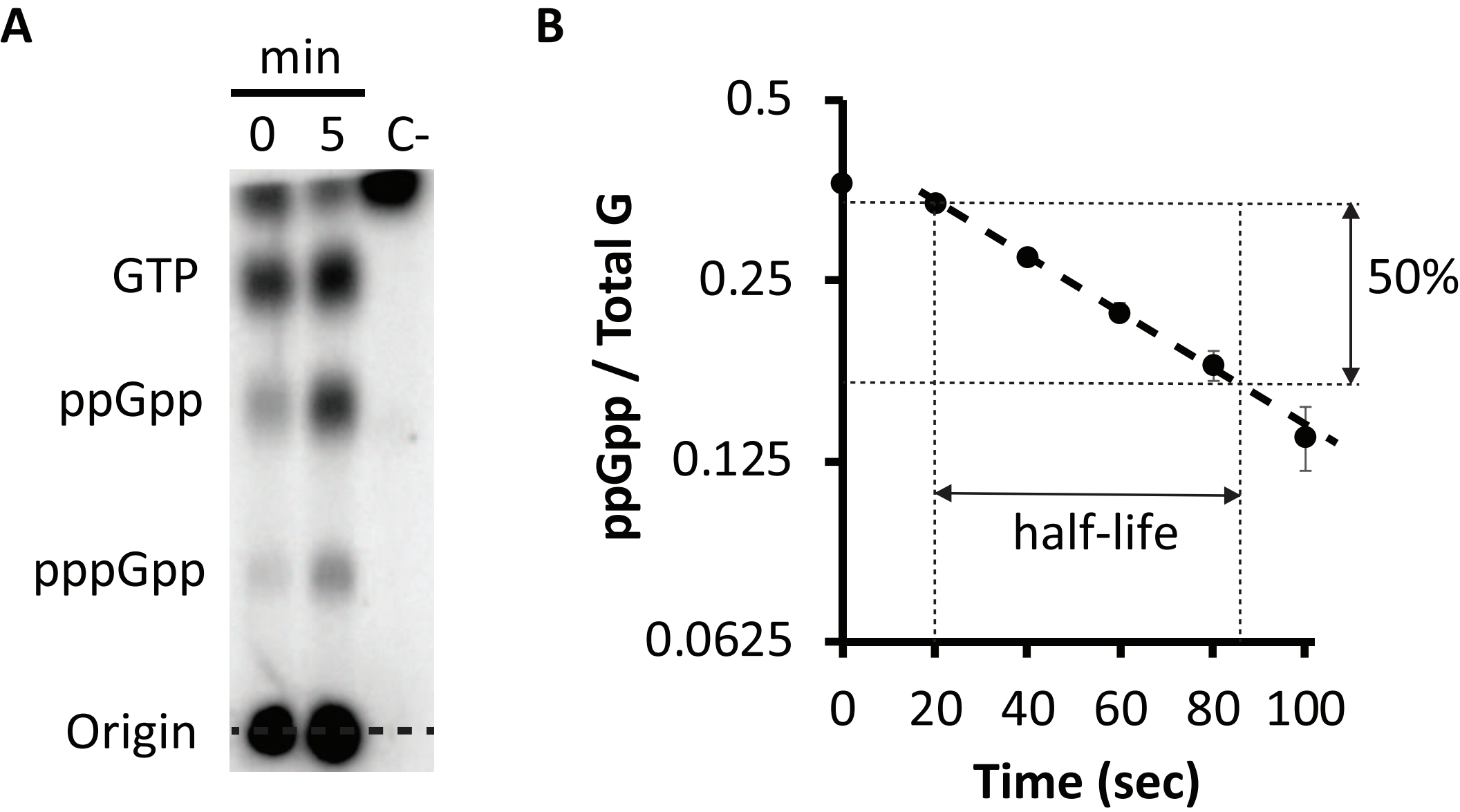


Figure 2

Click here to access/download;Figure;figure 2.eps



$$\text{Fractional amount of ppGpp} = \frac{\text{ppGpp (2)} - \text{blank (4)}}{\text{Total G ((1-4) + (2-4) + (3-4))}}$$



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
$(NH_4)_6(MO_7)_{24}$	Fisher Scientifics	A-674	
Autoradiography film	Denville scientific inc.	E3218	
$CaCl_2$	J.T.Baker	1-1309	
Chloramphenicol	RPI	C61000-25.0	
$CoCl_2$	Fisher Scientifics	C-371	
$CuSO_4$	J.T.Baker	1843	
$FeSO_4$	Fisher Scientifics	I-146	
Formic acid	Fisher Biotech	BP1215-500	
Glucose	Macron	4912-12	
H_3BO_4	Macron	2549-04	
H_3PO_4	J.T.Baker	0260-02	
K_2SO_4	Sigma	P9458-250G	
KH_2PO_4	Fisher Biotech	BP362-500	
L-Valine	Sigma	V-6504	
$MgCl_2$	Fisher Scientifics	FL-06-0303	
Microplate reader Synergy HT	Biotek	Synergy HT	
$MnCl_2$	Sigma	M-9522	
MOPS	Sigma	M1254-1KG	
Na_2HPO_4	Mallinckrodt	7892	
NaCl	J.T.Baker	3624-01	
NaH_2PO_4	Mallinckrodt	7917	
NH_4Cl	Sigma	A0171-500G	
P-32 radionuclide, orthophosphoric acid in 1 mL water (5 mCi)	Perkin Elmer	NEX053005MC	
Storage phosphor screen	Kodak	So230	
Thermomixer	Eppendorf	5382000015	
Thiamine	Sigma	T-4625	
TLC PEI Cellulose F	Merk-Millipore	1.05579.0001	
Tricine	RPI	T2400-500.0	

Typhoon 9400 imager	GE Healthcare
$ZnSO_4$	Fisher Scientifics Z-68

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: USE OF MICROTITER DISH RADIOLABELING FOR MULTIPLE IN VIVO MEASUREMENTS OF ESCHERICHIA COLI (P)PPGPP FOLLOWED BY TLC

Author(s): LLORENÇ FERNÁNDEZ-COLL AND MICHAEL CASHEL

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☐ The Author is **NOT** a United States government employee.

☒ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	LLORENÇ FERNANDEZ-COLL
Department:	INTRAMURAL RESEARCH PROGRAM
Institution:	NICHD, NIH
Title:	POSTDOCTORAL FELOW
Signature:	
Date:	12/20/2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

We believe that all the required changes were made in the manuscript in a traceable way, so it can be easily spotted by the editor and the reviewers. We agree in all the reviewer's comments and we think that they improve substantially our manuscript.

Editorial comments: all the format changed were performed as suggested.

Reviewer 1: all the suggested changes were addressed. We discussed with further detail the advantages of TLC method over other methods as suggested. A better description of the preparation of MOPS was provided. About comparing growth in thermomixer vs plate reader, in our case is quite similar, but we agree that it is a crucial step, so we included a comment on the discussion.

Reviewer 2: all the suggested changes were addressed. We actually use 100 µCi/well. We consider that for low basal levels 100 µCi with 0.2 mM carrier phosphate works well, but for measuring (p)ppGpp levels that are expected to become equivalent to GTP pools, we consider that it can be dropped to 25 or 50 µCi/well. Although it may seem too much, most of the signal will be discarded before exposing the film by removing the pH front that contains free ^{32}P (and even more if we remove any nucleotides on top of GTP). This has been addressed in the text.

We hope that this will satisfy all the comments and concerns.

Yours faithfully,

Llorenç Fernández-Coll and Michael Cashel