Journal of Visualized Experiments

Identifying the Binding Proteins of Small Ligands with the Differential Radial Capillary Action of Ligand Assay (DRaCALA)

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62331R1
Full Title:	Identifying the Binding Proteins of Small Ligands with the Differential Radial Capillary Action of Ligand Assay (DRaCALA)
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Biochemistry
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
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TITLE:

Identifying the Binding Proteins of Small Ligands with the Differential Radial Capillary Action of Ligand Assay (DRaCALA)

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KEYWORDS:

DRaCALA, ORFeome, ppGpp, cyclic AMP, c-di-AMP, c-di-GMP

SUMMARY:

The Differential Radial Capillary Action of Ligand Assay (DRaCALA) can be used to identify small ligand binding proteins of an organism by using an ORFeome library.

ABSTRACT:

The past decade has seen tremendous progress in the understanding of small signaling molecules in bacterial physiology. In particular, the target proteins of several nucleotide-derived secondary messengers (NSMs) have been systematically identified and studied in model organisms. These achievements are mainly due to the development of several new techniques including the capture compound technique and the differential radial capillary action of ligand assay (DRaCALA), which were used to systematically identify target proteins of these small molecules. This paper describes the use of the NSMs, guanosine penta- and tetraphosphates (p)ppGpp, as an example and video demonstration of the DRaCALA technique. Using DRaCALA, 9 out of 20 known and 12 new target proteins of (p)ppGpp were identified in the model organism, *Escherichia coli* K-12, demonstrating the power of this assay. In principle, DRaCALA could be used for studying small ligands that can be labeled by radioactive isotopes or fluorescent dyes. The critical steps, pros, and cons of DRaCALA are discussed here for further application of this technique.

INTRODUCTION:

Bacteria use several small signaling molecules to adapt to constantly changing environments[1,2]. For example, the autoinducers, *N*-acylhomoserine lactones and their modified oligopeptides, mediate the intercellular communication among bacteria to coordinate population behavior, a phenomenon known as quorum sensing[2]. Another group of small signaling molecules is the NSMs, including the widely studied cyclic adenosine monophosphate (cAMP), cyclic di-AMP,

cyclic di-guanosine monophosphate (cyclic di-GMP), and guanosine penta- and tetra phosphates (p)ppGpp[1]. Bacteria produce these NSMs as a response to a variety of different stress conditions. Once produced, these molecules bind to their target proteins and regulate several different physiological and metabolic pathways to cope with the encountered stresses and enhance bacterial survival. Therefore, identification of the target proteins is an unavoidable prerequisite for deciphering the molecular functions of these small molecules.

The past decade has witnessed a boom of knowledge of these small signaling molecules, mainly due to several technical innovations that unveiled the target proteins of these small molecules. These include the capture compound technique[3-5], and the differential radial capillary action of ligand assay (DRaCALA)[6] to be discussed in this paper. Invented by Vincent Lee and co-workers in 2011[6], DRaCALA deploys the ability of a nitrocellulose membrane to differentially sequester free and protein-bound ligands. Molecules such as proteins cannot diffuse on a nitrocellulose membrane, while small ligands, such as the NSMs, are able to.

By mixing the NSM (*e.g.*, ppGpp) with the protein to be tested and spotting them on the membrane, two scenarios can be expected (**Figure 1**): If (p)ppGpp binds to the protein, the radiolabeled (p)ppGpp will be retained in the center of the spot by the protein and will not diffuse outward, giving an intense small dot (*i.e.*, strong radioactive signal) under a phosphorimager. However, if (p)ppGpp does not bind to the protein, it will diffuse freely outward to produce a large spot with uniform background radioactive signal. Furthermore, DRaCALA can detect the interaction between a small molecule and an unpurified protein in a whole cell lysate if the protein is present in a sufficient amount.

This simplicity allows the use of DRaCALA in rapidly identifying protein targets by using an ORFeome expression library. Indeed, target proteins of cAMP[7], cyclic di-AMP[8], cyclic di-GMP[9,10], and (p)ppGpp[11-13] have been systematically identified by using DRaCALA. This video article uses (p)ppGpp as an example to demonstrate and describe the critical steps and considerations in performing a successful DRaCALA screening. Of note, a more thorough description of DRaCALA[14] is highly recommended to read in combination with this article before performing DRaCALA.

PROTOCOL:

1. Preparation of whole cell lysates

1.1. Inoculate the *E. coli* K-12 ASKA ORFeome collection strains[15] into 1.5 mL Lysogeny broth (LB) containing 25 µg/mL chloramphenicol in 96-well deep well plates. Grow overnight (O/N) for 18 h at 30 °C with shaking at 160 rpm. On the next day, add isopropyl β-d-1-thiogalactopyranoside (IPTG) (final 0.5 mM) to the O/N cultures to induce protein expression at 30 °C for 6 h.

1.2. Pellet cells at 500 x *g* for 10 min. Freeze the pellets at -80 °C until use. To lyse the cells, add 150 µL of lysis buffer L1 (40 mM Tris pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF), 40 µg/mL DNase 1, and 0.5 mg/mL lysozyme) to resuspend the pellet.

1.3. Freeze the cells at -80 °C for 30 min, and then thaw at 37 °C for 20 min. Repeat this cycle three times to lyse the cells. Store the lysates at -80 °C before use.

2. Purification of Rel_{seq} and GppA

NOTE: The recombinant proteins Rel_{seq} from *Streptococcus equisimilis* and GppA from *E. coli* K-12 are used to synthesize the radiolabeled pppGpp and ppGpp, respectively.

2.1. Grow and collect cells overexpressing each protein.

2.1.1. Grow the *E. coli* BL21 DE3 strain up to exponential phase (optical density (OD) ~0.3–0.4) in LB broth, and spin down 1 mL of culture at 6000 x *g* for 5 min. Decant the supernatant, and resuspend the cells with 100 µL of ice-cold TSB broth (LB broth supplemented with 0.1 g/mL PEG3350, 0.05 mL/mL dimethylsulfoxide, 20 mM $MgCl_2$).

2.1.2. Mix the plasmids bearing the histidine-tagged *rel_{seq}* and *gppA*, each 100 ng, with the above cell suspensions in TSB, and incubate on ice for 30 min. Heat-shock the cells at 42 °C for 40 s. Place the mixture on ice for 2 min, and add 1 mL of LB broth at room temperature to allow the cells to recover for 1 h at 37 °C with agitation at 160 rpm.

2.1.3. Plate the recovered cells on LB agar plates supplemented with the corresponding antibiotics (Rel_{seq}:100 µg/mL ampicillin; GppA: 30 µg/mL kanamycin).On the next day, inoculate the colonies in LB broth to start O/N precultures of both strains at 37 °C.

2.1.4. After 18 h, inoculate 500 mL of LB medium with 10 mL of the O/N cultures and the corresponding antibiotics. Grow the cultures by shaking at 160 rpm at 37 °C. When the OD_{600nm} reaches 0.5–0.7, induce protein expression by adding 0.5 mM IPTG and growing for 3 h at 30 °C with shaking at 160 rpm.

2.1.5. Collect the cells by spinning at 6084 x *g* for 10 min at 4 °C. Resuspend the pellet in 20 mL of ice-cold 1x phosphate-buffered saline (PBS), and re-centrifuge at 1912 x *g* for 20 min at 4 °C. Decant the supernatant, and freeze the pellets at -20 °C before use.

2.2. Nickel-nitrilotriacetic acid (Ni-NTA) affinity purification

NOTE: From this point onwards, ensure that the samples are cold.

2.2.1. Add 40 mL of ice-cold lysis buffer L2 (50 mM Tris pH 7.5, 150 mM NaCl, 5% glycerol, 10 mM imidazole, 10 mM β-mercaptoethanol supplemented with protease inhibitors (EDTA-free tablet; see the **Table of Materials**) to resuspend the pellet. Lyse the cells via sonication (60% amplitude, 2 s ON/ 4 s OFF for 8 min ON). Clear the lysate by spinning at 23,426 x *g* for 40 min at 4 °C, and continue with the supernatant for the purification.

2.2.2. During the above centrifugation, prepare the Ni-NTA resin.

2.2.2.1. Transfer 500 µL of homogenized Ni-NTA resin into a standing polypropylene chromatography column, and let it settle for 15 min and the storage solution drain through. Wash the resin with 15 mL of ultrapure water twice, and then wash the column with 15 mL of the lysis buffer L2.

2.2.3. Load the cleared supernatant of cell lysate from step 2.2.1 onto the column, and let it flow through. Wash the column with 30 mL of washing buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5% glycerol, 20 mM imidazole).

2.2.4. Elute the proteins with 400 µL of the elution buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5% glycerol, 500 mM imidazole) three times. Then, repeat elution with another 300 µL of the elution buffer. Combine the eluted proteins to a final volume of 700 µL.

2.3. Gel filtration

2.3.1. Prepare gel filtration buffer (50 mM Tris, pH 7.5; 200 mM NaCl; 5% glycerol). Wash the size exclusion column with one column volume (25 mL) of the gel filtration buffer.

2.3.2. Load the above 700 µL sample by using a 500 µL loop, run at 0.5 mL/min, and collect 2–3 fractions, each of 0.5 mL volume, containing the respective proteins.

2.3.3. Combine and concentrate the fractions containing each of the proteins using a spin column, and measure the protein concentration using the Bradford assay.

3. Synthesis of ^{32}P-labeled pppGpp and ppGpp

3.1. Assemble a small-scale Rel_{seq} reaction in a screw cap tube (see **Table 1**).

NOTE: Work with radioactive reagents only in a licensed place and with personal protective equipment.

3.2. Incubate the tube at 37 °C in a thermomixer for 1 h, then at 95 °C for 5 min, and place on ice for 5 min. Spin down the precipitated protein at 15,700 x *g* for 5 min, and transfer the supernatant (synthesized ^{32}P-pppGpp) to a new screw cap tube.

3.3. To synthesize ^{32}P-ppGpp from ^{32}P-pppGpp, transfer half of the ^{32}p-pppGpp product to a new screw cap tube, and add 1 µM GppA. Incubate the tube at 37 °C for 10 min, at 95 °C for 5 min, and then place on ice for 5 min.

3.4. Spin down the precipitated protein at 15,700 x *g* for 5 min, and transfer the supernatant (synthesized ^{32}P-ppGpp) to a new screw cap tube.

3.5. Analyze the ^{32}P-pppGpp and ^{32}P-ppGpp by running 1 µL of the samples on a thin layer chromatography (TLC) plate (polyethyleneimine-modified cellulose TLC plates) using the 1.5 M KH_2PO_4, pH 3.4, as mobile phase.

NOTE: Use the α-^{32}P-labeled guanosine 5'-triphosphate (^{32}P-α-GTP) as control.

3.6. Dry the TLC plate completely, place it between a transparent plastic folder, and expose it to a storage phosphor screen for 5 min. Visualize and quantify the signals by using a phosphorimager.

NOTE: When the ratios of ^{32}P-pppGpp and ^{32}P-ppGpp are higher than 85%, a large-scale reaction (500 µL, sufficient for screening 20 96-well plates) could be assembled and synthesized by using **Table 1**.

4. DRaCALA screening of the target proteins of (p)ppGpp

4.1. Thaw and transfer 20 µL of whole cell lysates to a 96-well V-bottom microtiter plate. Add 2.5 U/well of endonuclease from *Serratia marcescens*, and incubate at 37 °C for 15 min to reduce lysate viscosity. Place the lysates on ice for 20 min.

4.2. Mix the ^{32}P-pppGpp and ^{32}P-ppGpp in a 1:1 ratio, and add 1x lysis buffer L1 to make the final concentration of (p)ppGpp equal to 4 nM.

NOTE: Given the chemical similarity between pppGpp and ppGpp, a mix of both chemicals will simplify the screening process.

4.3. Use a multichannel pipette and filtered pipette tips to add and mix 10 µL of the (p)ppGpp mixture with the cell lysate. Incubate at room temperature (RT) for 5 min.

4.4. Wash the 96 x pin tool by placing in 0.01% solution of non-ionic detergent for 30 s, and dry on a tissue paper for 30 s. Repeat the washing of the pin tool 3x.

4.5. Place the pin tool in the above 96-well sample plates, and wait for 30 s. Lift the pin tool straight up, and place it straight down on a nitrocellulose membrane for 30 s.

NOTE: If a spot is missing, spot 2 µL of the corresponding samples with a pipette and filtered tips. It is advisable to make a duplicate spot of the same sample as indicated below.

4.6. Dry the membrane for 5 min at RT. Place the membrane between a transparent plastic folder, and expose it to a storage phosphor screen for 5 min. Visualize by using a phosphorimager.

5. Quantification and identification of potential target proteins

5.1. Use the analysis software associated with the phosphorimager to open the .gel file of the visualized plates. Use the **Array analysis** function to define the 96 spots by setting up a grid of 12 columns x 8 rows.

5.2. Define big circles to circumscribe the outer edge of the whole spots (see **Figure 1B**). Export the **Volumn+Background** and **Area** of the defined big circles, and save in a spreadsheet.

NOTE: If required, reposition each individual circle to perfectly overlap with the spots, and resize each individual circle to make it slightly bigger than the actual spot.

5.3. Size down the defined circles to circumscribe the small inner dots. Export the **Volumn+Background**, and **Area** of the defined small circles, and save in a spreadsheet.

5.4. Calculate the binding fractions in the spreadsheet by using the equation in **Figure 1B**, and plot the data. Identify the potential binding proteins in the wells that show high binding fractions in comparison to the majority of other wells.

REPRESENTATIVE RESULTS:

Following the above-described protocol will typically yield two types of results (**Figure 3**). **Figure 3A** shows a plate with relatively low background binding signals (binding fractions < 0.025) from the majority of wells. The positive binding signal from the well H3 gives a binding fraction of ~0.35 that is much higher than that observed for the other wells. Even without quantification, well H3 is remarkable, suggesting that a target protein expressed in well H3 binds to either pppGpp, ppGpp, or both. Indeed, the protein overexpressed in well H3 is the hypoxanthine phosphoribosyltransferase Hpt, which is known to bind (p)ppGpp[12,16]. The other typical result of the screening is shown in **Figure 3B,C**.

In this plate, several wells showed relatively higher background binding signals than those in **Figure 3A**. This is clearly visible from the relatively strong inner dots for many wells. Quantification also showed that many wells have binding fractions in the range of 0.02–0.04. A higher background of the binding signal is likely caused by the whole cell lysate being viscous despite the treatments with both DNase I and the endonuclease from *S. marcescens*, which degrade the released chromosomal DNA. For plates such as this one, it is important to compare the two replicate spots of the plate (step 4.5; **Figure 3B,C**). Quantification of both plates shows that the authentic positive targets (black circles, wells A10 PrfC, B11 NadR) tend to give consistently high binding fractions. Notably, some true targets could also give variable binding fractions (Well D5, HflX[12]) such as the false positives (red circles).

The reason for this variability lies in the fact that not all proteins in a library are expressed in soluble form and in required amounts. If the concentration of a protein is close to or just below the K_d value, variable binding results can be expected, even for the true targets. Indeed, large amounts of soluble HflX protein were not obtained from the ASKA strain[12]. To determine whether these proteins are true binders or not, the potential proteins must be purified to homogeneity and the binding confirmed by using a higher concentration (50–100 μM) of the proteins. Via this

screening, 9 out of 20 known target proteins of (p)ppGpp[12] were identified (see the discussion section), validating the usefulness of DRaCALA for this task. Additionally, 12 new targets of (p)ppGpp were discovered and confirmed, demonstrating that DRaCALA is a powerful technique to uncover novel target proteins of (p)ppGpp.

FIGURE AND TABLE LEGENDS:

Figure 1: The principle of DRaCALA. (**A**) Schematic of the DRaCALA assay. See the text for details. (**B**) Quantification and calculation of the binding fraction. See the text for details. Briefly, the DRaCALA spots will be analyzed by drawing two circles that circumscribe the whole spot and the inner dark dot (*i.e*., the retained (p)ppGpp due to the binding of the tested protein). The specific binding signal is the radioactive signal of the inner circle (S1) after subtracting the non-specific background signal (calculated by $A_1 \times ((S_2\text{-}S_1)/(A_2\text{-}A_1))$). The binding fraction is the specific binding signal divided by the total radioactive signal (S2). Abbreviations: DRaCALA = Differential Radial Capillary Action of Ligand Assay; (p)ppGpp = guanosine penta- and tetraphosphates; RT = room temperature.

Figure 2: Overall workflow of the DRaCALA screening process. Protein production from an *Escherichia coli* ASKA collection is induced, and the cells are lysed. Meanwhile, the recombinant proteins Rel_{seq}-His and GppA-His are purified and used to synthesize ^{32}P-labeled pppGpp and ppGpp from ^{32}P-α-GTP. The radioactively labeled (p)ppGpp molecules are then mixed with the lysates, and a 96 pin-tool is used to spot the mixtures onto a nitrocellulose membrane for subsequent exposure to a phosphor storage screen, imaging, and quantification of the radioactive signals. Abbreviations: DRaCALA = Differential Radial Capillary Action of Ligand Assay; (p)ppGpp = guanosine penta- and tetraphosphates; RT = room temperature; IPTG = isopropyl β-d-1-thiogalactopyranoside; GTP = guanosine 5'-triphosphate; SDS-PAGE = sodium dodecylsulfate-polyacrylamide gel electrophoresis ; TLC = thin layer chromatography.

Figure 3: Representative DRaCALA screening plates (to the left) and quantification (to the right). (**A**) DRaCALA spots of the ASKA Plate-50. The only positive hit, Hpt, gave a strong binding signal standing out in both the spot and the quantitation diagram. (**B,C**) Two replicate DRaCALA spots of the rearranged plate 31. Black broken circles and arrows indicate the true target proteins of (p)ppGpp, while the red broken circles indicate the false positives. See the text for details. Abbreviations: DRaCALA = Differential Radial Capillary Action of Ligand Assay; (p)ppGpp = guanosine penta- and tetraphosphates; RT = room temperature; IPTG = isopropyl β-d-1-thiogalactopyranoside; GTP = guanosine 5'-triphosphate; SDS-PAGE = sodium dodecylsulfate-polyacrylamide gel electrophoresis ; TLC = thin layer chromatography; Hpt = hypoxanthine phosphoribosyltransferase; Prfc = peptide chain release factor; NadR = NMN adenylyltransferase; HflX = translational GTPase.

Table 1: Assembling information for the small- and large-scale synthesis reactions of ^{32}P-labeled pppGpp. *10x Relseq buffer contains 250 mM Tris-HCl, pH 8.6; 1M NaCl; 80 mM $MgCl_2$. Abbreviation: pppGpp = guanosine pentaphosphate.

DISCUSSION:

One of the critical steps in performing DRaCALA screening is to obtain good whole cell lysates. First, the tested proteins should be produced in large amounts and in soluble forms. Second, the lysis of cells should be complete, and the viscosity of the lysate must be minimal. The inclusion of lysozyme and the use of three cycles of freeze-thaw are often enough to lyse cells completely. However, the released chromosomal DNA makes the lysate viscous and generates high background binding signal, resulting in false positives as shown in **Figure 3B,C**. To mitigate this, DNase 1 and/or endonuclease from *S. marcescens* can be used, and the samples can be incubated for a longer time to degrade the DNA. Both negative (empty plasmid vector) and positive (known target protein) controls should be used to optimize the conditions for whole cell lysate preparation and the DRaCALA binding assay before performing a large-scale screening. When such an optimal condition is found, it becomes unnecessary to include the controls in the actual screening of each plate.

This is because the screening procedure is very short and because the majority of the proteins in a plate are expected to not bind to (p)ppGpp. Therefore, these proteins serve as negative controls when quantifying the binding fractions (**Figure 3**). The production of purer ^{32}P-labeled pppGpp and ppGpp is also essential for DRaCALA screening. For instance, the conversion ratio from GTP to pppGpp could vary. Thus, it is important to optimize the pH, the concentrations of magnesium and enzymes used, and the reaction time. Small-scale reactions are therefore important to identify the best condition before setting up a large-scale reaction. DRaCALA clearly has some advantages and disadvantages (see Roelofs et al.[9] for more discussion) when compared to other techniques such as the capture compound technique. First, DRaCALA uses the ^{32}P-labeled (p)ppGpp, which does not affect the chemical structure of (p)ppGpp, thereby preserving its interaction with potential target proteins.

Second, once the ^{32}p-(p)ppGpp and cell lysates are prepared, it takes only a week to screen ~60 plates of the *E. coli* ORFeome library using DRaCALA. Indeed, the short experimental procedure (section 4) allowed the identification of even the proteins that degrade the (p)ppGpp (MutT, NudG)[12,17], which the capture compound technique missed[18]. However, the use of DRaCALA requires an ORFeome expression library, which is only available for a limited number of model organisms. Besides, the affinity purification tags, which are designed to facilitate protein purification in the ORFeome library, sometimes affect the expression or proper folding of the proteins, producing some false negatives. A new ORFeome library will ideally require the determination as to whether the majority (>80%) of the proteins are expressed in soluble form and in adequate amounts. Such a test also allows the researchers to evaluate the coverage and effectiveness of the DRaCALA screening results. Despite these limitations, DRaCALA is a powerful technique to successfully uncover novel target proteins of several nucleotide secondary messengers. In principle, DRaCALA could be used for studying any small ligand if it could be labeled by using either radioactive isotopes or even fluorescent dyes.

ACKNOWLEDGMENTS:

The work is supported by an NNF Project Grant (NNF19OC0058331) to YEZ, and a Marie Skłodowska-Curie grant (N^{o} 801199) to MLS.

DISCLOSURES:

The authors have no conflict of interest to disclose.

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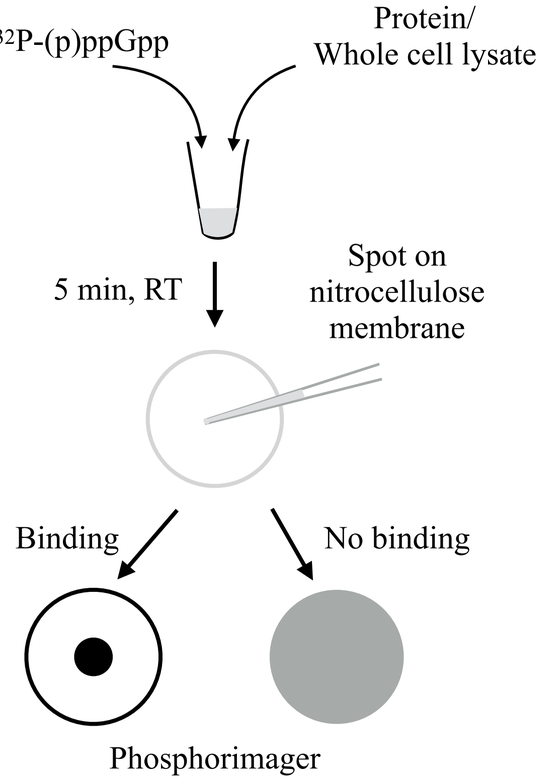
A.



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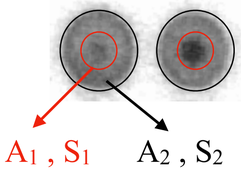


Binding fraction =

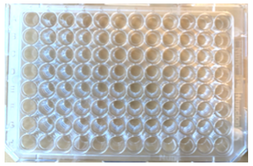
$$\frac{S_1 - \frac{S_2 - S_1}{A_2 - A_1} \times A_1}{S_2}$$

- A: area of the encircled spots
- S: radioactive signal of the area
- Binding fraction: specific signal of inner circle divided by the total signal

E. coli
ASKA collection

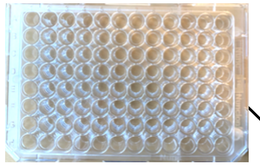
96-well deep well plate
IPTG 0.5 mM, 6 h, 30 ° C

Pellet cells; resuspend in lysis buffer; freeze-thaw for 3 times



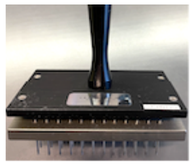
V-bottom microtiter plate
20 µL whole cell lysate

Endonuclease 2 U/ well, 37 ˚C 15 min, then on ice



Add 10 µL of (p)ppGpp in each well and mix

5 min, RT



96-pin tool

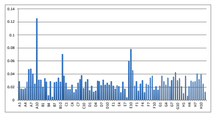
30 s
Inside the plate

Rest the pin tool on a nitrocellulose membrane for 30 s

Dry the membrane
10 min, RT

Expose to phosphor screen for 5 min

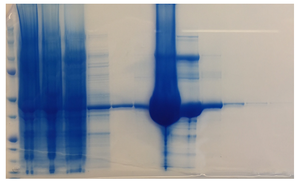
Visualize using Phosphorimager (2 min)



Quantification

BL21 DE3 containing pET28b-*relseq-his* or pET28b-*gppA-his*

Protein expression and purification



SDS-PAGE

Synthesis of ^{32}P-pppGpp and ppGpp

GTP
ppGpp
pppGpp

TLC

Mix pppGpp and ppGpp in 1:1 ratio, dilute to final 4 nM

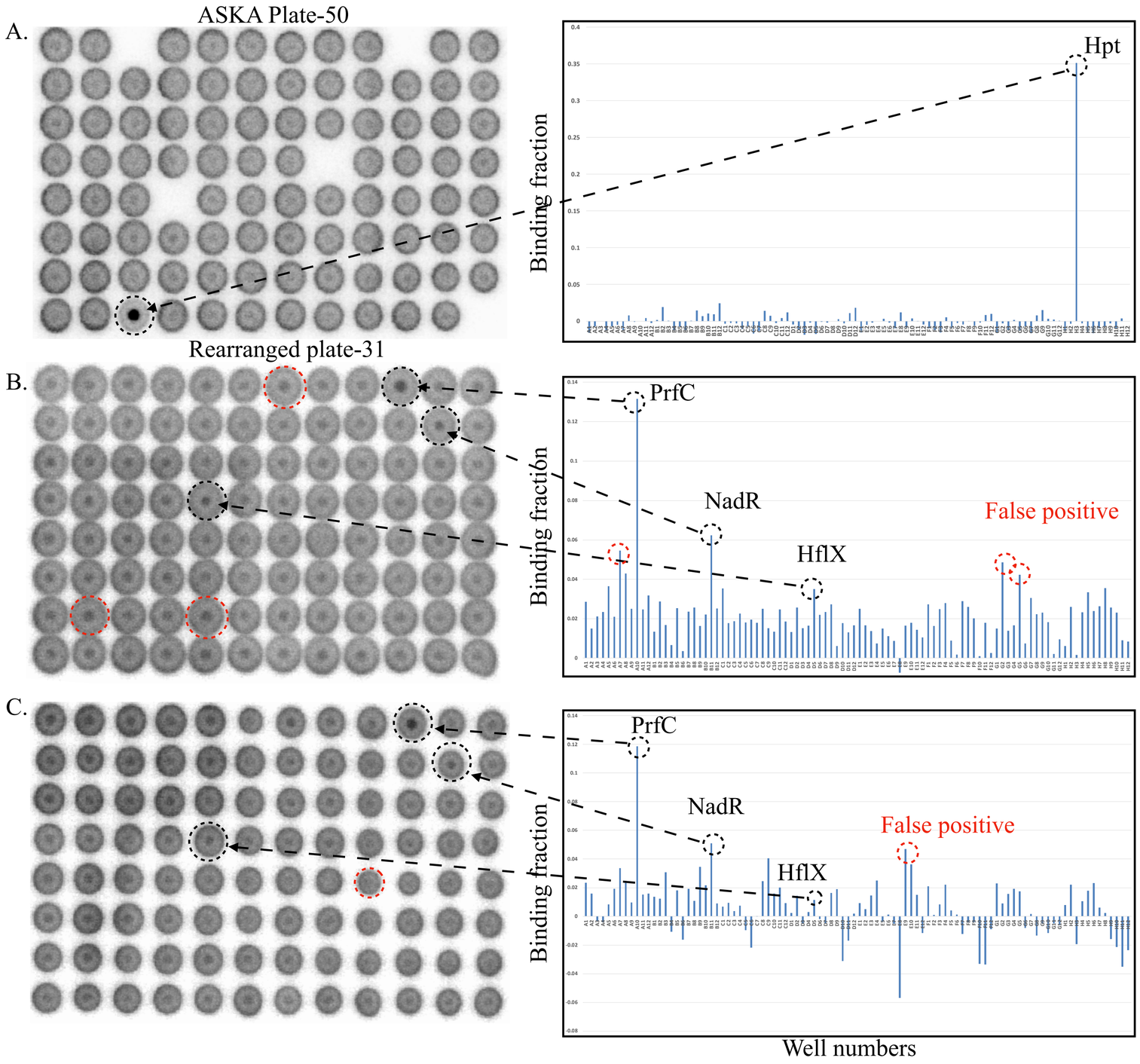

A.
ASKA Plate-50
Hpt
Binding fraction
B.
Rearranged plate-31
PrfC
NadR
HflX
False positive
Binding fraction
C.
PrfC
NadR
HflX
False positive
Binding fraction
Well numbers

Table 1

	Volume (μL)	
	Small scale	Large scale
Ultrapure water		
10x Rel_{seq} buffer*	2	50
ATP (8 mM final)		
Rel_{seq} (4 μM final)		
^{32}P-α-Guanosine triphosphate (GTP) (final 120 nM) (CAUTION)	0.2	5
total	20	500

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
^{32}P-α-GTP	Perkinelmer	BLU006X250UC	
96 x pin tool	V&P Scientific	VP 404	96 Bolt Replicator, on 9 mm centers, 4.2 mm Bolt Diameter, 24 mm long
96-well V-bottom microtiter plate	Sterilin	MIC9004	Sterilin Microplate V Well 611V96
Agar	OXOID - Thermo Fisher	LP0011	Agar no. 1
ASKA collection strain	NBRP, SHIGEN, JAPAN		Ref: DNA Research, Volume 12, Issue 5, 2005, Pages 291–299. https://doi.org/10.1093/dnares/dsi012
Benzonase	SIGMA	E1014-25KU	genetically engineered endonuclease from *Serratia marcescens*
Bradford Protein Assay Dye	Bio-Rad	5000006	Reagent Concentrate
DMSO	SIGMA	D8418	≥99.9%
DNase 1	SIGMA	DN25-1G	
gel filtration10x300 column	GE Healthcare	28990944	contains 20% ethanol as preservative
Glycerol	PanReac AppliChem	122329.1214	Glycerol 87% for analysis
Hypercassette	Amersham	RPN 11647	20 x 40 cm
Imidazole	SIGMA	56750	puriss. p.a., ≥ 99.5% (GC)
IP Storage Phosphor Screen	FUJIFILM	28956474	BAS-MS 2040 20x 40 cm
Isopropyl β-d-1-	SIGMA	I6758	Isopropyl β-D-thiogalactoside
Lysogeny Broth (LB)	Invitrogen - Thermo Fisher	12795027	Miller's LB Broth Base
Lysozyme	SIGMA	L4949	from chicken egg white; BioUltra, lyophilized powder, ≥98%
$MgCl_2$ (Magnesium chloride)	SIGMA	208337	
MilliQ water			ultrapure water
multichannel pipette	Thermo Scientific	4661110	F1 - Clip Tip; 1-10 ul, 8 x channels
NaCl	VWR Chemicals	27810	AnalaR NORMAPUR, ACS, Reag. Ph. Eur.
Ni-NTA Agarose	Qiagen	30230	
Nitrocellulose Blotting Membrane	Amersham Protran	10600003	Premium 0.45 um 300 mm x 4 m
PBS	OXOID - Thermo Fisher	BR0014G	Phosphate buffered saline (Dulbecco A), Tablets
PEG3350 (Polyethylene glycol 3350)	SIGMA	202444	
phenylmethylsulfonyl fluoride (PMSF)	SIGMA	93482	Phenylmethanesulfonyl fluoride solution - 0.1 M in ethanol (T)
Phosphor-imager	GE Healthcare	28955809	Typhoon FLA-7000 Phosphor-imager
Pipette Tips, filtered	Thermo Scientific	94410040	ClipTip 12.5 µl nonsterile
Poly-Prep Chromatography column	Bio-Rad	7311550	polypropylene chromatography column
Protease inhibitor Mini	Pierce	A32955	Tablets, EDTA-free
screw cap tube	Thermo Scientific	3488	Microcentifuge Tubes, 2.0 ml with screw cap, nonsterile
SLS 96-deep Well plates	Greiner	780285	MASTERBLOCK, 2 ML, PP, V-Bottom, Natural
spin column	Millipore	UFC500396	Amicon Ultra -0.5 ml Centrifugal Filters
Thermomixer	Eppendorf	5382000015	Thermomixer C
TLC plate (PEI-cellulose F TLC plates)	Merck Millipore	105579	DC PEI-cellulose F (20 x 20 cm)
Tris	SIGMA	BP152	Tris Base for Molecular Biology
Tween 20	SIGMA	P1379	viscous non-ionic detergent

β-mercaptoethanol	SIGMA	M3148	99% (GC/titration)

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done.

2. Please provide an institutional email address for each author.

Done.

3. Please define all abbreviations before use (IPTG).

Done.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

For example: Superdex, Thermo Mixer, ClipTip Pipette Tips, V&P Scientific, Typhoon FLA- 7000 Phosphor-imager, ImageQuant.

Corrected.

5. Line 90: Please include more details on transformation.

The details are added now.

6. For SI units, please use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2 (Lines: 72, 79, 80,93, 112, 115, 116,120,122, 124, etc.

Done.

7. Line 97/ 107/186: For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks

Done.

8. Line 132: Please mention how many fractions are collected. How long are the fractions collected?

Done.

9. Line 140-145: Please remove the embedded table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

Done.

10. Line 175: Please specify the duration for which the lysates are placed on ice.

Done.

11. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol (Figure Legends for Figure 2 and Figure 3).

Done.

12. Please do not use the &-sign or the word “and” when listing authors. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. End the list of authors with a period. Example: Bedford, C. D., Harris, R. N., Howd, R. A., Goff, D. A., Koolpe, G. A. Quaternary salts of 2-[(hydroxyimino)methyl]limidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998). Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and

should appear exactly as they were published in the original work, without any abbreviations or truncations
Done.
13. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.
Done.

14. Figure 2: please use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Please use the standard abbreviations for time units Examples: 10 mL, 8 s.
Done.

_____

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript by Schicketanz and colleagues is an easy-to-follow description of the DRaCALA approach. I have a few suggestions.
Major Concerns:
L233 It is disturbing that both false positives and "true" targets can yield variable results. Of your 12 new targets, how many of them have been validated, by for instance, L236 purification of "the potential target proteins to test if they indeed bind to (p)ppGpp or not."? I suggest adding more clarification and discussion on this topic.

Not all proteins could be expressed in soluble form and with decent amounts. If the concentration of the protein is just below the Kd value, one will expect to see variable binding results even for the true targets. This is a known caveat for DRaCALA technique and also why one should purify the potential proteins to homogeneity and confirm the binding again by using higher concentrations of the proteins. All of the 12 identified new targets were purified to homogeneity and confirmed this way for their genuine binding/degrading of the (p)ppGpp, as shown in our 2018 mBio article (PMCID: PMC5845004). Amendment is made in the text to clarify this further.

Minor Concerns:
Fig 1 change decided to divided
Corrected.
L270 "3C" should read "3B,C"
Corrected.

Reviewer #2:
Manuscript Summary:
This manuscript describes how to perform DRaCALA assays for the detection of protein:small molecule interactions. The manuscript also provides example data, using the model organism Escherichia coli.

Major Concerns:
Please add more detail about how the binding fraction is calculated. Are any no binding/free ligand controls used?
Actually, please add some information on what controls (positive or negative)

could/should be performed with this assay.
Before performing large scale DRaCALA screening, one should test with both negative and positive controls, to make sure the screening conditions actually work as expected. When such an optimal condition is found, we found unnecessary to include them in the actual screening of each plate. This is firstly because the screening procedure is very short. Secondly, the majority of the proteins in a plate is expected to not bind to (p)ppGpp and therefore they serve as negative controls when quantifying the binding fractions (Figure 3). This has been added in the Result and Discussion.
Also what is meant by 'substantially
higher' (section 5, lines 209/210).
The cutoff value can be arbitrary, but in general a binding fraction higher than 0.06 is considered substantially high, although this also varies with each of the plates one obtains. Rephrased.

Figure 3 also needs some clarification. The ability to distinguish true positive/negative hits is critical - and not currently clear. It is difficult to tell on the graphs which bar corresponds to which label. Please make the x-axes more clear. I would also recommend using coloured circles to highlight particular positive wells and negative wells (in different colours). In particular, examples of false positives should be highlighted in panels B,C. Squinting at the graph, I thought perhaps the false positives were G1 and G4 - but these (by eye) look identical to me in panels B,C?
Similarly, why is HflX considered a 'true positive' when it is is not replicated between panels B&C?
The Figure 3 is improved now.
The wells that show consistently high binding fractions are **probably** true positives, while those that show both high and low binding fractions in two replicates are **possible** true targets. The reason for the latter is that, not all proteins could be expressed in soluble form and with decent amounts. If the concentration of the protein is just below the Kd value, one will expect to see variable binding results even for the true targets, which is the case of HflX (we were unable to purify soluble HflX in large quantity). To discriminate, one should purify the latter ones (and actually also the former ones) separately and confirm the binding again with high concentrations of the proteins. So, large scale DRaCALA screening using whole cell lysates, to the most, will *indicate* whether a protein is a target of ppGpp or not. One still needs to confirm by using pure proteins. These have been added in Results and Discussion.

In the discussion of limitations, either expand or at least refer people back to reference 9 for more info on limitations..
Referred to ref 9.

Minor Concerns:
needs further proof-reading/copy-editing.
Done
Please provide complete figure legends for Figures 2 and 3.
Done
Please label panel 3c.
Done

Reviewer #3:
Manuscript Summary:

Dracala is a powerful method to identify protein-ligand parners, especially nucleotides that can be made in radiolabeled form. This article is a nice demonstration of the method and will make the method accessible to others.
Major Concerns:
None
Minor Concerns:
The original manuscript on Dracala has several names on it. It therefore does not serve the scientific process well to specifically state that Dracala was invented by Vincent T. Lee. It should be Invented by "Vincent T. Lee and co-workers". This is how proper scientific attribution should be given.
Thanks. Done.

Reviewer #4:
Manuscript Summary:
This manuscript describes the steps necessary to conduct an ORFeome binding screen using DRaCALA. The authors write out the steps to generating lysates, how to create the radiolabeled ligand, (p)ppGpp, using purified enzymes and 32P-GTP, and how to perform a DRaCALA. In the introduction, the authors explain the efficacy and importance of this protocol and cite a detailed methods paper from the lab of the originator of DRaCALA as a companion to this piece. In the discussions, the authors nicely summarize some of the major advantages and disadvantages of DRaCALA as compared to use of capture compounds. Overall, the methods are easy to follow and fairly clear. I have a few minor concerns that would aid in clarity.
Major Concerns:
I have no major concerns as the manuscript is by and large sufficiently detailed.
Minor Concerns:
-In the introduction (line 48-50) the writing implies that DRaCALA differentiates based on size. It is not size, but rather ability to be immobilized on nitrocellulose.
Rephrased to clarify.

-lines 90-92 - was antibiotic used?
Added.
-line 105 - how much protease inhibitor?
1 tablet per 40 mL buffer. Added.
-line 143 - Rel - is it also 4 µL final?
4 µM final. Added.
-line 148/155 - tiny nitpick that 95C is not boiling
Rephrased.
-line 163/193 - do you wrap the TLC plate and nitrocellulose in plastic before putting it on the screens? Or do you do something else to prevent accidental contamination with radioactivity?
The former one, added now.
-line167 - "large scale" is somewhat unclear. It would also be useful to know what volume of 32P-labeled ligand you need to screen 1 plate of a library. Do heating times remain the same for higher volume?
The heating was the same and shown to be enough to heat denature the proteins. The details of the large scale reactions are added now.
-line 184 - is it tissue paper or do you use filter paper or a paper towel?
It is tissue paper.
-line 265 (and maybe include in section 1) - how do you determine "good"? Is there

an expression test step you conduct to verify a good library?
A good library shall express the majority (ca. >80%) of the proteins of an organism in decent amount and in soluble forms. For a new library, ideally one should test if the majority of the proteins are expressed to decent levels. Such a test also allows the researchers to evaluate the coverage and effectiveness of the DRaCALA screening results. This has been added in the Discussion section.

-Figure 2 legend, the last sentence is hard to follow.
-Figure 2 and 3 legends should have at least a brief summary, even if the text provides the main information.
More detailed legends are added.
-The figures are a bit difficult to follow and there are a few typos. In 1B consider not using a semicolon to separate A and S since it is confusing.
Corrected.
In figure 2, the arrows
and layout make it hard to follow the path. Consider setting up the parallel culture/lysate path and the 32-P (p)ppGpp synthesis in two parallel vertical paths that lead to the screen. There are some arrows that just lead to more arrows rather than a graphic or text, which looks strange.
Improved.
Also, are any of the figures used in fig 2
copyrighted? They look like they were pulled from supplier webpages.
Improved.
-As noted, some copyediting of the manuscript and figure is required (for example, some buffer names are bold and some are not, periods are on some lines and not others, decided rather than divided in fig 1, u in place of μ in fig 2
Proofread and improved.