Chapter 7

Probing Pairwise Protein–Protein Interactions with the Bacterial POLAR Assay

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Abstract

The PopZ-linked apical recruitment (POLAR) assay is an in vivo two-hybrid assay performed in *Escherichia coli*, using epifluorescence microscopy to visualize the recruitment of a prey protein fusion by a bait protein fusion tethered artificially at the cell pole. Here, we present a standardized protocol of the POLAR assay, specifically designed to investigate protein–protein interactions (PPIs) in the cytoplasm. Notably, the POLAR assay has been successfully used to validate potential PPIs identified through proximity labeling, highlighting its relevance as a complementary tool within the broader context of studying protein interaction networks.

Key words POLAR assay, Protein-protein interaction (PPI), Two-hybrid assay, Epifluorescence microscopy, PopZ, Cell pole, Protein recruitment dynamics, Proximity labeling, *Escherichia coli*

1 Introduction

Pairwise protein–protein interaction (PPI) assays are powerful tools to get a better understanding of bacterial molecular processes, notably when used for the characterization of protein partners of a protein of interest (POI) after identifying its in vivo protein network through proximity labeling. Among these, in vivo two-hybrid assays are widely used since their readouts typically rely on simple reporter systems while not requiring protein purification. These assays are based on the co-production of two proteins of interest (POIs) in live cells, which, upon interaction, induce a specific phenotypic trait [1, 2].

The bacterial PopZ-linked apical recruitment (POLAR) assay, developed by Lim and Bernhardt, is an in vivo two-hybrid assay performed in *Escherichia coli*. It relies on epifluorescence microscopy to visualize the recruitment of a prey protein fusion by a bait protein fusion tethered to the cell pole [3]. The general principle of the POLAR assay is illustrated in Fig. 1. This assay exploits the

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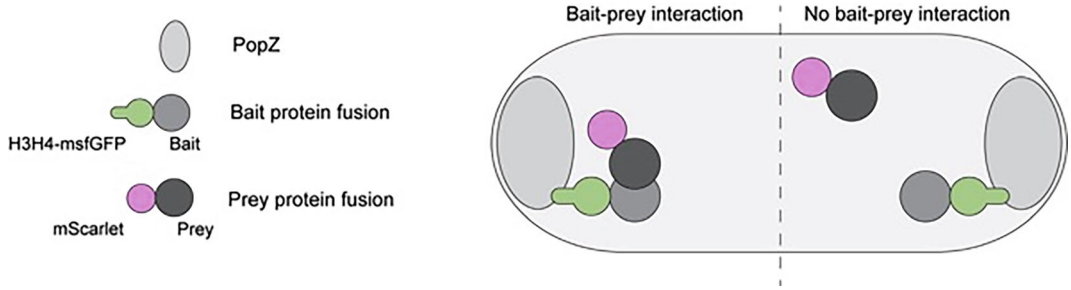

Fig. 1 General principle of the bacterial POLAR assay to investigate PPIs by epifluorescence microscopy. POLAR experiments are performed in *E. coli* cells producing (i) the PopZ protein from *C. crescentus*, which spontaneously self-assembles into clusters at the cell pole, (ii) the bait protein fused to msfGFP and the H3H4 domain from PopZ, which tethers the fusion protein at the cell pole, and (iii) the prey protein fused to mScarlet. Upon bait–prey interaction, the prey fusion is recruited to the cell pole by the PopZ-associated bait fusion (left cell part). In the absence of a PPI, the prey fusion remains diffuse in the cytoplasm (right cell part)

PopZ protein from *Caulobacter crescentus*, which spontaneously self-assembles into higher-order structures specifically located at the cell pole in *E. coli* through its H3H4 homo-oligomerization domain (named after α-helices 3 and 4 in the protein structure) [3, 4]. A putative PPI is investigated by fusing the bait protein to an msfGFP-PopZH3H4 domain fusion, which anchors the bait to polar PopZ clusters, and tagging the prey protein with the mScarlet fluorescent protein. Upon bait–prey interaction, the prey protein fusion is specifically recruited to the cell pole by the PopZ-associated bait protein, which is visualized using epifluorescence microscopy.

Initially, the POLAR assay was developed to study PPIs in the cell envelope, including interactions involving secreted proteins [3]. This capability represents a significant advantage over the widely used bacterial adenylate cyclase-based two-hybrid (BACTH) assay, which relies on the physical reconstitution of the *Bordetella pertussis* adenylate cyclase, preventing the investigation of POIs lacking cytoplasmic domains [3, 5, 6]. However, the POLAR assay can also be used for studying PPIs in the cytoplasm and offers several benefits compared to the BACTH assay [3]. First, visualization of protein fusions by epifluorescence microscopy allows for easy optimization of their expression levels by fine-tuning induction conditions, thus balancing bait and prey protein levels and limiting cell toxicity—typical drawbacks of the BACTH assay [3, 6]. Second, microscopy allows for monitoring recruitment dynamics through snapshots at specific time points or time-lapse imaging. Finally, because the POLAR assay does not depend on the reconstitution of a functional reporter protein, it is less prone to false negatives caused by steric hindrance.

Since its development, the POLAR assay has been successfully applied to investigate interactions between various protein pairs,

including periplasmic proteins involved in envelope stress responses in *E. coli* [7] and cytoplasmic ParA-ParB proteins implicated in the segregation of the *Bdellovibrio bacteriovorus* chromosome [8]. Recently, we used the POLAR assay to study the protein partners of the *B. bacteriovorus* polar hub protein RomR, following the mapping of its in vivo protein network by proximity labeling [9].

Here, we present a standardized protocol for detecting PPIs in the cytoplasm using the bacterial POLAR assay in *E. coli*. This protocol is intended to serve as a foundational guide and may require optimization depending on the POIs under investigation.

2 Materials

2.1 General Equipment

1. Static incubator for plates.
2. Shaking incubator for cell cultures.
3. Optical density (OD) meter.
4. Centrifuge for small volumes (1.5–2 mL tubes).
5. Refrigerated centrifuge for large volumes (50 mL tubes).
6. 0.1 cm bacterial electroporation cuvettes.
7. Electroporator.

2.2 Media and Solutions

1. 20% (w/v) casamino acids: Dissolve 2 g casamino acids in 10 mL sterile dH_2O, filter sterilize (0.2 μm syringe filter), and store at room temperature (RT).
2. Ampicillin stock (50 mg/mL): Dissolve 500 mg ampicillin in 10 mL sterile distilled water (dH_2O), filter sterilize (0.2 μm syringe filter), and store at 4 °C (short-term), or −20 °C (long-term).
3. Chloramphenicol stock (15 mg/mL): Dissolve 150 mg chloramphenicol in 10 mL 70% ethanol. Store at −20 °C.
4. Tetracycline stock (7.5 mg/mL): Dissolve 75 mg tetracycline in 10 mL 70% ethanol. Store at −20 °C.
5. LB broth and LB agar media, Miller formulations: 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. For LB agar, use 15 g/L Bacto agar. When antibiotic selection is required, supplement with the following antibiotics to reach the following final concentrations in LB broth or agar: chloramphenicol 15 μg/mL and/or tetracycline 7.5 μg/mL (LB + Cm, LB + Tet, or LB + Cm/Tet, respectively), or ampicillin 50 μg/mL (LB + Amp).
6. M9 minimal salts (M9) medium: 6.78 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, and 1 g/L NH_4Cl.

7. SOC medium: 5 g/L yeast extract, 20 g/L tryptone, 0.584 g/L NaCl, 0.186 g/L KCl, 2.4 g/L $MgSO_4$, 20 mM glucose (add glucose after autoclaving).
8. 10% (v/v) glycerol: Dilute 99 + % glycerol in sterile dH_2O. Store at 4 °C.
9. IPTG solution (100 mM): Dissolve 238 mg IPTG powder in 10 mL sterile dH_2O, filter sterilize (0.2 μm syringe filter), and store at 4 °C (short-term) or − 20 °C (long-term).
10. Arabinose solution (20% w/v): Dissolve 2 g arabinose in 10 mL sterile dH_2O, filter sterilize (0.2 μm syringe filter), and store at RT.

2.3 Strains and Plasmids

1. *E. coli* Top10 competent cells: Suitable for cloning and propagation of ColE1 plasmids (e.g., bait plasmid used in this study). Alternative competent cells capable of propagating ColE1 plasmids can also be used.
2. *E. coli* CC118 λpir competent cells: Used for cloning and propagation of plasmids with an R6K origin of replication (i.e., the prey plasmid used in this study). Alternative competent cells containing the *pir* gene may also be employed.
3. *E. coli* TB28 (*ΔlacZYA*) cells carrying pAH69 plasmid: TB28/pAH69 strain with a temperature-sensitive plasmid [3, 10].
4. Plasmids: pHCL150, pHCL149, pHCL147, and pHCL151 backbone vectors, developed in [3], and available from Addgene (#134456, #134457, #134454, and #134455, respectively).

2.4 Epifluorescence Microscopy

1. Epifluorescence microscope: Microscope equipped with classical red and green filters and a phase contrast objective for imaging cells and fluorescent proteins.
2. Glass microscopy slides and coverslips.
3. 1.2% (w/v) M9 agarose pads: Dissolve 0.06 g of molecular biology-grade agarose in 5 mL of M9 medium. Melt the agarose and prepare pads as routinely performed [11].
4. Vaseline-Lanolin-Paraffin (VALAP) Sealing Agent: Mix equal amounts of vaseline, lanolin, and paraffin, and melt to combine. Let solidify and store at RT. Melt as needed before use.

3 Methods

3.1 Construction of E. coli Cells Producing Bait and Prey Protein Fusions

The bacterial POLAR assay is performed in *E. coli* TB28 [3]. For each POLAR experiment, a TB28 strain carrying a bait and a prey plasmid, producing the bait and prey protein fusions, respectively, must be constructed.

This process involves two main steps: (i) constructing bait and prey plasmids and (ii) co-transformation of *E. coli* TB28 with bait and prey plasmids.

3.1.1 Construction of Bait and Prey Plasmids

Bait and prey plasmids are constructed using designated backbones (Fig. 2). Bait plasmids (e.g., pHCL149 and pHCL150) are medium-copy replicative plasmids conferring chloramphenicol resistance. They enable the expression of (i) *popZ* and (ii) the *bait* gene fused to *msfGFP* and the *H3H4* domain under the arabinose-inducible promoter *Para* (Fig. 2a). pHCL149 is used for N-terminal bait fusions to H3H4-msfGFP, while pHCL150 is used for C-terminal fusions to msfGFP-H3H4 [3]. Prey plasmids (e.g., pHCL151 and pHCL147) confer tetracycline resistance and integrate as a single copy into the chromosome upon transformation of TB28 (detailed in Subheading 3.1.2). The *prey* gene is cloned in pHCL151 or pHCL147 to obtain N- or C-terminal

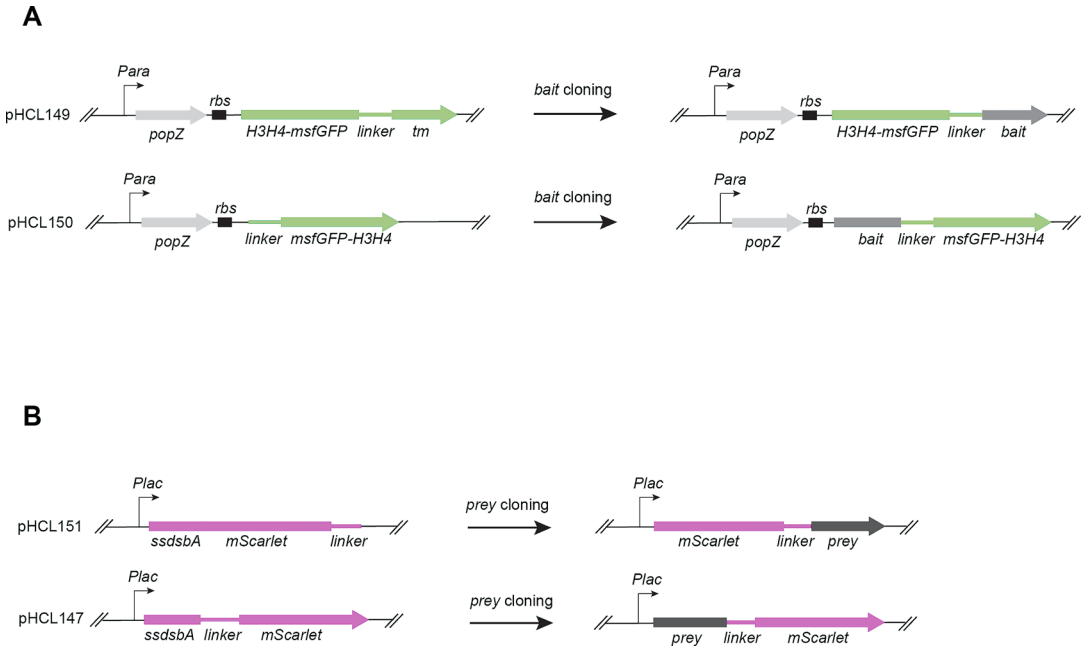

Fig. 2 Schematic representation of bait and prey plasmids. (**a**) Bait plasmids are constructed by cloning the *bait* gene into the pHCL149 and pHCL150 empty backbones for N-terminal or C-terminal fusions to msfGFP-H3H4, respectively. The bait and PopZ are expressed under the control of the arabinose-inducible *Para* promoter. When cloning *baits* into pHCL149, the *tm* sequence encoding the TM domain is excluded, as inner membrane anchoring is unnecessary for cytoplasmic baits. *rbs:* ribosome-binding site. (**b**) Prey plasmids are generated by cloning the *prey* gene into the pHCL151 and pHCL147 empty backbones for N-terminal or C-terminal fusions to mScarlet, respectively. The prey fusion is expressed under the control of the IPTG-inducible *Plac* promoter. The signal sequence *ssdsbA* is omitted during cloning to prevent secretion, as this protocol focuses on cytoplasmic POIs. Flexible linkers are included in all bait and prey fusions to ensure proper folding of the POI and to minimize steric interference during interaction detection

prey fusion to mScarlet, respectively, and expressed under the IPTG-inducible promoter *Plac* (Fig. 2b) [3].

1. Determine the bait and prey proteins to be tested (*see* **Note 1**).
2. Select appropriate bait and prey plasmid backbones. Choose N- or C-terminal fusions based on the characteristics of the bait and prey proteins and their preliminary characterization (*see* **Note 2**).
3. Clone the genes of interest into the selected backbones using standard DNA cloning techniques. When investigating soluble cytoplasmic proteins, design primers to exclude the sequences encoding the ssDsbA signal sequence or transmembrane domain from the pHCL149 bait backbone (originally used for envelope protein studies [3, 12]).
4. Transform *E. coli* Top10 cells with the bait plasmid and *E. coli* CC118 λpir cells with the prey plasmid. Select transformants on LB + Cm or LB + Tet agar plates, respectively.
5. Validate constructs by sequencing DNA isolated from individual colonies using sequencing primers annealing upstream and downstream of the generated gene fusions.

3.1.2 Preparation of Electrocompetent E. coli TB28/pAH69 Cells

E. coli TB28/pAH69 must be co-transformed with bait and prey plasmids. The pAH69 helper plasmid expresses the HK022 integrase-encoding gene under a temperature-sensitive promoter to enable prey plasmid integration into the HK022 phage attachment site of the TB28 chromosome (*see* **Note 3**) [3, 10].

Hereafter we describe the procedure, based on [3], for the preparation of TB28/pAH69 electrocompetent cells. Bacterial co-transformation with prey and bait plasmids by electroporation is presented in Subheading 3.1.3.

1. Grow *E. coli* TB28/pAH69 cells at 30 °C on LB agar or in liquid LB broth to prevent loss of the pAH69 helper plasmid.
2. Prepare a culture of *E. coli* TB28/pAH69 in 4 mL LB + Amp. Grow overnight (O/N) at 30 °C with shaking.
3. Dilute the O/N culture 1:100 in 50 mL LB + Amp (*see* **Note 4**). Incubate at 30 °C with shaking.
4. Monitor growth until OD_{600} reaches 0.15.
5. Shift the incubation temperature to 42 °C for 30 min to induce HK022 integrase expression.
6. Transfer the culture to a 50 mL tube and chill it on ice for 30 min (*see* **Note 5**).
7. Harvest cells by centrifugation at 4000× *g* for 10 min at 4 °C.
8. Discard supernatant and resuspend the pellet in 50 mL of ice-cold 10% glycerol.

9. Harvest cells by centrifugation at 4000× *g* for 10 min at 4 °C.
10. Discard supernatant and resuspend the pellet in 20 mL of ice-cold 10% glycerol.
11. Repeat the centrifugation step and resuspend the pellet in 1 mL ice-cold 10% glycerol.
12. Aliquot 100 μL into sterile 1.5 mL tubes.
13. Snap freeze in liquid nitrogen and store at −80 °C for up to several weeks (*see* **Note 6**). Wear safety goggles and cryogenic gloves when handling liquid nitrogen.

3.1.3 Co-transformation of E. coli *with Expression Plasmids*

1. Thaw an aliquot of electrocompetent cells on ice.
2. Add 100 ng of each plasmid (bait and prey) in a 1:1 ratio. Mix gently by stirring with the pipette tip, being careful not to pipette the mixture to avoid shearing the cells (*see* **Note 7**).
3. Incubate on ice for 15 min.
4. Transfer to a chilled 0.1 cm electroporation cuvette (*see* **Note 8**).
5. Electroporate at 1.7 kV, 100 Ω, and 25 μF.
6. Immediately add 900 μL SOC medium and transfer to a sterile 2-mL tube (*see* **Note 9**).
7. Incubate with shaking at 37 °C for 1 h to allow recovery and simultaneously initiate the loss of the pAH69 helper plasmid.
8. Concentrate the cells by centrifugation at 4000× *g* for 5 min at RT and resuspend the resulting cell pellet in 100 μL liquid LB medium.
9. Plate the cells onto LB + Cm/Tet agar plates.
10. Incubate O/N at 37 °C to select for transformants and ensure the loss of the pAH69 plasmid.

3.2 Induction of Protein Fusions and Visualization of PPIs by Microscopy

The key step of this protocol involves inducing the expression of genes encoding prey and bait protein fusions in *E. coli* TB28 to monitor prey protein recruitment by epifluorescence microscopy. Compared to the initial procedure described in [3], here a two-step induction and imaging protocol has been optimized to sequentially induce prey and bait fusions and visualize PPIs using time-course microscopy, taking snapshots at two distinct time points (an overview of this procedure is shown in Fig. 3).

First, the production of the prey fusion is induced with IPTG to confirm that it does not localize at the cell pole in the absence of the bait fusion (*see* **Note 10**). Next, the production of PopZ and the bait fusion is induced with arabinose to assess whether the PopZ-anchored bait recruits the prey fusion (indicating a bait–prey interaction). Alternatively, time-lapse microscopy can be used to track the dynamics of prey recruitment upon bait induction by imaging cells at regular intervals (*see* **Note 11**).

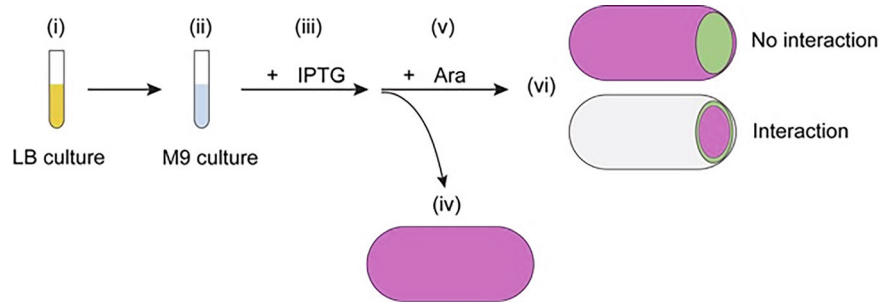

Fig. 3 Overview of the two-step induction and imaging procedure used to investigate cytoplasmic PPIs with the POLAR assay in *E. coli* TB28. (i) *E. coli* TB28 cells carrying bait and prey plasmids are grown in LB broth (LB culture) until the early exponential phase. (ii) Cells are harvested and resuspended in M9 medium supplemented with casamino acids (M9 culture). (iii) Production of the prey fusion alone is induced with IPTG. (iv) Following IPTG induction, TB28 cells are imaged to confirm that the prey fusion remains diffuse in the cytoplasm in the absence of the bait fusion. (v) Production of PopZ and the bait fusion is induced by adding arabinose (Ara). (vi) Following this second induction step, TB28 cells are imaged to assess bait–prey interaction, characterized by the recruitment of the prey fusion to the bait tethered at the cell pole (co-localization of msfGFP and mScarlet fluorescent signals)

For each prey protein investigated, include a control condition using an empty bait plasmid to confirm that prey recruitment does not occur in the absence of a specific bait protein *(see* **Note 12**).

1. Prepare an O/N culture from a single colony of *E. coli* TB28 in 4 mL of LB + Cm/Tet.
2. Dilute the O/N culture 1:100 in 3 mL of LB + Cm/Tet.
3. Incubate at 37 °C with shaking.
4. Monitor growth until the culture reaches an OD_{600} of 0.2.
5. Pellet the cells by centrifugation at 4000× *g* for 5 min at RT.
6. Resuspend the cell pellet in 3 mL of M9 medium supplemented with 0.2% (v/v) casamino acids. From this point, induce prey and bait fusions sequentially as described below.

3.2.1 Induction of Prey Fusion

1. Add 3 µL of IPTG 100 mM to the cell suspension (100 µM final concentration).
2. Incubate for 2 h at 37 °C with shaking.
3. Collect 1 mL of the culture in a sterile 1.5 mL tube and pellet cells by centrifugation at 4000× *g* for 5 min at RT.
4. Remove the supernatant and resuspend the cell pellet in 500 µL of M9 medium to concentrate cells.
5. Spot 1.5 µL of cells on a 1.2% M9 agarose pad, cover with a coverslip, and seal with melted VALAP. For time-lapse microscopy, use an agarose pad containing 100 µM IPTG and 0.2% (v/v) arabinose to maintain prey fusion induction and initiate bait expression.

6. Image cells by phase contrast microscopy (for cell outline visualization) and epifluorescence microscopy. The mScarlet fluorescence signal should be diffused in the cytoplasm in the absence of bait induction (*see* **Notes 10** and **13**). If performing a time-course experiment, continue with Subheading 3.2.2. For time-lapse microscopy, image cells at regular time intervals (e.g., every 2 min) to track prey recruitment upon bait induction.

3.2.2 Induction of Bait Fusion

1. Add 20 μL of 20% arabinose (0.2% final concentration) to the remaining 2 mL of culture and incubate at 37 °C with shaking for an additional hour.
2. Collect a 1 mL sample in a sterile 1.5 mL tube and pellet cells by centrifugation at 4000× *g* for 5 min at RT.
3. Remove the supernatant and resuspend the cell pellet in 700 μL of M9 medium to concentrate cells.
4. Spot 1.5 μL of cells on a 1.2% M9 agarose pad, cover with a coverslip, and seal with melted VALAP.
5. Image cells by phase contrast microscopy and epifluorescence microscopy. The msfGFP signal should localize at the cell pole. In case of interaction between the bait and prey protein, the mScarlet signal, which was diffuse in **step 6** of Subheading 3.2.1, will now co-localize with the msfGFP signal at the cell pole (*see* Fig. 3 and **Note 14**).
6. Quantify co-localization of bait and prey signals at the single-cell or population levels using software tools such as Oufti, custom MATLAB codes [3, 8, 9], or the FIJI plugin MicrobeJ [13].

4 Notes

1. To assess the applicability of the POLAR assay for strain construction and visualization of protein recruitment by epifluorescence microscopy, using a previously characterized PPI is recommended.
2. If the POI has been studied previously (e.g., through subcellular localization using a fusion to a fluorophore or in vivo protein network mapping via proximity-tagging enzymes), adopt the same fusion design for POLAR assays. For uncharacterized POIs, test both N- and C-terminal fusions, as tag positioning may impact protein folding and interaction potential.
3. Successful integration of the prey plasmid into the TB28 chromosome is a critical step in the transformation protocol.

Transformation efficiency can vary significantly depending on the DNA concentration, purity, and the nature and size of the *prey* gene cloned within the plasmid.

4. For larger culture volumes, split the culture into multiple 50 mL flasks rather than using a single larger flask to ensure efficient temperature shifts during the protocol.
5. Cells can remain on ice for more than 30 min without affecting the procedure.
6. Transformation efficiency typically decreases after the competent cells have been stored for several weeks.
7. At least 100 ng of each bait and prey plasmid is generally required for electroporation. However, the optimal DNA quantity may depend on the bait-prey plasmid combination and on the quality of TB28/pAH69 competent cells.
8. When using 0.2 cm electroporation cuvettes, adjust the electroporation settings accordingly.
9. Use 2 mL tubes for post-electroporation culture steps to ensure adequate aeration.
10. If the prey fusion consistently localizes to the pole, this may indicate interactions with native *E. coli* proteins or an intrinsic propensity to localize at the pole in a heterologous host.
11. If the prey fusion is recruited at the pole in the absence of a specific bait, consider testing it as a bait rather than a prey or using alternative (two-hybrid) assays to confirm PPIs.
12. Importantly, although positive hits in the POLAR assay indicate the presence of a PPI, bridging interactions by *E. coli* proteins cannot be excluded. Therefore, biochemical in vitro assays with purified POIs are required to confirm the direct nature of the interaction [14]. In cases where heterologous POIs have no homologs in *E. coli*, bridging interactions are less likely, and prey recruitment is more likely to indicate a direct PPI [3, 9].
13. If the fluorescent signal of the prey fusion is too weak or too strong, adjust the IPTG concentration or incubation period accordingly. Assess prey induction and integrity by Western blotting using an anti-RFP antibody in addition to epifluorescence microscopy.
14. If the bait fusion fluorescent signal is too weak or too strong, adjust the arabinose concentration or incubation period accordingly. Evaluate bait induction and integrity by Western blotting using an anti-GFP antibody.

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