RoboPop™ Solubility Screening Kit

Table of Contents

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About the Kit

RoboPop Solubility Screening Kit 71255-3

Description

The RoboPop Solubility Screening Kit is designed for protein solubility screening of bacterial cultures in a 96-well format (1). The kit takes advantage of PopCulture® and Lysonase™ Reagents for efficient preparation of protein extracts from *E. coli* directly from the culture medium. An innovative Filter Plate retains insoluble inclusion bodies while soluble proteins are collected for rapid analysis. Insoluble proteins retained by the filtration plate are solubilized with 4% SDS, collected, and quantified separately. The Filter Plate is compatible with standard vacuum manifolds for manual processing and the protocol has been validated for robotic sample processing. Two Collection Plates with Sealers are provided for convenient storage of separated soluble and insoluble proteins. RoboPop Solubility Screening Kit together with Novagen's RoboPop Purification Kits (2) allow rapid optimization of the host-vector combinations, expression conditions, and purification parameters toward high-throughput production of proteins for structural or functional analysis.

Components

- 15 ml PopCulture Reagent
- 1 350 µl 96-Well Filter Plate
- 2 Collection Plate with Sealer (450 µl wells)
- 20 ml 4% SDS
- 0.2 ml Lysonase Bioprocessing Reagent

Storage

Store PopCulture Reagent, 4% SDS, 350 µl 96-Well Filter Plate, and Collection Plate with Sealer at room temperature. Store Lysonase Bioprocessing Reagent at –20°C.

Notes: *Storage of PopCulture Reagent below 4°C may cause precipitation. Incubate PopCulture in a room temperature water bath with gentle swirling or inversion to redissolve.*

Lysonase Bioprocessing Reagent should not be stored at –70°C. Freezing may result in loss of activity.

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Protocol

A quick protocol is given below followed by detailed protocols in the subsequent sections. If manual processing is desired, an 8- or 12-tip multichannel pipettor can be used instead of programming a robotic liquid handling system. An optimized robotic program for the Packard-brand MultiPROBE® HT EX liquid handling workstation from PerkinElmer Life Sciences using WinPrep® software can be downloaded from http://www.novagen.com/RoboPopsoftware. Note that the WinPrep software requires the user to establish Gripper™ positions for each instrument. For Tecan Genesis Freedom™ workstation software, please contact your Tecan Sales Representative.

Quick protocol

1. Culture *E. coli* cells in a sterile deep well culture plate using air-permeable sealers under conditions for target protein expression. LB, TB or YT media formulations can be used (see page 7).
2. Add 0.1 ml PopCulture® Reagent and 2 µl of Lysonase™ Bioprocessing Reagent per milliliter culture volume. Mix and incubate 10 min at room temperature. This extract contains total cell protein. A sample may be removed prior to fractionation and stored at 4°C until further analysis.
3. Mount the 350 µl 96-Well Filter Plate on a Collection Plate.
4. Transfer a 200 µl sample from the culture extract to the 350 µl 96-Well Filter Plate and apply gentle vacuum to separate the soluble from the insoluble fractions. Collect the filtrate containing the soluble proteins in the Collection Plate.

Note: The Filter/Collection Plates assembly may be centrifuged at 2,000 × g for 5 min as an alternative to vacuum filtration for collection of soluble proteins.

5. Seal the Collection Plate containing the soluble fraction with a plate Sealer and store samples at 4°C until further analysis.
6. Mount the Filter Plate on a fresh Collection Plate.
7. Add 200 µl 4% SDS to each well of the Filter Plate. Mix and incubate 10 min at room temperature.
8. Collect the filtrate containing the solubilized inclusion body proteins into the Collection Plate using the vacuum manifold.

Note: The Filter/Collection Plates assembly may be centrifuged at 2,000 × g for 5 min as an alternative to vacuum filtration for collection of solubilized inclusion body proteins.

9. Seal the Collection Plate containing the solubilized inclusion body proteins with a plate Sealer and store the samples at 4°C until further analysis.
10. Analyze soluble and insoluble fractions by SDS-PAGE and/or Western blot. Soluble target protein may be quantified by ELISA or activity assays.

Instructions for the Collection Plate Sealer

The two-layer Collection Plate Sealer has positioning tabs at both ends. After the Sealer is fully secured on the culture plate, you can remove positioning tabs or layers on the tabs. Do not remove positioning tabs or layers from the tabs until the sealer is fully secured on the Collection Plate. The bottom layer of the Sealer is brown and when removed exposes the adhesive. The top layer is foil and covers the purified protein samples in the Collection Plate.

1. Remove the brown bottom layer to expose the adhesive. Use the positioning tabs for handling.
2. Position the Sealer on the Collection Plate.
3. Secure the Sealer by rubbing across the foil to ensure the adhesive is attached between each well.
4. To remove the Sealer from the plate, lift from a corner and gently pull.
5. To remove a sample from a well, cut the foil above the well with a razor blade to allow the pipet tip to reach the well. Sealers are single-use and after removal cannot re-seal the wells.

General cell culture guidelines

Induction using IPTG

These conditions may require optimization depending on the expression system, target protein, host strain, growth medium temperature and orbital-shaking incubator used. The following protocol is based on culturing Tuner™(DE3) cells. Cultures can be grown in a 96-well (1 ml/well) or 24-well format (5 ml/well). Sterile 96-Well Deep Well Culture Plates are available from Novagen (Cat. No. 71111-3). 24-well cultures plates (10 ml capacity) are available from Whatman (Cat. No. 7701-5102). To avoid cross-contamination between wells, use air-permeable BugStopper™ Venting capmats during cell culturing (VWR International, Cat. No. 14217-208).

1. Streak a glycerol stock or plate freshly transformed *E. coli* containing the recombinant plasmid and incubate overnight at 37°C on LB agar plates containing appropriate antibiotics.
2. The following day, pipet pre-warmed (24°C) medium containing appropriate antibiotics into each well of a sterile deep well culture plate.

   24-well plate: Use 500 µl pre-warmed medium per well.
   96-well plate: Use 100 µl pre-warmed medium per well.

Note: *Dispense the medium immediately prior to inoculation (step 3). Use of pre-warmed medium avoids a lag phase in bacterial growth*

3. Using a sterile pipet tip, inoculate 1 colony into each well and swirl briefly to disperse the cells.
4. Cover the inoculated plate with an air permeable sealer or lid.
5. Incubate at 24°C for approximately 16 h.

   24-well plate: Shake culture at 200 rpm.
   96-well plate: Shake culture at 300 rpm.

The initial 24°C growth temperature was chosen so the cells could be incubated for 16 h without reaching saturation phase. Subsequent growth at 30°C rather than 37°C may enhance target protein solubility. These temperatures can be optimized for individual target proteins.

6. Remove the sealer and add pre-warmed (30°C) medium containing appropriate antibiotics into each well.

   24-well plate: Add 4.5 ml pre-warmed medium per well.
   96-well plate: Add 1.0 ml pre-warmed medium per well.

7. Reseal the culture plate with a new sealer or lid.
8. Incubate at 30°C, shaking at 250 rpm until the OD_{600} is 1–1.5. To determine the OD, cut the membrane above a well or remove the lid and remove a sample from the well. Note that OD variation between individual wells can occur.

   24-well plate: Incubation for 2–3 h is generally needed to reach an OD_{600} of 1–1.5.
   96-well plate: Incubation for 1–1.5 h is generally needed to reach an OD_{600} of 1–1.5.

9. Induce target protein expression by adding a sterile stock solution of IPTG and incubate approximately 3 h at 30°C. Use a final concentration of IPTG appropriate for the expression vector. Optimize induction conditions as required for the expression system. The OD_{600} after induction in our experiments is typically 4–5. Final optical densities may vary.

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Induction using Overnight Express™ Autoinduction System 1

The Overnight Express Autoinduction System 1 (Cat. No. 71300) is designed for high-level protein expression with the pET bacterial expression system without monitoring cell growth (3). In λDE3 lysogenic hosts, uninduced cells are grown to a high cell density followed by spontaneous induction of T7 RNA polymerase expression without monitoring cell density or adding IPTG. The Overnight Express Autoinduction System 1 is compatible with traditional glucose-free media and results in maximum yields of target proteins with the pET system (4). See Technical Bulletin 383 for more information.

The conditions described below may require optimization depending on the expression system, target protein, host strain, growth medium, temperature, culture volume and orbital-shaking incubator used. The following protocols is are based on culturing BL21(DE3) cells. Cultures can be grown in a 96-well (1 ml/well) or 24-well format (5 ml/well). Sterile 96-Well Deep Well Culture Plates are available from Novagen (Cat. No. 71111-3). 24-well cultures plates (10 ml capacity) are available from Whatman (Cat. No. 7701-5102). To avoid cross-contamination between wells, use air-permeable BugStopper™ Venting capmats during cell culturing (VWR International, Cat. No. 14217-208).

Note: *Because lactose is used for induction, expression hosts should produce functional Lac permease (encoded by the lacY gene) and β-galactosidase (encoded by the lacZ gene) for consistent results in both complex and defined media. Strains that are lacY mutants (i.e., the Tuner™, Origami™ B and Rosetta-gami™ B strains) or lacZ mutants should not be used. See "Additional Guidelines" for more information.*

1. Streak a glycerol stock or plate freshly transformed *E. coli* containing the expression plasmid and incubate overnight on LB agar plates containing appropriate antibiotics.
2. Prepare Overnight Express Autoinduction System 1 medium aseptically by combining sterile glucose-free medium with 0.02 vol OnEx™ Solution 1, 0.05 vol OnEx Solution 2, and 0.001 vol OnEx Solution 3 (see Table below). Add appropriate antibiotics for the host strain and plasmid.

Note: *Overnight Express™ Autoinduction System 1 is compatible with glucose-free media such as Terrific Broth (TB), Luria-Bertani (LB) broth, and 2X YT. See "Additional Guidelines" for media recipes.*

Overnight Express System 1 medium
Aseptically combine the following reagents
Per liter:
20 ml OnEx™ Solution 1
50 ml OnEx Solution 2
1 ml OnEx Solution 3
929 ml sterile glucose-free medium

3. The following day, pipet pre-warmed Overnight Express System 1 medium plus appropriate antibiotics into each well of a sterile deep well culture plate.

   24-well plate: Use 5 ml pre-warmed medium per well.
   96-well plate: Use 1 ml pre-warmed medium per well.

Note: *Dispense the medium immediately prior to inoculation (step 4). Use pre-warmed medium to avoid a lag phase in bacterial growth.*

4. Using a sterile pipet tip, inoculate 1 colony into each well and swirl briefly to disperse the cells.
5. Cover the inoculated plate with an air permeable sealer or lid.

6. Incubate at 30°C approximately 16 hours.

   24-well plate: Shake culture at 200 rpm.
   96-well plate: Shake culture at 300 rpm.

Note: It is important to culture cells to stationary phase when using the Overnight Express System 1. See "Additional Guidelines" for more information.

Cell extract preparation and fractionation

Tip: Disposable pipet tips should be used where appropriate to prevent cross-contamination between sample wells.

1. After induction is complete, transfer the culture plate(s) to the robotic platform.
2. Remove the culture plate sealer.
3. Add 0.1 ml PopCulture® Reagent and 2 µl Lysonase™ Bioprocessing Reagent per milliliter culture volume.

Note: Lysonase Bioprocessing Reagent can be pre-mixed with PopCulture Reagent for single-step addition. Screening of 96 × 1 ml cultures will require approximately 10 ml PopCulture and 200 µl Lysonase. Pre-mixed PopCulture and Lysonase should be used the same day and stored at 4°C until use.

4. Pipet up and down to mix and incubate with gentle shaking for 10–15 min at room temperature. This extract contains the total cell protein. A sample can be removed prior fractionation and stored at 4°C until further analysis

Note: Protease Inhibitors may be added with the PopCulture Reagent if desired.

5. Mount the 50 µl 96-Well Filter Plate on a Collection Plate.
6. Transfer a 200 µl sample from the culture extract to the 350 µl 96-Well Filter Plate and apply gentle vacuum to separate the soluble from the insoluble fraction. Unused wells must be sealed prior to vacuum application. Seal Plate Film (VWR Cat. No. 10011-16) may be cut to the appropriate size for this purpose.
   Caution: Do not pull excess air through the Filter Plate after the filtrate has been removed. Drying can reduce yield of the insoluble protein fraction. Vacuum parameters should be adjusted to optimize the filtrate removal without drying.

Note: The Filter/Collection Plates assembly may be centrifuged at 2,000 × g for 5 min as an alternative to vacuum filtration for collection of soluble proteins.

7. Seal the collection plate containing the soluble proteins with a Sealer and store the sample at 4°C until further analysis.
8. Mount the Filter Plate on a fresh Collection Plate.
9. Add 200 µl 4% SDS to each well of the Filter Plate. Mix and incubate 10 min at room temperature.
10. Collect the filtrate containing the solubilized inclusion body proteins into the second Collection Plate using the vacuum manifold.

Note: The Filter/Collection Plates assembly may be centrifuged at 2,000 × g for 5 min as an alternative to vacuum filtration for collection of solubilized inclusion body proteins.

11. Seal the Collection Plate containing the denatured insoluble proteins with a Sealer and store the samples at 4°C until further analysis.
12. Analyze soluble and insoluble fractions by SDS-PAGE and/or Western blot. Soluble target protein may be quantified by ELISA or activity assays. See the following section.

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Analysis of the Samples after Fractionation

SDS-PAGE and Western blot analysis

SDS-PAGE or Western blot analysis can be used to determine the relative levels of protein expression in the soluble and insoluble fraction. Analysis of the extracts on SDS-PAGE gels with Coomassie blue staining generally requires a maximum load of extract containing a highly expressed target protein, combined with loading buffer. The insoluble fraction now solubilized in SDS can be combined with SDS loading buffer. Excess amounts of SDS in the sample will not cause interference with SDS-PAGE or Western blot analysis.

Note: SDS may precipitate when stored at 4°C. Warm at room temperature with gentle shaking to redissolve.

Soluble fraction

PopCulture® extracts are compatible with the GST•Tag™ Assay, S•Tag™ Rapid Assay, FRETWorks™ S•Tag Assay and BCA protein assays. Because proteins from the soluble fraction generally retain their activities and conformation, other protein-specific activity and immunoassays are likely to work. However, optimal buffer conditions for specific enzyme assays, ligand binding, or subunit assembly may require dilution or dialysis into the desired buffer.

The soluble protein fraction can be concentrated or the buffer changed by one of several methods. The storage buffer for your purified protein is often determined through an empirical process. Inappropriate storage buffer may lead to precipitation or inactivation of the protein.

1. Dialyze into the buffer of choice. A 3-fold concentration can be achieved by dialysis into storage buffer containing 50% glycerol. Glycerol often stabilizes proteins for long-term storage. 96-well dialysis plates are available from Harvard Apparatus to facilitate processing.
2. Use plastic disposable microconcentrator units (e.g. Microcon-Amicon) as directed by the manufacturer to both desalt and concentrate the sample by difiltration. Desalt the sample by gel filtration on Sephadex (MicroSpin G-25, MicroSpinG-50; Amersham Biosciences).

GST•Tag Assay

The GST•Tag Assay Kit (Cat. No. 70532-3) is designed to perform quantitative colorimetric assays of glutathione-S-transferase (GST) fusion proteins in crude extracts or purified fractions (5). The GST activity assay is simple to perform using the supplied 1-chloro-2, 4-dinitrobenzene (CDNB) substrate. A sample is combined with CDNB substrate in the supplied reaction buffer and the absorbance of the reaction is monitored at 340 nm. The rate of change in A_{340} is proportionate to the amount of GST activity present in the sample. The assay detects as little as 8 pmol of functional GST, which corresponds to approximately 250 ng unfused GST. Typically using 2–25 µl of the soluble extract in the assay produces readings in the linear range. For further details, see Technical Bulletin 236.

S•Tag Assays

The FRETWorks S•Tag Assay (Cat. No. 70724-3) enables extremely sensitive detection of S•Tag fusion proteins in minutes with a homogenous format. The interaction of the 15 amino acid S•Tag fusion peptide with purified S-protein reconstitutes RNase activity, which is measured using the FRET ArUAA Substrate. Fluorescence of the uncleaved substrate is inhibited by the interaction of the fluorophore and quencher; upon cleavage at the ribonucleotide residue, the fluorophore is released and becomes highly fluorescent. The specificity of the substrate permits this assay to be performed with crude extracts. PopCulture extract samples should be diluted approximately 1:2,500 in 1X FRET Assay Buffer. For further details, see Technical Bulletin 251.

With the S•Tag Rapid Assay (Cat. No.69212-3), a sample is added to a buffer containing the ribonuclease substrate poly(C). The reaction is started by adding purified S-protein. After 5 min incubation, the reaction is stopped with trichloroacetic acid. The resulting precipitate is removed by centrifugation. Activity is measured by reading the absorbance of the supernatant at 280 nm, which increases as the poly(C) is broken down into acid-soluble nucleotides by the enzyme. By comparing the results with a known S-peptide standard included in the kit, the molar concentration of target protein in the sample can be determined. See Technical Bulletin 082.

Cell culture

Culture Medium: PopCulture® Reagent is compatible with Terrific Broth (TB)(6), Luria-Bertani (LB) and 2X YT media. Experiments using TB have promoted rapid growth and high cell density (2).

LB: Per liter:

To 950 ml deionized water add:

Bacto® tryptone	10 g
Bacto yeast extract	5 g
NaCl	10 g

Stir until dissolved. Adjust the pH to 7.0 with 5 N NaOH (~0.2 ml). Adjust the volume of the solution to 1 liter with deionized water and autoclave.

TB: Per liter:

To 900 ml deionized water add:

Bacto tryptone	12 g
Bacto yeast extract	24 g
glycerol	4 ml

Stir until dissolved and sterilize by autoclaving for 20 min. Allow the solution to cool to < 60°C and then add 100 ml of sterile 0.17 M KH_2PO_4, 0.72 M K_2HPO_4 solution (dissolve 2.31 g KH_2PO_4 and 12.54 g K_2HPO_4 in 90 ml of deionized water. After the salts have dissolved, adjust the volume of the solution to 100 ml with deionized water and autoclave).

2X YT: Per liter:

To 900 ml deionized water add:

Bacto tryptone	16 g
Bacto yeast extract	10 g
NaCl	5 g

Stir until dissolved. Adjust the pH to 7.0 with 5 N NaOH. Adjust the volume of the solution to 1 liter with deionized water and autoclave.

Glucose: Inclusion of 0.5% glucose to the medium is recommended to minimize expression prior to induction for IPTG-inducible expression systems susceptible to catabolite repression (7). The inclusion of glucose may also increase the growth rate of the bacteria.

Important: Do not add glucose to the growth medium when using Overnight Express™ Autoinduction System 1.

Overnight Express Autoinduction System 1

Aeration: Efficient growth to saturation and utilization of carbon sources in OnEx™ Solution 1 requires vigorous agitation and proper aeration. Optimized culture volume:vessel dimension ratio is required in order to achieve proper aeration.

Temperature and length of incubation: It is important to grow the cultures to stationary phase when using the Overnight Express System 1. Using the cell culture guidelines above, stationary phase is usually reached as quickly as 8–10 hours, if the cultures are incubated at 37°C. When lower incubation temperatures are used, stationary phase may not be reached until 24 hours. Continued incubation for several hours after stationary phase appears to not be deleterious. Growth and induction at 25°C or 30°C may be optimal if you want to export the target using the signal sequence leaders present in a number of pET vectors or improve the yield of soluble protein.

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Bacterial strains: Because lactose is used for induction, expression hosts should produce functional Lac permease (encoded by the *lacY* gene) and β-galactosidase (encoded by the *lacZ* gene) for consistent results in both complex and defined media. *lacY* mutant strains will not efficiently transport lactose for induction and *lacZ* mutants will not convert a portion of the transported lactose into the allolactose inducer. Elevated levels of target gene expression in *lacY* and *lacZ* mutant strains may occur as cells approach stationary phase in some complex media. However, this induction will be variable depending upon medium composition, cell growth stage, and nutrient availability, all of which affect pH and the levels of cyclic AMP and acetate (8).

PopCulture® Reagent compatibility and optimization

Bacterial strain: Protein extraction is efficient for both B (e.g. BL21) and K-12 (e.g. NovaBlue) strains. Other B and K-12 strains should be compatible; however, it is possible that strain-dependent differences could occur. It is important to consider the growth rate of the bacteria if performing the entire experiment in one day. In general, BL21 and its derivatives have a vigorous growth rate and are ideal for this extraction method.

Volume of PopCulture Reagent: There are no adverse effects to using higher ratios of PopCulture Reagent per ml of culture.

Temperature of extraction: PopCulture extraction along with Lysonase™ Bioprocessing Mix treatment can be performed at room temperature or at 4°C. However, incubation times at 4°C may need to be increased because Lysonase activities are decreased at lower temperatures.

pH of extraction: Acidic pH (< 5.0) can degrade components of PopCulture.

Protease inhibitors: Protease inhibitors may be added with the PopCulture Reagent to the culture medium.

Lysonase™ Bioprocessing Reagent

Lysonase Bioprocessing Reagent is an optimized blend of rLysozyme™ Solution and Benzonase® Nuclease and is effective at 2 µl per 1 ml of original culture volume. The addition of lysozyme increases the efficiency of cell lysis and protein extraction (2, 7, 9). Cell extracts may become viscous from nucleic acids released during cell lysis from a high density culture and or when lysozyme is present. Benzonase Nuclease degrades all forms of DNA and RNA (single stranded, double stranded, linear and circular) to 5'-monophosphate terminated oligonucleotides 2–5 bases in length (10, 11). Lysonase treatment is not generally recommended for extracts that must be nuclease free. For further details on Lysonase see Technical Bulletin 361.

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