# Rapid Proline Transport Assay

**Strains:**

| | |
|---|---|
| LT2 | Wild-type *Salmonella enterica* sv Typhimurium ($PutP^+$ control) |
| MS1209 | LT2 putP1024::Mud J (PutP- control) |

**Strain Preparation:**

1. Grow LT2 and MS1209 overnight (O/N) 37°C shaker 2 ml rich medium.
2. Subculture 1 drop into 2 ml NCE + 0.6% succinate O/N 37 C
3. Subculture 0.5 ml into 25 ml NCE + 0.6% NaSuccinate (noninduced) and 25 ml NCE + 0.6% NaSuccinate + 0.2% proline (induced) in 300 ml baffle bottom flasks. Grow at 37°C to mid-late log phase (ca. 100 KU.
4. Pellet 20 ml cells (5000 RPM SS34 for 10 min 4 C). Wash cells 2x with ice-cold NCE. Resuspend final pellet in 10 ml NCE + 1 mM dextrose + 50 $\mu$g/ml chloramphenicol (cells are 2X concentrated).
5. Place 2 ml aliquots in 25 ml erlenmeyer flasks (one per assay). Cover with parafilm and store on ice (up to 3 hours with no appreciable effects).
6. Measure OD650 of remaining cells. We determined that most *Salmonella* strains grown under these conditions have 97.7 $\mu$g/ml cell protein (0.039 mg protein/OD650 in a 0.4 ml reaction.). We determined this by measuring the protein concentration at different OD of cells during growth curves, and found that the plot of [protein] vs OD is linear. Alternatively, you could determine the protein in each sample directly to avoid any assumptions about protein content. The Lowry assay gives better reproducibility for whole cell protein determinations than the Bradford (BioRad) assay which interacts with many of the detergents used to lyse the cells and solubilize the membrane proteins.

**Transport Assays:**

1. Place 0.2 ml aliquots of 2x reaction mix (see buffer worksheet) in 7 ml plastic mini-vials. We do the assays in small plastic scintillation vials that have a raised dimple in the bottom. The vials are inexpensive, disposable, and the dimple seems to help the samples mix thoroughly despite the small reaction volume (0.4 ml). We use 12 vials/assay (triplicate measurements at 4 time points).
2. Before initiating the assay, allow cells to warm up to room temperature ("25°C"). Remove parafilm and swirl on a rotary shaker 100 RPM 15 min at 25°C to starve for proline.
3. Remove 0.2 ml starved cells and add to mini-vial containing 2X Rx Mix. Quickly expel cells into 2x Rxn Mix. Swirl once upon addition (Final concentrations in NCE):

| | |
|---|---|
| 1 mM | Dextrose |
| 50 $\mu$g/ml | Chloramphenicol |
| 9 $\mu$M | L-proline |
| 1 $\mu$M | L-$^{14}$C-proline |
| ~0.125 mg/ml | cells (dry weight) |

4. At appropriate time, dispense 5 ml stopping buffer (see buffer work sheet) into mini-vial (we use Repipet brand dispensers with supple plastic hoses and caps at the ends to minimize leakage). Time points: 0" 5" 10" 15" give good initial rates for kinetic studies. We find that proline transport is "linear" over this time course but starts to taper off after 30 sec. For the 0" time point, dispense 5 ml stopping buffer into mini-vial containing 2x Rxn Mix before addition of cells.

5. Within 10 min of stopping the reaction, filter the stopped mix through a cellulose nitrate filter (Sartorius 0.2 $\mu$m, usually gives <100 CPM $^{14}$C-proline background counts at specific activities in the neighborhood of 0.285 $\mu$Ci/nmol proline. The background can vary by a factor of 5 from lot to lot, so note the lot # if you would like to order that particular lot of filters) that has been previously wet with ~1-2 ml stopping buffer. Immediately filter under 20 inches Hg vacuum. Rinse out the mini-vial with 5 ml stopping buffer and pour over filtered sample under vacuum.
6. Don't allow the filters to dry out under vacuum (to avoid sample loss). After samples are filtered, remove filters and place on a window screen to dry (we use screens that can be adjusted depending on the number of samples).
7. Place dried filters in 7 ml plastic mini-vials (filters will be crunchy). Add 5 ml of your favorite scintillation cocktail -- we typically use BioSafe II (~95% efficient in $^{14}$C quench curves). Count the samples.

**Radioactivity worksheet**

L-[U-$^{14}$C]-proline (Amersham, NEN, or ICN)
285 mCi /mmol 50 $\mu$Ci/ml 175 mM proline

For a 12 ml 2x Rxn Mix stock at 20 $\mu$M proline:
136.8 $\mu$l = 2 $\mu$M = 6.84 $\mu$Ci
20 $\mu$M = 0.24 $\mu$mol proline/12 ml
Specific Activity = 6.84 $\mu$Ci/0.24 $\mu$mol proline = 28.5 $\mu$Ci/$\mu$mol (2.2x 106 DPM/$\mu$Ci) so:

Specific Activity = 62,700 DPM/nmol proline

**Buffer worksheet**

Cell Resuspension Buffer
(50 ml) 45 $\mu$l dextrose (20% stock in dd$H_2O$)
1.25 ml chloramphenicol (2 mg/ml stock in dd$H_2O$)
48.7 ml NCE medium pH 7.0

2X Reaction Mix (20$\mu$M proline)

(12 ml) 10.8 $\mu$l dextrose (20% stock in dd$H_2O$)
300 $\mu$l chloramphenicol (2mg/ml stock in dd$H_2O$)
216 $\mu$l L-proline (1 mM stock in dd$H_2O$)
136.8 $\mu$l L-[U-$^{14}$C]-proline (see radioactivity worksheet)
11.3364 ml NCE pH 7.0

Stopping Buffer (5 mM MES, 5mM Tris, 300 mM KCl, 2 mM $HgCl_2$, pH 7.0)

(1000 ml) 0.976 g MES
0.6055 g Tris
22.365 g KCl
0.543 g $HgCl_2$
ddH2O

Dissolve MES, Tris, and KCl to ~900 ml dd$H_2O$. Adjust to pH 7.0 with concentrated HCl, if necessary. Bring to 1000 ml and transfer to a plastic bottle that has a closely fitting lid. Add $HgCl_2$ and stir on a magnetic stir plate for about 2 hr to dissolve $HgCl_2$.

WARNING: $HgCl_2$ can mess with your nervous system, so be careful. Wear gloves and a lab coat. Dispose of waste appropriately. Contact your local toxic waste team for suggestions. We use old Tris bottles to store Stopping Buffer. It will keep at 4°C indefinitely, but make sure $HgCl_2$ remains in solution. If you need to mess with the buffer composition, avoid phosphate buffer ($HgCl_2$ is insoluble in $PO_4$). Even though NCE medium in the reaction mix is $PO_4$ buffer, the dilution factor appears to avoid solubility problems.

**Suppliers**

Mini-vials:
Danville Scientific Inc., P.O. Box 304 Danville, NJ 07836
cat # V 9971, 17 x 55 $\mu$m Beta vials 7 ml 1000/bulk

Cellulose nitrate filters:
Sartorius, 1775 W. Cullom St., Chicago, Il 60613
cat # 11207.025N, Cellulose nitrate filters 0.2 $\mu$m 15 mm diameter 100/pkg