# Chapter 6

# Membrane Protein Production in *Lactococcus lactis* for Functional Studies

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## Abstract

Due to their unique properties, expression and study of membrane proteins in heterologous systems remains difficult. Among the bacterial systems available, the Gram-positive lactic bacterium, *Lactococcus lactis*, traditionally used in food fermentations, is nowadays widely used for large-scale production and functional characterization of bacterial and eukaryotic membrane proteins. The aim of this chapter is to describe the different possibilities for the functional characterization of peripheral or intrinsic membrane proteins expressed in *Lactococcus lactis*.

Key words *Lactococcus lactis*, Membrane proteins, Expression, Transport assays

## 1 Introduction

In the past decades, *Lactococcus lactis*, a Gram-positive bacterium traditionally used in food fermentations, has emerged as a useful system for functional expression of prokaryotic and eukaryotic membrane proteins (MPs) [1]. *L. lactis* is an attractive alternative host for *Escherichia coli*, especially for eukaryotic MPs, because of (1) its moderate proteolytic activity, (2) the absence of inclusion body formation and endotoxin production [2, 3], (3) the efficient targeting of MPs into a single glycolipid cytoplasmic membrane [2, 4, 5], and (4) its ability to express MPs in their oligomeric state [2, 6]. This facultative anaerobe-aerobe lactic acid bacterium (LAB) grows at 30 °C with a doubling time of 35–60 min [7]; it is easy and inexpensive to grow and genetic methods and vector systems are well developed [8]. In addition to the classical cloning techniques, different strategies have been developed to obtain a larger number of recombinant clones [9, 10].

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The expression of heterologous proteins in *L. lactis* has been facilitated both by advances in genetic methods and by new developments in molecular biology techniques. Using these tools, various vectors containing either constitutive or inducible promoters have been developed to obtain increased levels of proteins and to control their production. They currently constitute the basis of all expression systems in *L. lactis* [11]. The tightly regulated nisin-controlled gene expression (NICE) system is the most commonly used [12]. This promising and powerful expression system is based on genes involved in the biosynthesis and regulation of the antimicrobial peptide, nisin. When a gene of interest is placed upstream of the inducible promoter PnisA on a plasmid, its expression can be induced by the addition of subinhibitory amounts of nisin (0.1–5 ng/mL) to the culture medium [13]. The NICE system has proved to be highly versatile and is widely used in pharmaceutical, medical, and bio- and food-technology applications [14]. This well-characterized system is nowadays widely used for functional studies of homologously and heterologously expressed soluble and membrane proteins from diverse origins (prokaryotic or eukaryotic), topologies, and sizes (for reviews, see [1] and [14]). Moreover, in the last years, three structures have been obtained after expression of MPs using the NICE system [15–17] as well as several domain structures of human membrane proteins [18, 19].

In this chapter, we will give some examples of eukaryotic MPs for which functional analysis has been carried out after their expression in *L. lactis* using the NICE system. These functional characterizations can be performed on: (1) whole-cell bacteria, (2) membrane extracts, (3) fused membrane vesicles, (4) proteoliposomes after reconstitution of the MPs in phospholipids, using radioactive substrates, or (5) directly solubilized and purified membrane proteins [2, 3, 10, 12, 18, 19].

## 2 Materials

### 2.1 Growth of Recombinant Bacteria and Expression of the Proteins of Interest

1. *Lactococcus lactis* NZ9000 and nisin-producing NZ9700 strains (NIZO; *see* **Notes 1** and **2**).
2. M171GChl medium: M17 broth, 1% [w/v] glucose, 10 μg $mL^{-1}$ chloramphenicol (*see* **Note 3**).
3. Laboratory glassware bottles (Schott bottles).
4. Incubator for cell growth.
5. Appropriate buffers for bacterial resuspension (*see* **Note 4**).

### 2.2 Isolation of Lactococcal Membranes with the Cell Disruptor

1. One Shot (Constant Cell Disruption Systems, Northants, UK) (*see* **Note 5**).
2. Appropriate buffers for protein resuspension (*see* **Note 4**).

### 2.3 SDS-PAGE and Detection of Recombinant Protein

1. Acrylamide-bis ready-to-use solution, 30% [w/v] (37.5:1).
2. 8× Laemmli resolving gel buffer: 3 M Tris–HCl pH 8.8 (60.6 g Tris–HCl resuspended in Milli-Q water; adjust to pH 8.8 at 25 °C with 12 N HCl. Store at room temperature).
3. 4× Laemmli stacking gel buffer: 0.5 M Tris–HCl pH 6.8 (363 g Tris–HCl resuspended in Milli-Q water; adjust to pH 6.8 at 25 °C with 12 N HCl. Store at room temperature).
4. Aqueous solution 20% [w/v] sodium dodecyl sulfate (SDS).
5. Ammonium persulfate: Prepare 10% [w/v] solution in water and immediately freeze in single-use (200 µL) aliquots at −20 °C.
6. Tetramethylethylenediamine (TEMED).
7. Resolving gels (10% acrylamide): 3.3 mL of 30% [w/v] acrylamide solution, 1.25 mL of 8× Laemmli resolving gel buffer, 50 µL of 20% [w/v] SDS, 5.3 mL of water, 10 µL of TEMED, and 100 µL of 10% [w/v] ammonium persulfate (*see* **Note 6**).
8. Stacking gels (5% [w/v] acrylamide): 2.8 mL of 30% [w/v] acrylamide solution, 1.25 mL of 4× Laemmli stacking gel buffer, 25 µL of SDS 20% [w/v], 2.8 mL of water, 5 µL of TEMED, and 50 µL of 10% [w/v] ammonium persulfate.
9. Laemmli running buffer (10×): For 1 L, 144.2 g of glycine (192 mM), 30.3 g of Tris–HCl (25 mM); add 50 mL of 20% [w/v] SDS (0.1% final concentration) and Milli-Q water. Store at room temperature.
10. Molecular weight marker.
11. Reducing sample buffer (4×): 0.08 M Tris–HCl, pH 6.8, 40% [v/v] glycerol, 1% [w/v] SDS, 0.1 mM bromophenol blue, 10 mM dithiothreitol. Store at −20 °C.
12. Sample buffer: 100 mL reducing buffer and 20 mL of 20% [w/v] SDS.
13. Control protein: Recombinant *Strep*-tag II fusion protein, MW about 28 kDa (0.1 mg $mL^{-1}$) (IBA, Goettingen, Germany).
14. System for protein transfer to nitrocellulose membranes (central core assembly, holder cassette, nitrocellulose filter paper, fibber pads, and cooling unit).
15. Protein transfer buffer: dilute running buffer 1× with ethanol to a final concentration of 20% [v/v]. Store at 4 °C.
16. Nitrocellulose or polyvinylidene difluoride (PVDF) membranes.
17. 3 MM paper from Whatman.
18. Protein-specific antibody or conjugate specific to the affinity tag (*see* **Note 7**).
19. Bio-Safe Coomassie (Biorad).
20. Electrochemiluminescence (ECL) detection kit.

### 2.4 Functional Characterization of Membrane Proteins Expressed in L. lactis

#### 2.4.1 Dehydrogenase Assay on Purified Protein (ceQORH)

1. Solubilization buffer: 50 mM MOPS pH 7.8 containing 0.5 or 1 M NaCl.
2. 10 mM Tris–HCl pH 8.0.
3. Ni-NTA resin (Qiagen).
4. Binding buffer: 5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl pH 7.9.
5. Wash buffer: 60 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl pH 7.9.
6. Elution buffer: 0.5 M imidazole, 0.25 M NaCl, 10 mM Tris–HCl pH 7.9.
7. Dehydrogenase reaction buffer: 100 μM NADPH, 100 μM nitroblue tetrazolium (NBT), 10 mM Tris–HCl pH 8.0.
8. PD10 column (GE Healthcare).
9. Eppendorf centrifuge.
10. Lipids (P3644, Sigma).
11. Spectrophotometer.

#### 2.4.2 Phosphorylation Assays with AtHMA6 and AtHMA8 Using L. lactis Membranes

1. ATP phosphorylation buffer: 20 mM Hepes pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 300 mM sucrose (*see* **Note 8**).
2. Pi phosphorylation buffer: 20 mM Hepes pH 6.0, 10 mM $MgCl_2$, 20% [v/v] DMSO (*see* **Note 9**).
3. Metal solutions: prepare solutions at concentration ranging from 1 to 100 μM in phosphorylation buffer or water (*see* **Note 10**).
4. 1 mM $^{32}Pi$ (10–100 μCi $nmol^{-1}$, Perkin Elmer, 7 μL): Add 1 mL of 1 mM $H_3PO_4$ (prepared in 100 mM Hepes pH 5.6) directly in the tube containing the isotope $^{32}Pi$. Filter the solution through a 0.2 μm membrane. To avoid loss of the solution in the filter, push the volume of the solution stayed in the filter using an empty syringe. Use 10 μL/reaction. Store at 4 °C.
5. 10 μM [$\gamma$-$^{32}P$]ATP (50–500 μCi $nmol^{-1}$, Perkin Elmer, 7 μL): for 10 reactions, add 1 μL of [$\gamma$-$^{32}P$]ATP to 110 μL of a solution of 10 μM ATP (*see* **Note 11**). Prepare a stock solution of 400 mM ATP in $H_2O$, aliquot in small volumes, and store at −20 °C. 10 μM ATP is prepared freshly from one stored aliquot of 400 mM ATP.
6. Stop buffer: 1 mM $KH_2PO_4$ in 7% [v/v] trichloroacetic acid (TCA). Store at 4 °C.
7. Denaturing buffer: 5 mM Tris-$PO_4$ pH 5.0, 6.7 mM urea, 400 mM DTT, 5% [w/v] SDS, 0.004% [w/v] orange G (*see* **Note 12**).
8. Chelator mix: 1 mM bicinchoninic acid (BCA) and 100 μM bathocuproine disulfonate (BCS) (*see* **Note 13**).

9. Resolving gel: 6 mL of Acrylamide/Bis 24/0.8% [w/v] solution (*see* **Note 14**), 4.5 mL of 4× resolving gel buffer (260 mM Tris-$H_3PO_4$ pH 6.5, 0.4% SDS), 7.4 mL of $H_2O$, 18 µL of TEMED, and 100 µL of 10% [w/v] ammonium persulfate.
10. Stacking gel: 1.2 mL of Acrylamide/Bis 24/0.8% [v/v] solution, 2 mL of 4× stacking gel buffer (260 mM Tris-$H_3PO_4$ pH 5.5, 0.4% SDS), 4.64 mL of $H_2O$, 7.5 µL of TEMED, and 160 µL of 10% [w/v] ammonium persulfate.
11. Running buffer: 0.17 mM MOPS (pH 6.0 adjusted with 2 M Tris), 0.1% [w/v] SDS. Store at 4 °C before use (do not store for more than 1 week) (*see* **Note 15**).
12. Acetic acid 15% [v/v].
13. Gel staining medium: acetic acid/isopropanol/water, 10/25/65 [v/v/v], supplemented with 2.5 $g.L^{-1}$ of Coomassie Brilliant Blue R250.
14. Gel destaining medium: 30% [v/v] ethanol.
15. Eppendorf centrifuge.
16. Gel electrophoresis apparatus (Bio-Rad Protean 3 or equivalent), with the various accessories needed for protein separation by electrophoresis (combs, plates, and casting apparatus).
17. Phosphorimaging device (*see* **Note 16**).

*2.4.3 Transport Assays with Whole Cells Expressing AtAATP1/NTT1*

1. 50 mM potassium phosphate buffer pH 7.0.
2. 3.33 nM [$\alpha$-$^{32}$P]ATP (3000 mCi $mmol^{-1}$, Perkin Elmer). Add 1 µL to 50 mM potassium phosphate buffer pH 7.0 containing 1.5 mM cold ATP (A9062; Sigma) to obtain a 50 µM solution. Store on ice until use.
3. Filter membranes 0.45 µm (HAWP02500; Millipore).
4. Scintillation vials.
5. d.d. water.
6. Polymeric Vacuum Filter Holder (1225 Sampling Manifold; Millipore).
7. Multi-Purpose Scintillation Counter.

*2.4.4 Transport Assays with Whole Cells and Fused Membrane Vesicles Expressing Mitochondrial Carriers*

1. *E. coli* polar lipid extract (Avanti Polar Lipids).
2. Egg yolk phosphatidylcholine (Avanti Polar Lipids).
3. Nitrogen.
4. Diethyl ether.
5. Substrate/inhibitor, 10× stock (*see* **Note 17**).
   - ADP to give a final concentration of 5 mM
   - Carboxyatractyloside (CATR; Sigma) to give a final concentration of 2 µM

- Bongkrekic acid (BKA; Sigma) to give a final concentration of 2 μM

6. Extruder Set (Avanti Polar Lipids).
7. 1 μm polycarbonate filter (Whatman); filter supports (Whatman).
8. PD-10 column (GE Healthcare).
9. 2 mL Eppendorf tubes.
10. Hamilton robot (with vacuum manifold).
11. 96-well MultiScreenHTS-Hi Flow-FB opaque, Barex plastic plates (pore size = 1 μm; Millipore).
12. 96-well MultiScreenHTS-HA opaque, Barex plastic plates (pore size = 0.45 μm; Millipore).
13. 1.5 μM [$^{14}$C]-ADP (60 mCi mmol$^{-1}$ = 2.22 GBq mmol$^{-1}$; Perkin Elmer) prepared in PIPES buffer to start the transport assays with mitochondrial carriers.
14. BackSeal black backing paper (Perkin Elmer).
15. MicroScint-20 (Perkin Elmer).
16. MultiScreen sealing tape, clear backing paper (Perkin Elmer).
17. TopCount (Perkin Elmer).

## 3 Methods

### 3.1 Growth of Recombinant Bacteria and Expression of the Proteins of Interest

The gene(s) of protein(s) of interest have to be cloned first into an expression vector containing nisin-inducible promoter either through classical cloning methods [2] or other strategies developed in the last years to facilitate cloning [9, 10]. First trials could be performed following protocols already described [2, 10, 20–22]. Here we present protocols that can be optimized for each protein of interest.

1. Inoculate M17G1Chl precultures with concentrated glycerol stocks or frozen cell stocks of recombinant bacteria carrying the gene of protein of interest and bacteria carrying the empty vector as negative control.
2. Incubate overnight at 30 °C without shaking (*see* **Notes 18** and **19**).
3. Inoculate M17G1Chl with 1/40$^{e}$ precultures (*see* **Note 20**).
4. Incubate cultures at 30 °C; measure $OD_{600nm}$ every 45 min to construct growth curve (doubling roughly every 45 min).
5. Induce protein expression by addition of homemade nisin (*see* **Note 21**) at $OD_{600nm}$ from 0.5 to 0.8 depending on proteins of interest (*see* **Note 22**).

6. Swirl immediately to prevent cell lysis; return the flasks to the 30 °C incubator for further 2.5–4 h (*see* **Note 23**).
7. Depending on functional tests, bacteria are either centrifuged, resuspended with an appropriate buffer (*see* **Note 4**) and centrifuged again before storage at −20 °C (*see* Subheading 3.2), or directly used (*see* Subheading 3.4.1) or snap-frozen and stored in liquid nitrogen (*see* Subheading 3.4.4).

### 3.2 Isolation of Lactococcal Membranes with the Cell Disruptor

1. Resuspend the bacteria into the appropriate buffer (*see* **Note 4**).
2. Disrupt the bacteria by twofold passages through a One Shot at 35,000 p.s.i. (2.3 kbars).
3. Centrifuge 100,000×*g*, 15 min, 4 °C and transfer the supernatant into ultracentrifuge tubes.
4. Centrifuge 100,000×*g*, 1 h, 4 °C; resuspend the pellet and homogenize into the appropriate buffer (*see* **Note 4**).
5. Snap-freeze and store at −80 °C or in liquid nitrogen until use.

### 3.3 Detection of the Recombinant Protein Produced

1. Prior to the experiment, prepare acrylamide gels for protein electrophoresis, the gel apparatus according to the manufacturer's specifications, and the different gel solutions (stacking gel, acrylamide separating gel; *see* **Note 6**).
2. Heat the protein samples at 95 °C for 5 min to solubilize the proteins (*see* **Note 24**). Load protein samples, one molecular weight marker, and positive controls in defined quantities.
3. Run gels for 1 h at room temperature at 150 V with constant voltage (*see* **Note 25**).
4. After electrophoresis, perform the transfer for 1 h 30 min at 100 V in protein transfer medium prior to Western blotting analysis.
5. Recover the nitrocellulose membrane and rinse the membrane with water. The following incubation and washing steps require agitation on a rocking plate at room temperature.
6. Perform Western blotting analysis and/or Coomassie blue staining using protocols already established or given by manufacturers.
7. Perform ECL detection (Figs. 1 and 2)

### 3.4 Functional Characterization of Membrane Proteins

#### 3.4.1 Dehydrogenase Assay on Purified Protein (ceQORH)

The chloroplast envelope Quinone OxidoReductase Homologue (ceQORH) protein from *Arabidopsis thaliana* is a peripheral protein associated with the chloroplast envelope through electrostatic interactions [24]. This nuclear-encoded protein is devoid of a classical and cleavable transit peptide and uses an alternative targeting pathway for its import into the chloroplast [25, 26]. The ceQORH protein is structurally related to bacterial, fungal, and animal proteins with known quinone oxidoreductase function. In an earlier

12. Follow the formation of Formazan at 560 nm using a Spectrophotometer.
13. Deduce the enzymatic activities from the OD measurement using the molar absorption coefficient (*see* **Note 30**, Fig. 3c).

### 3.4.2 Phosphorylation Assays with AtHMA6 and AtHMA8 Using L. lactis Membranes

$P_{IB}$-ATPases (reviewed in [28]) belong to the large family of P-type ATPases that are transmembrane proteins responsible for the transport of ions and phospholipids across plasma and organelle membranes using the energy provided by ATP hydrolysis. Like all P-type ATPases, $P_{IB}$-ATPases (or HMA for Heavy Metal ATPase) are composed of a transmembrane domain M containing the transport site and determining ion selectivity, and of three cytosolic loops constituting the catalytic domain. $P_{IB}$-ATPases have six to eight predicted transmembrane helices and have been classified into several subgroups according to their ionic specificity [29]. Recently, the crystal structure of a prokaryotic $P_{IB}$-ATPase, LpCopA, has been solved providing new topological information on these enzymes [30]. In *Arabidopsis* species, AtHMA6 and AtHMA8 are two chloroplastic ATPases of the PIB-1 subgroup involved in Cu transport across the chloroplast envelope and the thylakoid respectively [31, 32]. The enzymatic properties of these two transmembrane proteins could be assessed using in vitro biochemical assays [23, 33] after their successful and efficient expression in *Lactococcus lactis*. Phosphorylation assays performed on lactococcal membranes expressing these exogenous $P_{IB}$-ATPases (*see* **Note 31**) could provide information about (1) the kinetic parameters of the enzyme using phosphorylation from ATP (*see* Fig. 4a–c), and (2) the apparent affinity for the translocated metal using phosphorylation from Pi (Fig. 4b). These phosphorylation assays can be performed on all $P_{IB}$-ATPases whatever their ionic specificity to assess their enzymatic properties.

*Phosphorylation from ATP:*

1. Prepare a mix containing 0.5 mg $mL^{-1}$ of *L. lactis* membranes, with metals or chelators at the desired concentration (*see* **Note 32**) and complete with ATP phosphorylation buffer to a final volume of 90 µL.
2. Start the reaction by addition of 10 µL of 10 µM [$\gamma$-$^{32}$P]ATP (1 µM final). Vortex the suspension.
3. Stop the reaction 30 s later (*see* **Note 33**) by addition of 1 mL ice-cold Stop buffer. Vortex the suspension and incubate 30 min on ice.
4. Centrifuge for 15 min, at 15,000 × *g*, 4 °C and keep the pellet (*see* **Note 34**).
5. Wash the pellet with 1 mL of ice-cold Stop buffer and centrifuge for 15 min, at 10,000 × *g*, 4 °C.

*Phosphorylation assays from Pi:*

1. Prepare a mix containing 1 mg $mL^{-1}$ of *L. lactis* membranes, metals, or chelators (*see* **Note 32**) in Pi phosphorylation buffer to a final volume of 90 µL.
2. Incubate the mix 5 min at 30 °C.
3. Start the reaction by addition of 10 µL of 1 mM $^{32}Pi$ (100 µM final). Vortex the suspension and incubate the reaction mix at 30 °C for 10 min.
4. Stop the reaction by addition of 1 mL ice-cold stop buffer. Vortex the suspension and incubate 30 min on ice.
5. Then proceed as described in **steps 4–12** for phosphorylation assays from ATP (Fig.4b).

### *3.4.3 Transport Assays with Whole Cells Expressing AtAATP1/NTT1*

The nucleotide transporter 1, AtAATP1/NTT1, a highly hydrophobic membrane protein with 12 predicted transmembrane domains [34], is localized within the inner membrane of the chloroplast envelope [35]. This translocator imports ATP in exchange of ADP and Pi [36] in contrast to mitochondrial ATP/ADP translocators [37]. It supplies energy to chloroplasts used by storage plastids required for starch synthesis and to allow nocturnal anabolic reactions in chloroplasts [38].

1. Centrifuge 4350×*g*, 10 min twice and resuspend recombinant bacteria into 30 mL to a final concentration of 100 mg $mL^{-1}$ (3 mg/30 µL) in ice-cold 50 mM potassium phosphate buffer pH 7.0.
2. Add 50 µM [α-$^{32}P$]ATP (3000 mCi $mmol^{-1}$; Perkin Elmer) diluted in ice-cold 50 mM potassium phosphate buffer pH 7.0 to each sample (*see* **Note 36**).
3. Incubate at 25 °C for planned time periods and stop nucleotide uptake by addition of 1 mL of ice-cold potassium phosphate buffer.
4. Filtrate the cells through a 0.45 µm filter under vacuum.
5. Wash three times with 1 mL of ice-cold potassium phosphate buffer.
6. Transfer filter to a scintillation vial and add 3.5 mL of d.d. water
7. Measure the radioactivity retained on the filters in a scintillation counter.
8. Generate graphs using the KaleidaGraph version 4.02 (Synergy Software) and fit experimental data with the appropriate curve (i.e., single exponential) (Fig. 5).

### *3.4.4 High-Throughput Transport Assays of Mitochondrial Carriers*

Mitochondrial carriers link the biochemical pathways of the cytosol and the mitochondrion matrix by transporting metabolites, nucleotides, inorganic ions, and cofactors across the mitochondrial inner

***High-throughput transport assays*** can be carried out using a Hamilton MicroLab Star robot. The first five steps are programmed to be carried out by the robot in 96-well format, allowing eight different uptake experiments to be performed simultaneously.

1. Pipet 100 µL bacteria ($OD_{600nm} = 25$) into the wells of a 96-well $MultiScreen_{HTS}$-Hi Flow-FB plate (pore size = 1 µm) or fused membrane vesicles (5 µg) into a 96-well $MultiScreen_{HTS}$-HA plate (pore size = 0.45 µm), while the plate is placed on a vacuum manifold.
2. Initiate transport with the addition of 100 µL PIPES buffer containing 1.5 µM of $^{14}C$-labeled nucleotide.
3. Incubate at room temperature for planned time periods, the longer time points being added first.
4. Stop transport by filtration followed immediately by the addition of 200 µL of ice-cold PIPES buffer to all wells.
5. Wash wells two times with 200 µL ice-cold PIPES buffer.
6. Leave the plates to dry overnight.
7. Stick black backing paper on the underside of the filter plate.
8. Add 200 µL of MicroScint-20 to each well using the robot.
9. Stick clear backing paper on the topside of the filter plate; stand for at least 4 h to allow filter dissolution.
10. Load the plates into the TopCount Scintillation Counter. Initial rates are determined from the linear part of the uptake curves (first 60 s) (*see* Fig. 6).

## 4 Notes

1. Nisin can be either commercial or produced by the nisin-producing strain NZ9700. We have noticed that homemade nisin gave rise to higher amounts of proteins compared to commercial one [21].
2. Recombinant bacteria are generated through transformation with an expression vector containing the gene of interest; in our studies, we use pNZ8148.
3. The concentration of antibiotic (chloramphenicol) could vary from 5 to 10 µg $mL^{-1}$.
4. The buffers for bacterial and protein resuspension have to be adjusted to the protein of interest (50 mM Tris–HCl pH 8.0 for ceQORH [10] and 20 mM HEPES pH 6.0 for AtHMA6 and AtHMA8 [33] containing 6 or 20% glycerol for protein resuspension respectively; 50 mM potassium phosphate buffer pH 7.0 for AtNTT1 [10]; 10 mM PIPES pH 7.0, 50 mM NaCl for mitochondrial carriers [3]).

37. Quickly thawing the pellets is vital for transport activity.
38. The syringes are not airtight at 4 °C for prolonged periods of time and will leak.

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