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### The COS-7 Cell In Vitro Paradigm to Study Myelin Proteolipid Protein 1 Gene Mutations

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#### 1. Introduction

Despite many shortcomings, a reductionist approach using cell culture paradigms to define basic principles underlying disease processes has considerable merit. One example of the utility of this approach is the expression of mutant forms of proteolipid protein 1 (PLP1) in transiently transfected COS-7 cells. In humans, the *PLP1* gene is located on the long arm of the X-chromosome and deletion, duplication, or coding region mutations in this gene cause the leukodystrophy, Pelizaeus-Merzbacher disease (PMD). Clinically, PMD is a heterogeneous disease that generally becomes apparent within the first year of life and is associated with hypomyelination in the central and peripheral nervous systems (CNS/PNS), breathing difficulties, poor motor coordination and paraparesis or paraplegia (1–3). From simple beginnings using a transfection paradigm to express missense mutant gene products identified in PMD patients, we have developed an hypothesis to account for the cellular (4–8) and molecular pathogenesis of disease (9) and we have made use of several excellent mouse models of PMD to confirm our in vitro findings in vivo (10).

The *PLP1* gene gives rise to two protein isoforms by alternative splicing of a cryptic splice-site in exon 3. Messenger RNAs (mRNAs) encompassing all of exon 3 (i.e. exon 3a and 3b) encode the 276 amino acid protein, PLP1. On the other hand, mRNAs in which exon 3b is absent encode the 242 amino acid protein, DM-20. The 35 amino acids encoded by exon 3b form part of a cytoplasmic loop near the center of PLP1 between transmembrane-spanning domains II and III. The presence of this polypeptide does not alter the topology of PLP1 compared to DM-20; both proteins exhibit 4 transmembrane domains with the amino- and carboxyl-termini exposed to the cytoplasm (11). Although the function of this PLP1-specific peptide is unknown, it arose recently in evolution with the emergence of terrestrial vertebrates (12), confers properties on PLP1 that cannot be recapitulated by DM-20 (13), and clearly renders this protein more susceptible than DM-20 to the deleterious effects of missense mutations.

The initial phase of our research was to obtain cDNAs for wild-type and mutant forms of PLP1 and DM-20 and to examine the behavior of the encoded proteins in

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transfected COS-7 cells (4–8). From wild-type PLP1 and DM-20 cDNA templates, we selected known mutations from the literature and generated the mutations for experimentation using site-directed mutagenesis. Thereafter, we sequenced and subcloned these cDNAs and constructed heterologous genes that force transfected cells to express extremely high levels of protein under transcriptional regulation of the human cytomegalovirus immediate-early promoter (14).

A major motivation behind the choice of expression cassette was that during myelinogenesis in the CNS, 10% of the mRNA present in *PLP1* gene-expressing cells, called oligodendrocytes, encodes PLP1 and DM-20 (15). This proportion exceeds that of other highly expressed genes, such as tubulin and actin, by two orders of magnitude. Accordingly, we wanted to recapitulate these levels as closely as possible in COS-7 cells. In this regard, the SV-40 T antigen expressed by these cells replicates the pCMV5 plasmid to high copy number from the SV-40 origin of replication site in the plasmid. Two other factors are also advantageous for high level expression: 1) the use of the human cytomegalovirus immediate-early promoter; and 2) transient transfections, which in general provide significant increases in expression compared to stably transfected cells. Finally, an additional benefit of transient transfection is the presence of nontransfected cells in all dishes, which serve as convenient internal negative controls for morphology, cell survival, and immunocytochemical labeling with antibodies.

Protein behavior in transfected fibroblasts was assessed by immunocytochemistry using a monoclonal antibody (MAb) raised against the carboxyl-terminus of PLP1/DM-20. As expected for polytopic membrane proteins, wild-type PLP1 and DM-20 are detected in all major compartments of the secretory pathway, the cell surface, and the endocytic pathway. In contrast, all single amino acid changes identified in PMD patients and animal models cause PLP1 to accumulate in the endoplasmic reticulum, which is consistent with a protein trafficking defect brought about by the inability of the mutant proteins to adopt stable three-dimensional conformations. Surprisingly, a portion of the same mutations introduced into DM-20 do not interrupt trafficking to the cell surface; these mutations are associated with mild forms of disease (6).

The principle findings defined in the in vitro COS-7 cell transfection analyses were tested in vivo in mouse models of PMD (10). Mutant PLP1 and DM-20 expressed in *jimpy<sup>msd</sup>* and *jimpy<sup>rsh</sup>* oligodendrocytes is confined to the perinuclear region of oligodendrocytes which likely reflects protein accumulation in the endoplasmic reticulum. In contrast, other myelin proteins such as myelin basic protein (MBP) are transported out to the myelin membranes that ensheath nearby axons. Thus, these data are consistent with earlier studies showing that PLP1 and DM-20 accumulate in the endoplasmic reticulum of *jimpy* mice (16–18), and afford a novel interpretation of other published data for mild and severe PMD, that of a defect in protein trafficking (9). Thus, the use of a simple in vitro paradigm to ask simple processes about PMD has led to new insights into the pathogenesis of this disease. Finally, the strong correlation between mild forms of disease and the trafficking of mutant DM-20 through the secretory pathway of transfected cells suggests that such an assay may be used to predict pathogenesis and severity of novel coding region mutations for the purposes of future genetic counseling. Towards this goal, we describe in detail the protocols we use to explore the disease mechanisms of PMD in COS-7 cells in culture.

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### 2. Materials

#### 2.1. Restriction Digest of Plasmids

1. Plasmid containing the cDNA for study. Store indefinitely at  $-20^{\circ}\text{C}$ .
2. Plasmid, pUC19. Store indefinitely at  $-20^{\circ}\text{C}$ .
3. Restriction enzyme, 10–20 U/ $\mu\text{L}$ , 10X restriction enzyme buffer, and 10  $\mu\text{g}/\text{mL}$  bovine serum albumin (BSA) (New England Biolabs, MA, USA).
4. Gel loading dye, 6X: 4 mL of glycerol, 2.5 mL of 0.4 M EDTA, 3.5 mL distilled water, 0.25% bromophenol blue. Store indefinitely at room temperature (RT).

#### 2.2. Agarose Gel Electrophoresis

1. Agarose, 0.5 g (Gibco-BRL).
2. Ethidium bromide (EtBr) in distilled water, 10 mg/mL stock. Store away from light for up to 1 yr at RT. Caution: EtBr is a powerful mutagen and should be handled with gloves.
3. Tris-acetate buffer, pH 8.3, 50X stock: 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL of 0.5 M EDTA (pH 8.0). Store indefinitely at RT.
4. EtBr-TAE buffer, pH 8.3: dilute 50X stock to 1X with distilled water. Add 30  $\mu\text{L}$  of EtBr stock per liter of buffer. Stable for 1 mo at RT.
5. DNA molecular weight markers, 0.5  $\mu\text{g}/\mu\text{L}$ , 1 kb ladder (New England Biolabs).

#### 2.3. Subcloning the cDNA

1. QIAquick gel extraction kit.
2. T4 DNA ligase, 4000 units/ $\mu\text{L}$  and 10X buffer (Stratagene).
3. XL-1 blue competent *Escherichia coli*,  $\geq 10^6$  transformants/ $\mu\text{g}$ , (Stratagene).
4. Luria broth (LB): 1% bacto-tryptone (Fisher), 0.5% bacto-yeast extract, 1% NaCl. Autoclave and store at RT for up to 6 mo.

#### 2.4. Growth of Transformed Bacteria on Ampicillin Plates

1. LB (see **Subheading 2.3., item 4**).
2. Bacto-agar (Fisher).
3. Ampicillin in distilled water, 50 mg/mL stock. Filter through 0.45- $\mu\text{m}$  Millipore membrane to sterilize. Store away from light up to 2 mo at  $4^{\circ}\text{C}$ .
4. Sterile 100 mm polystyrene petri dishes.
5. X-gal (Fisher) in N,N-dimethylformamide, 50 mg/mL stock. Store away from light up to 3 mo at  $-20^{\circ}\text{C}$ . Discard if the solution turns noticeably yellow.
6. IPTG (Fisher) in distilled water, 0.1 M stock. Filter through 0.45- $\mu\text{m}$  Millipore membrane to sterilize. Store indefinitely at  $-20^{\circ}\text{C}$ .
7. Ethanol in a glass beaker. Caution: flammable, keep away from Bunsen flame.

#### 2.5. Purifying Plasmids from Mini- and Maxi-Prep Cultures

1. Sterile tooth picks.
2. Ampicillin in distilled water, 50 mg/mL stock.
3. LB (see **Subheading 2.3., item 4**)
4. LB-ampicillin: to 500 mL of sterile LB at RT add 0.5 mL of ampicillin stock. Store away from light for up to 2 mo at  $4^{\circ}\text{C}$ .
5. Qiagen mini-prep kit, standard purity DNA.
6. Restriction enzyme (see **Subheading 2.1., item 3**).
7. Qiagen Endofree Plasmid Megakit.

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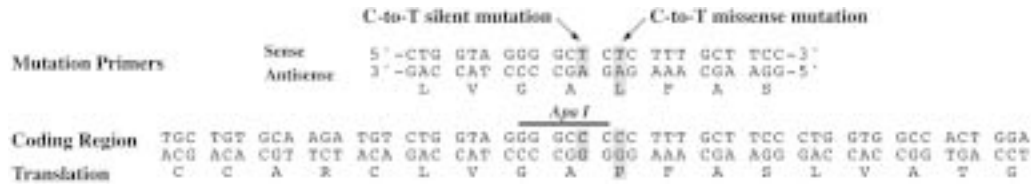


Fig. 1. Mutagenesis of the human *PLP1* coding region in exon 2. Codons 6–23 of the human *PLP1* cDNA are shown, which encode amino acids 5–22 of the mature PLP1 molecule. To generate the mutation identified in a connatal PMD patient, two PCR mutation primers were designed with single base changes (gray boxes) to effect the missense mutation, P14L, at codon 15 and to ablate an *Apa I* restriction enzyme site at codon 14. Note that the amino acid, alanine, encoded by codon 14 is unchanged by the single base change and that the new base triplet used, G-C-T, is one that commonly appears in mammalian cDNAs. Finally, the primers are of sufficient length so that 10–15 bases of perfect complementarity to the cDNA template flank the base changes on either side.

**2.6. Site-Directed Mutagenesis to Generate the P14L Mutation in PLP1**

1. Two overlapping and complementary oligodeoxynucleotide mutation primers. Store indefinitely at –20°C.
2. QuikChange mutagenesis kit (Stratagene).
3. LB (see Subheading 2.3., item 4)
4. LB-ampicillin (see Subheading 2.5., item 4).
5. Qiagen mini-prep kit.
6. *Apa I* restriction enzyme, 10X buffer, 10 µg/µL BSA (New England Biolabs) (see Fig. 1).

Fig. 1

**2.7. Subclone the cDNA into an Expression Cassette**

1. pCMV5 expression plasmid.
2. Components listed in Subheadings 2.1.–2.5.

**2.8. Maintaining COS-7 Cells in Culture**

1. T75 flask of COS-7 cells between 10–100% confluence.
2. Sterile 9" Pasteur pipets.
3. Phosphate buffered saline (PBS) without Ca<sup>2+</sup> or Mg<sup>2+</sup> (BioWhittacker) (see Note 1M).
4. 0.25% trypsin /1 mM EDTA (Ginco-BRL). Store indefinitely in 10 mL aliquots at –20°C.
5. Dulbecco's Minimal Essential Medium (DMEM; Gibco-BRL).
6. 200 mM glutamine, 100X. (Ginco-BRL). Store indefinitely in 5-mL aliquots at –20°C.
7. Penicillin (10,000 U/mL)/streptomycin (10,000 U/mL), 100X. (Ginco-BRL). Store indefinitely in 5-mL aliquots at –20°C.
8. Fetal calf serum (Sigma). Heat-inactivate this reagent by incubating at 56°C for 30 min. Store indefinitely in 50-mL aliquots at –20°C.
9. Culture medium: 500 mL of DMEM, 50 mL of fetal calf serum (FCS), 5 mL of 100X penicillin/streptomycin, 5 mL of 100X glutamine added fresh every mo. Store medium at 4°C for up to 4 mo.
10. T75 culture flasks and individually wrapped sterile polystyrene pipets (Falcon).

**2.9. Transfection of COS-7 Cells**

1. Ethanol, 70%, in a spray bottle.
2. Culture medium (see Subheading 2.8., item 9).

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3. PBS without  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ .
4. Confluent T75 culture flask of COS-7 cells.
5. T75 culture flasks, 100-mm and 60-mm culture dishes for mammalian cells, individually wrapped sterile polystyrene pipets, 35 mm NUNC culture dishes (Fisher) (*see Note 2*).
6. DMEM.
7. FUGENE 6 (Stratagene).
8. Expression plasmid DNA for transfection. Store indefinitely at 4°C.

### 2.10. Fixation and Immunocytochemistry

1. DMEM warmed to 37°C.
2. Sodium phosphate buffer, 0.1 M, pH 7.4. Autoclave and store indefinitely at RT.
3. Paraformaldehyde powder (Sigma).
4. Stericup vacuum filter units, 100 mL capacity, 0.45- $\mu\text{m}$  pore size (Millipore, MA, USA).
5. Tris-buffered saline (TBS), 10X stock: 250 mM Tris base, 1.5 M NaCl. Dilute with distilled water and adjust pH to 7.4 with conc. HCl in a fume hood. Add 50 mg sodium azide/100 mL and store indefinitely at RT. Cautions: HCl fumes are harmful to skin and lungs; sodium azide is extremely toxic, handle with gloves in a fume hood.
6. Saponin (Sigma) in distilled water, 10% stock. Add 50 mg sodium azide /100 mL and store indefinitely at RT. Caution: saponin is cardiotoxic, prepare in a fume hood.
7. Grease pen (e.g., Super HT PAP pen, Research Products International Corp.).
8. Gelatin (Type A, 300 Bloom, Sigma).
9. Bovine serum albumin (BSA), fraction V (Sigma).
10. Sodium azide (Sigma). Caution: extremely toxic, handle with gloves in a fume hood.
11. Normal goat serum (Sigma).
12. Combine reagents from **Subheading 2.10., items 5,8–10** as follows to make 500 mL of TBSGBA solution. Add 0.5 g of gelatin (*see item 8*) to 450 mL of distilled water at RT and place in a water bath also at RT. Heat the solution to 45°C. Gently mix the gelatin crystals until they dissolve. Allow the solution to come to RT and add 5 g of BSA (*see item 9*). This component dissolves most easily if the solution is left unstirred for approx 30 min. With moderate stirring, add 50 mL of a 10X stock of TBS (*see item 5*). Add 250 mg of sodium azide (*see item 10*). Pass this TBSGBA solution through a 0.45- $\mu\text{m}$  filter to remove small protein aggregates and store in 50-mL aliquots at RT for at least 1 y.
13. Blocking solution is made by combining the following reagents from **Subheading 2.10., items 11 and 12**. To 10 mL of TBSGBA (*see item 12*), add 0.3 mL of normal goat serum (*see item 11*). This solution is stable at RT for 1 mo and should be centrifuged in a microfuge for 2 min immediately before use.
14. Humidified chambers (*see Note 3*).
15. DAPI (4,6, Diamidino-2-phenylindole, Sigma) in distilled water, 0.1 mg/mL, 100X stock.
16. 0.5 M Tris base in distilled water, pH 8.0.
17. DTG: dissolve 1 g of DABCO (Sigma) in 45 mL of glycerol at 45°C. Add 5 mL of 0.5 M Tris, pH 8.0 (*see item 16*) and aliquot for indefinite storage at -20°C. (*see Note 4*).
18. Soldering iron, 20–40 W, with a sharpened electrode to cut NUNC culture dishes.
19. Glass microscope slides, 22 mm square coverslips (No. 1, Fisher).
20. Superglue to attach NUNC dishes to glass slides.

### 3. Methods

#### 3.1. Restriction Digest of Plasmids

1. Combine 1–2  $\mu\text{g}$  of a plasmid containing the cDNA plus distilled water to 7  $\mu\text{L}$ . Add in order: 1  $\mu\text{L}$  of 10X restriction enzyme buffer, 1  $\mu\text{L}$  of 1  $\mu\text{g}/\text{mL}$  BSA, and 10–20 units of restriction enzyme (*see Note 5*).

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2. Combine 0.5  $\mu\text{g}$  of pUC19 with distilled water to 7  $\mu\text{L}$ , then add the buffer, BSA, and enzyme.
3. Incubate samples for 1 h at the recommended temperature for the enzyme. Add 2  $\mu\text{L}$  of 6X gel loading dye to each sample.

## 3.2. Agarose Gel Electrophoresis

1. Add 0.5 g of agarose to 50 mL of EtBr-TAE buffer in a 200 mL conical flask. Microwave 1 min to dissolve, cool for several min, and pour into  $2 \times 25$  mL gel molds. Use a 5-well comb (preparative gel) in one gel and a 10-well comb (analytical) in the other (see **Note 6**).
2. Place gels at 4°C for 20 min to solidify.
3. Place the gels into the electrophoresis tank with sufficient EtBr-TAE buffer to submerge them by 0.5 cm.
4. Load digested DNAs into the preparative gel.
5. Combine 0.5  $\mu\text{g}$  of DNA ladder, 1  $\mu\text{L}$  of 6X loading dye and distilled water to 6  $\mu\text{L}$ . Load onto gel.
6. Electrophorese at 80–100 V until the dye reaches the bottom of the gel or until the bands of interest are well separated from other bands on the gel.
7. Photograph the gel on a UV transilluminator. Caution: the UV illuminator can cause severe burns to exposed skin within 30 s. Wear a face mask and a laboratory coat.

## 3.3. Subcloning the cDNA

1. Excise the linearized pUC19 DNA and cDNA fragments from the gel using a razor blade and place slices into microfuge tubes.
2. Use the Qiagen gel extraction kit as directed to purify the DNAs.
3. Electrophorese 5  $\mu\text{L}$  of each sample and 0.5  $\mu\text{g}$  of DNA ladder on the analytical gel for 15 min to determine the DNA concentrations (see **Note 7**).
4. Combine the plasmid and cDNA, in a 1:3 molar ratio, into a microfuge tube and add distilled water to 8  $\mu\text{L}$ . Add the same volume of either plasmid or cDNA into separate tubes and bring to 8  $\mu\text{L}$  with distilled water. (see **Note 8**).
5. To each tube for ligation, add in order: 1  $\mu\text{L}$  ligase buffer and 1  $\mu\text{L}$  of ligase. Incubate for 1–2 h at RT or 16°C overnight (see **Note 9**).
6. Transform competent *E. coli* as recommended by the manufacturers.
7. After heat shocking the bacteria, add 10 competent cell-volumes of LB and incubate at 37°C for 1 h with gentle shaking (see **Note 10**).

## 3.4 Growth of Transformed Bacteria on LB-Agar Ampicillin Plates

1. Add 7.5 g bacto-agar per 500 mL of LB (LB-agar). Autoclave for 20 min, cool to 55°C in a water bath. Add 0.5 mL of ampicillin stock and pour 10–15 mL of LB-agar into sterile 100-mm polystyrene petri dishes. Leave covered plates to cool overnight at RT (see **Note 11**).
2. Spread 40  $\mu\text{L}$  of X-gal onto 6 plates and allow to dry. Spread 20  $\mu\text{L}$  of IPTG and allow to dry (see **Note 12**).
3. Spread 5, 15, and 80% of the *E. coli* transformed with plasmid + cDNA onto separate ampicillin plates. For plasmid-only and cDNA-only transformations, use amounts of bacteria equivalent to 15% of the plasmid + cDNA transformation. For the positive control, spread 10% of the pUC19-transformed *E. coli* (see **Note 13**).
4. Grow the bacteria overnight at 37°C or until colonies grow to 0.5–1 mm diameter.
5. Place plates at 4°C for 2 h (see **Note 14**).

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### 3.5. Purifying Plasmids from Mini- and Maxi-Prep Cultures

1. Pick 8 white colonies from one of the plasmid + cDNA plates with a sterile tooth pick and place into 3 mL of LB-ampicillin (*see Note 15*).
2. Grow mini-prep cultures at 37°C overnight in a shaking incubator.
3. Purify the plasmid DNA from the cultures using a Qiagen mini-prep kit following the manufacturers recommendations (*see Note 16*).
4. Digest 0.5–1 µg of each plasmid with the restriction enzyme from **Subheading 3.1.** and electrophorese on an analytical gel to ensure that colonies have the cDNA insert.
5. Choose one correct colony to inoculate 500 mL of LB-ampicillin in a 2 L flask, and grow at 37°C overnight in a shaking incubator.
6. Purify the plasmid DNA using a Qiagen Megakit and following the manufacturer's recommendations.
7. Sequence the 5' untranslated region and coding region of the cDNA in both directions. Retain this stock for all mutagenesis experiments (*see Note 17*).

### 3.6. Site-Directed Mutagenesis to Generate the P14L Mutation in PLP1

1. The design of two overlapping and complementary oligodeoxynucleotide primers that harbor mutations at the center of each primer is shown in **Fig. 1**.
2. Use the mutation primers with the QuikChange mutagenesis kit as recommended by the manufacturers to generate a mutated plasmid using the Megakit DNA as template.
3. Choose 8 colonies and grow overnight at 37°C in separate tubes containing 3 mL of LB-ampicillin.
4. Use Qiagen mini-prep kit to purify plasmid DNA from overnight cultures.
5. Digest 0.5–1 µg of each mini-prep DNA with 10 U of *Apal* and electrophorese on agarose gel (*see Note 18*).
6. Sequence the 5' untranslated and coding regions of two plasmids in both directions to ensure that the PCR did not introduce random point mutations (*see Note 17*).

### 3.7. Subclone the cDNA into an Expression Cassette

1. Subclone the mutagenized, sequenced cDNA into the polycloning site of the expression vector of choice (*see Subheadings 3.1.–3.5.*, and **Note 19**).

### 3.8. Maintaining COS-7 Cells in Culture (*see Note 20*)

1. Grow a flask of COS-7 cells to 80–100% confluence. These cells are easily maintained in T75 flasks at 37°C in 95% air, 5% CO<sub>2</sub>. Replace culture medium every 3 d.
2. To passage the cells, remove the medium by suction using a Pasteur pipet and rinse the cells for a few s with PBS. Rinse the walls of the flask to remove traces of medium.
3. Add 5 mL of trypsin/EDTA to the cells and remove all but 0.5 mL by suction. Incubate for 3–5 min at 37°C (*see Note 21*).
4. Suspend the cells in 10 mL of culture medium (*see Note 22*).
5. Transfer 1 mL of cells to a fresh T75 flask containing 14 mL of fresh medium.
6. Grow the cells to confluence 37°C (*see Note 23*).

### 3.9. Transfection of COS-7 Cells

1. D 1 (afternoon). Begin with a T75 culture flask of COS-7 cells grown to confluence. Split the cells equally into 60-mm culture dishes. (*see Note 24*).
2. D 2 (morning). For each transfection, add 90 µL of DMEM (i.e., no serum) to a sterile microfuge tube. Add 6 µL of FUGENE 6 incubate for 5 min at RT (*see Note 25*).

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3. Add the DMEM/FUGENE 6 solution, dropwise, to a sterile microfuge tube containing 4  $\mu\text{g}$  of plasmid DNA. Tap the bottom of the tube gently and incubate for 15 min at RT.
4. Pipet the DNA solution, dropwise, across the COS-7 cells in the 60-mm dishes and culture overnight at 37°C. (*see Note 26*).
5. Day 3 (morning). Add 1.5 mL of culture medium to 35 mm NUNC culture dishes (*see Note 27*).
6. Wash the cells briefly with PBS, trypsinize for 5 min, and suspend in up to 4 mL of culture medium (*see Note 28*).
7. Add 0.5 mL of cell suspension to each 35 mm dish and culture the cells overnight at 37°C (*see Note 29*).

### 3.10. Fixation and Immunocytochemistry

1. Rinse cells twice for several s, by swirling, with 1 mL of DMEM. Add 1 mL of fixative to each dish and incubate for 30 min at RT (*see Note 30*).
2. Wash out excess fixative with 2 changes of TBS over 5 min.
3. Permeabilize cells for 30 min in 1 mL of 0.1% saponin in TBS. (*see Note 31*).
4. Suction away the buffer and clear the cells in a 2–3 mm track around the perimeter of each dish. (*see Note 32*).
5. Apply a PAP pen to the cleaned surface and quickly pipet 50  $\mu\text{L}$  of a 0.1% saponin/TBSGBA block solution onto the cells and incubate for 15–30 min at RT (*see Note 33*).
6. Exchange the 0.1% saponin/block solution with 50  $\mu\text{L}$  of primary antibody diluted in block solution and incubate overnight in a humidified chamber at RT.
7. Wash the cells 2  $\times$  5 min with 1 mL of 0.1% saponin/TBS. Add 50  $\mu\text{L}$  of secondary antibody diluted in 0.1% saponin/block solution for 1 h at RT. (*see Note 34*).
8. Wash the cells 2  $\times$  5 min with 1 mL of 0.1% saponin/TBS. Remove buffer with suction and add 100  $\mu\text{L}$  of DTG with a wide mouth tip.
9. Coverslip and stand for 30 min. Remove excess DTG and seal the coverslips with clear nail polish (*see Note 35*).
10. Cut away the wall of each NUNC dish using the soldering iron in a fume hood (*see Note 36*).
11. Mount each dish bottom onto a glass slide with drops of superglue at two points on the outer edge (*see Note 37*).
12. View cells using epifluorescence or confocal microscopes (*see Note 38*).

### 4. Notes

1.  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  reduce the effectiveness of cell detachment from culture dishes and cause detached cells to clump.
2. NUNC dishes have thin walls and low intrinsic fluorescence, which are important advantages for the immunocytochemistry protocol described herein. If necessary, coating these dishes with poly-[scl]-lysine or poly-[scl]-arginine (Sigma) will improve the adhesion of transfected cells to the dish but will increase the background staining somewhat. To coat dishes, dissolve either amino acid at 5  $\mu\text{g}/\text{mL}$  in water and filter sterilize (0.45- $\mu\text{m}$  filter). This solution is stable at 4°C for 1 yr. Dilute this stock 100-fold in PBS (50  $\mu\text{g}/\text{mL}$ , stable at 4°C for 1 yr) and add 1 mL to each culture dish. After 3 h, rinse each dish with 3  $\times$  2 mL of PBS. Coated dishes can be stored sterile for 1 mo at 4°C.
3. Place several wet paper towels into a 150 mm culture dish with a lid. Each chamber accommodates up to 6  $\times$  35 mm dishes and will remain moist overnight at RT.
4. To maximize fluorescence signals from labeled cells, the pH of this solution is very important. DABCO is alkaline in solution, so the DTG should be approx pH 8.6 after

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the addition of Tris. The pH can be easily measured after diluting the DTG 10-fold in distilled water.

5. Enzyme volume should be no more than 10% of the total.
6. Use protective gloves when handling the hot agarose solution, which can superheat and boil over, causing serious burns.
7. The known concentration of the ladder is used to estimate the concentrations of the samples by comparing band intensities under UV illumination.
8. Use 20–50 ng of plasmid DNA for the ligation. Increasing the molar ratio to more than 1:5 may cause 2 or more copies of the cDNA to be ligated into the pUC19 vector.
9. The 16°C incubation slows the enzyme activity and, theoretically, increases the ligation efficiency. However, for routine cloning the shorter ligation times yield sufficient numbers of bacterial colonies.
10. Do not use LB-ampicillin in this step because the bacteria need time to transcribe and translate the  $\beta$ -lactamase gene on the introduced plasmid. The 1 h incubation period can be eliminated to reduce processing time, which will reduce the number of colonies on the plates by two- or three-fold.
11. Ampicillin is heat labile and it is important to ensure the correct temperature of the LB-agar, which takes 1–2 h to cool to 55°C with occasional swirling of the bottle. After adding the ampicillin, mix thoroughly by swirling. When pouring the agar plates, large and small bubbles will collect on the liquid surface. Burst the bubbles by flaming the plates with a blue-yellow Bunsen flame for a few s. 500 mL of LB-agar makes up to 40 plates and can be stored for up to 1 mo at 4°C. Discard plates if fungal contamination is present.
12. To make a spreader, use a Bunsen burner to make a right-angle bend in the tapered end of a 9-in. Pasteur pipet. Sterilize with ethanol and briefly flame.
13. Spreading more than 100  $\mu$ L of bacteria onto a plate should be avoided. Reduce the volume by microfuging the bacterial suspension for 20 s, withdrawing excess supernate to 100  $\mu$ L and resuspending the soft pellet with a pipet.
14. The 4°C incubation increases the intensity of blue colonies. If the concentration of ampicillin in the plates is low, or if the transformed bacteria grow vigorously, true transformants will be ringed by small satellite colonies. Avoid picking the satellite colonies, which do not harbor plasmid DNA and will not grow in LB-ampicillin medium. In general, the longer the time of growth on the plates, the greater the number of satellite colonies. Thus, avoid growing transformants for longer times than is necessary to obtain 0.5–1 mm diameter colonies.
15. Colonies should be large, round, smooth and well-isolated.
16. The DNA concentration will be approx 0.1–0.2  $\mu$ g/ $\mu$ L.
17. Although small, there is a finite risk of introducing point mutations into the cDNA during cloning and culturing the bacteria. Such mutations could confound all future work with the plasmid. Thus, the cDNA should be sequenced to ensure that random mutations are absent.
18. Include the PCR template DNA as a positive control. Correctly mutagenized plasmids will harbor one less *Apa* I site than the parent plasmid.
19. To achieve very high expression levels, pCMV5 is an ideal vector because it harbors an SV-40 origin of replication which the SV-40 T antigen expressed by COS-7 cells uses to replicate the plasmid to high copy number.
20. Use 70% ethanol to sterilize the surfaces of the culture hood, benches, Gilson pipets and outer surfaces of the culture-medium bottle. Allow the ethanol to evaporate.
21. Strike the side of the flask several times, which dislodges the cells when trypsinizing is complete.

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22. Gently pipet cells up-and-down 5–10 times with a 10 mL pipet to break up cell clumps.
23. Growth time is generally 3–4 d if the cells are healthy. Slow growing cells may be due to a viral infection (e.g., mycoplasma), which will make the cells more difficult to transfect and will yield cells with poor morphology under the microscope. Discard the cultures and obtain a fresh flask of cells.
24. Cell number will approx double by d 2.  $1 \times T$  75 flask at 100% confluence on d 1 yields approx  $8 \times 60$  mm dishes at 50% confluence on d 2. Transfections also can be performed directly in individual 35-mm dishes rather than using a 60-mm dish intermediate. A disadvantage of this approach is that replicate dishes may have greater variability due to differences in transfection efficiencies. However, an advantage is that transfection times can be reduced to 15 h before fixation and staining which will yield higher apparent transfection efficiencies if the mutant protein being expressed is particularly toxic to the cells. We have used this approach to examine trafficking of a *PLP1* splice site mutation (**19**) that results in skipping exon 6.
25. The FUGENE 6 must be added directly into the DMEM, not down the wall of the tube.
26. Gently move the dishes several times from side-to-side to evenly distribute the DNA on the cells. Be careful not to move dishes in a circular motion, which will concentrate reagents in the centers of the dishes.
27. The number of dishes prepared depends on the number of DNAs for transfection and the number of replicate dishes for each DNA. Each 60 mm dish will provide sufficient cells for up to  $8 \times 35$  mm dishes.
28. The levels of confluence in each 60 mm dish should approximate 100% if the transfected plasmid is not toxic to the cells. However, proportionately less medium should be used for subconfluent dishes (e.g., use 2 mL of medium for cells at 50% confluence).
29. Gently move the dishes from side-to-side to evenly distribute the cells. The desired level of confluence when the cells adhere is 20–30%. After overnight culturing, a confluence of 40–50% is expected, which provides sufficient transfected cells to examine and plenty of room for the cells to flatten out for good morphology.
30. Keep 35 mm dishes on a rocking platform (e.g., Bellco, NJ, USA) during staining. Warm DMEM and fixative minimize changes to cell morphology before the cells are fixed. Add solutions to the edge of each dish to minimize cell damage. Use a low vacuum to hasten solution changes.
31. Detergents such as Triton X-100 and other permeabilizing agents such as methanol can extract some proteins, such as PLP1, from the membranes of fixed cells, which reduces the staining intensity.
32. Attach a wide-mouth P200 or P1000 Gilson pipette tip to a vacuum line. It is most important that all cells and liquid be removed in preparation for applying the PAP pen. These pens are very expensive and are easily damaged by contact with debris and aqueous solutions.
33. This and subsequent steps use small volumes of antibody-containing solutions to label antigens in transfected cells spread over a wide area. Therefore, it is important to ensure that the culture dishes are level. If antibody solutions are in plentiful supply, larger volumes, e.g., 100 or 200  $\mu$ L can be used.
34. In this step, the DNA-binding fluor, 1  $\mu$ g/mL 4,6, Diamidino-2-phenylindole (DAPI, Sigma, MO, USA), can be included for visualizing the nuclei. This compound strongly labels nuclei and aids the location of cells that are unlabeled or weakly labeled by antibodies. Some antigens are expressed at sufficiently low levels to require signal amplification using a biotinylated secondary antibody and a streptavidin-conjugated fluorophore. Simply repeat this step with the necessary tertiary reagents.
35. Colored nail polish contains compounds that quench the fluorescence and can bleed into the DTG with time.

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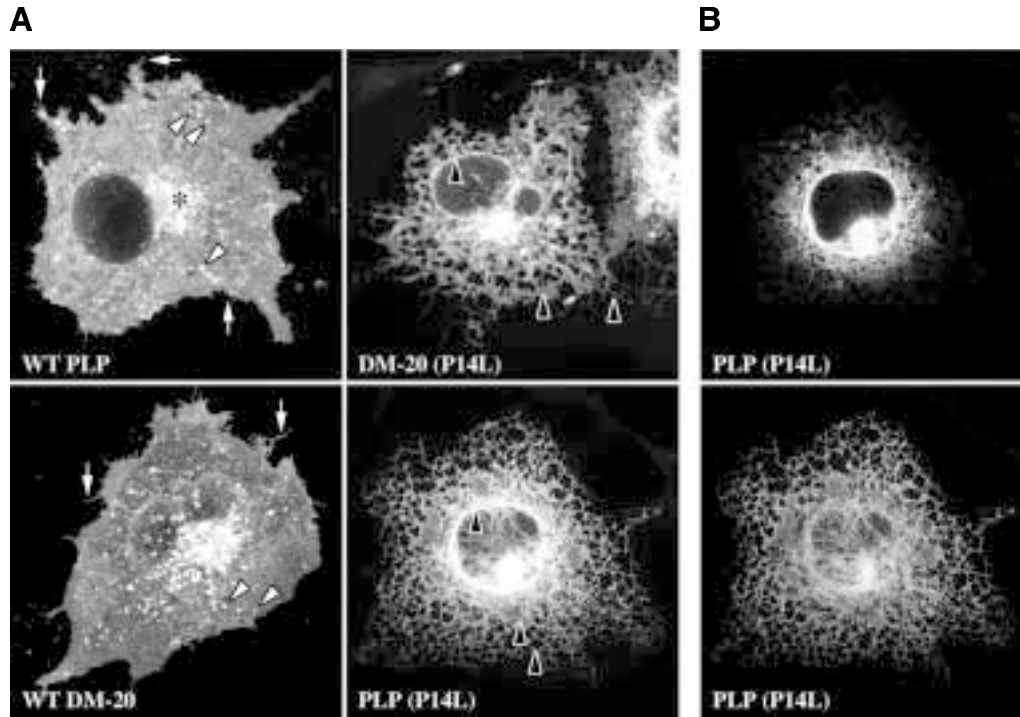


Fig. 2. Intracellular trafficking defect for PLP1 and DM-20 harboring the P14L mutation identified in a patient with severe PMD. (A) Extended-focus confocal series showing wild type PLP1 expressed in a transfected COS-7 cell. The protein is present in the Golgi (asterisk), on the cell surface (white arrows) and in lysosomes (white arrowheads). DM-20 expression in COS-7 cells is indistinguishable from PLP1. In contrast, mutant DM-20 (P14L) accumulates in the endoplasmic reticulum (black arrowheads) and does not reach the cell surface or lysosomes. The trafficking of PLP1 (P14L) is similar to that of DM-20. (B) Single optical slices through the middle of the nucleus (upper panel) and near the bottom of the nucleus (lower panel) from the extended focus series used in (A). These slices illustrate the intracellular detail gleaned from confocal microscopy.

36. This process is optional, but only takes approx 1 min per dish and increases the viewable field under the microscope by several-fold.
37. Dishes are stored at RT or 4°C for several wk without loss of signal. Strong, fluorescence signals remain visible for 6–12 mo.
38. Regular epifluorescence or confocal microscopes equipped with 20× or 40× lenses will usually provide sufficient resolution to document cell morphology and the general subcellular localization of heterologous proteins in transfected cells. However, 63× or 100× lenses may be necessary for high resolution imaging of organelles such as the endoplasmic reticulum and Golgi stacks. The shape of a COS-7 cell usually resembles that of a fried egg and many organelles of interest are located toward the bottom of the nucleus in the thick portion of the cell. Thus, confocal microscopy yields superior images to epifluorescence microscopy because optical sections obtained at the bottom of the cell are not contaminated with stray fluorescence from overlying structures (*see Fig. 2*).

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## Myelin PLP1 Mutations

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