

# Improved Protocols For Preparing Lipid-Linked And Related Saccharides For Fluorophore-Assisted Carbohydrate Electrophoresis (FACE) Analysis

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## **A. Cell Harvest As A Methanolic Sonicate**

The goal is to preserve cellular saccharides as much as possible by rapid disruption with methanol.

1. Transfer a bottle of PBS from the refrigerator to ice.
2. Add ice (half full) to a suitable large container with enough surface area to lay out 2 or 4 plates at a time. Level the ice surface as flat as possible. (*We recommend adding cold water so that it just reaches the surface of the ice, to aid leveling and contact with dishes*).
3. Transfer plates from incubator onto the ice surface.
4. Using suction, remove media from the plates while still on ice.
5. For a 15 cm plate, add 10-15 ml ice cold PBS, swirl briefly, and then suction off as much PBS as practical. Repeat once.
6. For a 15 cm plate, add 8 ml of room temperature methanol. Then, transfer the plates from ice to a room temp surface.
7. Vigorously scrape cells from the surface of the plate into the methanol using a disposable plastic scraper (we use the Corning "Cell Lifter", #3008). *Once you have finished scraping, the saccharides are preserved and you can stop the procedure at any convenient point later on.*
8. Transfer the methanolic cell suspension to a 16x100 conical glass tube labeled "P" using a plastic disposable pipet.
9. Add an additional 4 ml of methanol to the plate and scrape any remaining debris, then transfer to tube P as in step (8).
10. Cap and sonicate tube P until cells are thoroughly disrupted and dispersed into a suspension. We use a bath sonicator that can hold many tubes at once, but probe sonicators can also be used. This methanolic cell suspension (designated #1) in tube P can now be used in the next extraction process to generate many different saccharide fractions (Figure 1), shipped at room temperature, or stored at -20 °C for extended periods for later use.

## **B. Extractions Of Saccharides From Methanolic Suspensions**

This greatly improved process is designed to remove the methanol from the initial methanolic suspension (CH<sub>3</sub>OH Suspension #1) and retaining all methanol solutes, but without drying of the methanol-insoluble material (Figure 2). Over-drying this material is problematic, and gives a tough insoluble pellet that resists later extraction. This process also yields various saccharide fractions in a single simple step, replacing earlier procedures involving multiple sequential extractions.

1. Label four 16x100 glass round-bottom tubes (VWR #47729-576): M (methanolic supernatant), U (upper/aqueous layer), L (lower/organic layer), & LLO (lipid-linked oligosaccharides). If not yet done (section A), label a 15 ml conical tube (Kimble # 73790-15, ordered with plastic caps #73837-1) containing your harvested methanolic suspension P (pellet). *Note: quality of glass conical tubes varies from lot to lot. Although rated for certain centrifugal forces, flawed tubes will crack in the centrifuge. To prevent loss of sample, we routinely pre-spin empty tubes 1 hr approximately 1850 x g in a swinging bucket rotor (with rubber bases inside each bucket) to ensure their durability.*
2. Alternatively sonicate and vortex the methanolic cell suspension in conical tube P until completely suspended (suspension #1).
3. Centrifuge the suspension in conical tube P with a plastic cap at ~1500 x g in a swinging-bucket rotor for 20 min at approximately room temperature. In our lab, we centrifuge at 2500 RPM in a Beckman Allegra X-15R centrifuge with an SX4750A rotor set at 25 °C. The rubber base inside each bucket is important to prevent damage to the glass tubes. Unless stated otherwise, all other 1500 x g steps are done this way.
4. Decant supernatant in conical tube P to round bottom tube M. Remove as much supernatant as possible, but pellet should remain moist.
5. Set aside pellet in conical tube P for later use in step 8. Freeze if desired.
6. Dry supernatant in round bottom tube M in a speed vac (2 to 3 hours on medium heat).
7. Add 1.8 ml water to round bottom tube M, sonicate /vortex, and spin for 2 min at 1500 x g to pull all liquid off the wall of tube.
8. Transfer the water supernatant to conical tube P.
9. To round bottom tube M add 3 ml methanol, then sonicate /vortex (suspension #2). *Do not spin.*
10. Transfer the entire methanolic suspension to conical tube P, sonicate /vortex. Discard round bottom tube M.
11. Add 6 ml chloroform to conical tube P, affix a plastic cap, vortex, spin for 60 min (not 20 min) at 1500 x g. This should result in 2 well-formed liquid layers (upper and lower) and a semi-solid interface.
12. With a pipet, transfer upper layer to round bottom tube U and the lower layer to round bottom tube L, leaving the interface behind in tube P.
13. To tube P, add 5 ml chloroform:methanol:water (CMW; 10:10:3). Affix a plastic cap (with pinholes to release pressure of volatilized chloroform during sonication), then alternatively sonicate and vortex to solubilize LLOs. Spin 20 min, 1500 x g. Transfer supernatant to a round-bottomed tube labeled "LLO". Repeat extraction 1 more time and pool the supernatants. The residual pellet in tube P contains glycoproteins. *If it is necessary to dry this pellet for further analysis, excessive hardening can be avoided by suspending the pellet (while still moist) with 0.5 methanol, sonication, addition of 3 ml chloroform, more sonication, and then drying with a "speed vac" or "N-evap" as described below. The residual material will be powder-like and amenable to further analysis.*
14. Dry round bottom tubes U, L, & LLO with a centrifugal evaporation system ("speed vac") on medium heat. We centrifuge in a Thermo SC210A (modified with a corrosion-resistant Thermo SUMAX400 upper magnet assembly) with a -105 °C trap (Thermo RVT4101) and a diaphragm-

type vacuum pump with corrosion-resistant components (Vacuubrand PC201 NT). When dry, place all samples at -20 °C (along with conical tube P w/ glycoprotein pellet) for later analysis. *For LLO preps needing additional purification with DEAE-cellulose, do not dry. The liquid will be applied directly to the DEAE-cellulose column.*

### **C. Weak Acid Hydrolysis Of Dolichol-linked Saccharides**

1. Dissolve the LLO residue in 0.1N HCl/50% isopropanol with vortexing. Heat at 50 °C, 1 hr, capped loosely with a marble.
2. Dry in speed vac, or with mild heat under a stream of nitrogen gas (such as with a “N-evap”).
3. Add 1 ml n-butanol-saturated water and vortex. *A well-equilibrated mixture of n-butanol and water can be used as a source of each solvent saturated with the other.*
4. Add 1 ml water-saturated n-butanol and vortex. *Be sure that the two layers intermix and do not merely vortex on top of each other. This can be helped by hold the tube at a slight angle while vortexing.*
5. Centrifuge 1500 x g for 20 min at room temp to reform two layers.
6. Collect bottom layer (aqueous), transfer to a 1.5 ml microcentrifuge tube, and dry in a speed vac.
7. Dissolve the dried sample in 1 ml water.
8. Add 100 µl bed volume of AG50W-X8 (hydrogen form, *X8 beads are used for batch binding in tubes*). Shake at room temperature for ~10 min.
9. Pellet the beads in a microcentrifuge at top speed for ~30 seconds. Collect the supernatant (rinse the beads once with water if desired), then add 100 µl bed volume of AG1-X8 (formate form). Shake at room temperature for ~10 min.
10. Pellet the beads in a microcentrifuge at top speed for ~30 seconds. Collect the supernatant. To ensure removal of all beads, repeat the microcentrifugation. Collect the supernatant taking care not to disturb any pellet, and begin drying in a 1.5 ml microcentrifuge tube. If the sample is expected to be less than 200 pmol, then during the drying when the liquid volume drops to 0.5 ml or so, transfer the liquid to a 0.5 ml tube. *If the desalting step is performed properly, no more than a very small transparent pellet should be visible after drying (approximately the size of a 0.5 µl droplet with samples less than 200 pmol, or a 2 µl droplet with 1 nmol samples). An unusually large or opaque pellet indicates poor desalting which could be due to poor mixing, insufficient desalting time, or insufficient resin, in which case steps 7-10 should be repeated.*
11. Store the fully dried sample at -20 °C, to be used for later labeling with ANDS or AMAC.

#### **D. Ion Exchange Separation Of Neutral and Anionic Monosaccharides**

1. Pack AG1-X2 (formate form; bed volume 0.5 ml; *X2 beads are used for columns*) in a BioRad Poly-Prep disposable column with a 10-ml upper reservoir. To help direct flow into collection tubes, attach to the outlet of the column a long gel loading tip (of the type used for manual DNA sequencing gels) with the top trimmed to permit secure attachment to the Poly-Prep column.
2. Wash the column with 20 ml 1 M formic acid, then 20 ml deionized water.
3. If standards need to be made to validate the separation, prepare 1 ml of a sugar mixture (1  $\mu$ M = nmol/ml each sugar) containing GalNAc, M1P, M6P, G1,6-bisP, and UDP-GlcNAc. *For each 20 ml step following, collect as two 10 ml portions in 16x100 round bottom tubes.*
4. Load 1 ml of the experimental sample, or the sugar mixture. Wash with 20 ml water and collect the flow-through (*free neutral sugars and free neutral glycans*). Dry in a speed vac.
5. Elute the column with formic acid solution (sugar monophosphates). For the initial experiment with a new bottle of AG1-X2, elute the column with 20 ml each of 0.5 M, 1.0M, 2.0 M and 4.0 M formic acid, sequentially. If the column is working properly, elution of sugar monophosphates should be complete with 0.5 M formic acid. Dry in a speed vac.
6. Elute the columns with 20 ml 0.5 M pyridine acetate, pH 5.0 (*bisphosphate sugars and nucleotide-sugars*). *Store the reagent in dark bottles at room temperature.* If desired, 1.0 M pyridine acetate can be used instead to ensure rigorous elution, but this will extend drying time. Dry in a speed vac.
7. 1-phospho linkages are labile in the prior formic acid solvents. However, to ensure complete removal of 1-phospho linkages in preparation for AMAC labeling, dissolve all the dried samples in 1 ml 0.1 N HCl. Transfer to a 1 ml plastic tube, and heat at 100 °C (a pre-warmed electric hot-block works well) for 15 min. Dry in a speed vac.
8. Label monosaccharides with AMAC, or free glycans with ANDS, as described below.

### **E. DEAE-Cellulose Fractionation of Dolichol-linked Saccharides**

DEAE fractionation of lipids is done to remove glycogen contamination from large LLO preps (large numbers of cells, or whole tissues), and to separate monophosphate lipids from pyrophosphate lipids (Figure 3). Contaminating glycogen fragments will consume fluorophore, and appear on FACE gels. For LLOs isolated from small amounts of material, this step is optional.

1. Prepare Poly-Prep columns with ~0.5 - 1 ml bed volume of DEAE-cellulose equilibrated in CMW (10:10:3). First, firmly attach a 200- $\mu$ l flat-end tip (of the type used for loading DNA sequencing gels), trimmed at top to fit the poly-prep column snugly, to the outlet to slow the flow rate. Add dry DEAE-cellulose (see below) to the column, swell with 10 ml methanol, then equilibrate with 10 ml CMW. For these and subsequent steps, it is suitable to allow the solvent to run under gravity flow and expose the surface of the DEAE-cellulose. *The DEAE-cellulose is converted to the acetate form ahead of time by these steps: (i) soak 200 g DEAE-cellulose in 1 liter 0.5 N HCl for 1 hr. (ii) wash thoroughly with water on a Buchner funnel until neutral. (iii) soak in 1 liter 0.5 N NaOH for 1 hr. (iv) Repeat step ii. (v) Soak in 1 liter 1 N acetic acid for 1 hr. (vi) Repeat step ii. (vii) Wash the DEAE-cellulose with methanol, allow to air-dry, and store dry at room temperature.*
2. Typically, a CMW extract containing dolichol-linked saccharides is loaded.
3. Wash with 10 ml. CMW. *The following steps require addition of a 1 M ammonium acetate stock to CMW to reach the desired concentration. The 1 M stock is prepared by dissolving ammonium acetate in methanol, supplemented with glacial acetic acid to a concentration of 1.5% (v/v). This stock can be added directly to CMW for the desired concentration of ammonium acetate, and changes in the final ratios of chloroform/methanol/water can be ignored.*
4. Elute with 10 ml. CMW/3 mM  $\text{NH}_4\text{OAc}$ . Normally, this fraction is discarded. *However, if the column was loaded with a chloroform-methanol (CM; (2:1) extract enriched in mannose-P-dolichol (MPD) and glucose-P-dolichol (GPD), they will be recovered in this fraction and should be collected in a 10 ml round bottom glass tube.*
5. Elute with 20 ml. CMW/300 mM  $\text{NH}_4\text{OAc}$  into two round bottom tubes (10 ml each). Most of the lipid will be in the first 10 ml. When starting with a CMW extract, this fraction contains mature LLOs plus LLO intermediates with three or more monosaccharides. *When starting with a CM extract, this fraction contains LLO intermediates with one or two monosaccharides.*

### **F. Clean-up of DEAE-Cellulose Eluted Samples**

1. Dry in a speed vac. *N-evap is not recommended because of the difficulty of drying with the high concentration of salt. As indicated in Figure 3, simply redissolve and reevaporate the sample once each with methanol and then CMW.*
2. Hydrolyze with 0.1 N HCl, 50 °C for 1hr as described in section C.

### **G. Amylase Digestion To Remove Glycogen Fragments**

Can be used in place of, or in addition to, DEAE cellulose to eliminate trace glycogen fragments from LLO glycans. Start with dried LLO glycans obtained by acid hydrolysis of LLOs.

1. Dissolve glycans in 20  $\mu$ l 0.1 M NaOAc (pH 5.0).
2. Add 2 U (2  $\mu$ l) amylase (Sigma-Aldrich # A6211). Incubate 30 min, 37 °C.
3. Add 1 ml water and 200  $\mu$ l AG50W-X8 (hydrogen form). Mix with shaking for 10 min.
4. Spin, collect supernatant, add 200  $\mu$ l AG1-X8 (formate form). Mix with shaking for 10 min.
5. Spin, collect supernatant, dry, and proceed with fluorophore labeling.

## **H. Fluorophore Labeling Of Saccharides**

1. If AMAC-labeled standards are needed, prepare a sugar standard “working solution” (1 mM) from a stock solution (for example, 100 mM each of glucose, glc-1-P, mannose, man-1-P).
2. Ahead of time, prepare these reagents:

**2-aminoacridone (AMAC) labeling reagent, 0.1M:** Dissolve an entire dry aliquot (25 mg) of AMAC (MW 210.23; Fluka) in 950  $\mu$ l DMSO, then add 50  $\mu$ l 1N HCl. Aliquot in 100  $\mu$ l portions, store at -80 °C

**7-amino-1,3-naphthalenedisulfonic acid (ANDS) labeling reagent, 0.15 M:** Weigh out ANDS (MW 303.31, Aldrich) and dissolve in 15% acetic acid. Aliquot in 100  $\mu$ l portions, store at -80 °C.

**1M NaBCNH<sub>3</sub>:** Dissolve 63 mg NaBCNH<sub>3</sub> in 1 ml DMSO, aliquot to 100  $\mu$ l tubes, store at -80 °C.

3. Transfer 25  $\mu$ l of the experimental sample, or standard working solution (25 nmol of each sugar standard), to a 1 ml microcentrifuge tube. Dry in a speed vac.
4. Add 5  $\mu$ l 0.1 M AMAC (for monosaccharides) or 0.15 M ANDS (for oligosaccharides) and vortex to dissolve the dried saccharides to be labeled. *If the sample is expected to be less than 200 pmol, then use 1  $\mu$ l of AMAC or ANDS reagent, see step C10.*
5. Add 5  $\mu$ l 1M NaBCNH<sub>3</sub> to the tube, vortex, and spin briefly to get all contents to the bottom of the tube. *If the sample is expected to be less than 200 pmol, then use 1  $\mu$ l of NaBCNH<sub>3</sub> reagent.*
6. Incubate at 37 °C for ~18 hrs (typically overnight).
7. Dry in a speed vac (medium heat, with speed vac in dark or covered with foil).
8. Dissolve experimental samples in a small volume of 100% DMSO as desired. For sugar standards, dissolve in 50  $\mu$ l of 100% DMSO to make 500 pmol/ $\mu$ l AMAC-labeled sugar standard stock solution. Store at -80 °C.

## **I. Mixing Samples With FACE Gel Loading Buffer**

Labeled samples are prepared in 1x loading buffer. Actual volumes depend upon the amount of sample available and the sensitivity needed. Two examples are given here:

### Maximum sensitivity (for low abundance samples)

Dry sample, then dissolve in 2  $\mu$ l 1x loading buffer (0.005% Thorin I in 20% glycerol). Load entire sample onto gel.

### Minimum sensitivity (for standards)

To 10  $\mu$ l labeled standard in DMSO (500 pmol/ $\mu$ l), add 40  $\mu$ l water and 50  $\mu$ l 2x loading buffer (0.01% Thorin I in 40% glycerol). Final standard concentration is 50 pmol/ $\mu$ l. Load 1-2  $\mu$ l.

## **J. Gel Buffers**

Gels are prepared and run as outlined in the Methods In Enzymology (2006) 415:3-210. We emphasize that concentrations and pH of resolving and stacking gel buffers must be accurate, and suggest these minor modifications:

1. All 4X resolving and stacking gel buffers should be filtered through a 0.22 micron membrane.
2. The borate-containing 4X resolving and stacking buffers used for monosaccharide gels may form precipitates during storage at room temperature or 4 °C. For long term storage, aliquots can be stored at -20 °C. Thawed aliquots are stable at room temperature for up to 4 weeks. Discard if white precipitates are visible.

## **K. Imagers**

Before purchasing or upgrading a fluorescence imager, we strongly recommend contacting the manufacturer to verify that it will work for these excitation and emission wavelengths of AMAC (420 ex., 538 em.) and ANDS (310 ex., 450 em.). *Not all fluorescence "gel doc" systems can meet these specifications.* For optimal sensitivity, we also recommend that the imager be equipped with a CCD camera cooled to at least -20 °C. Currently, we are using a UVP ChemiDoc-Ilt2 imager with a #510 camera, with filter sets recommended by the supplier. Data analysis can be done with combinations of functions in the imager's own software and in ImageJ.

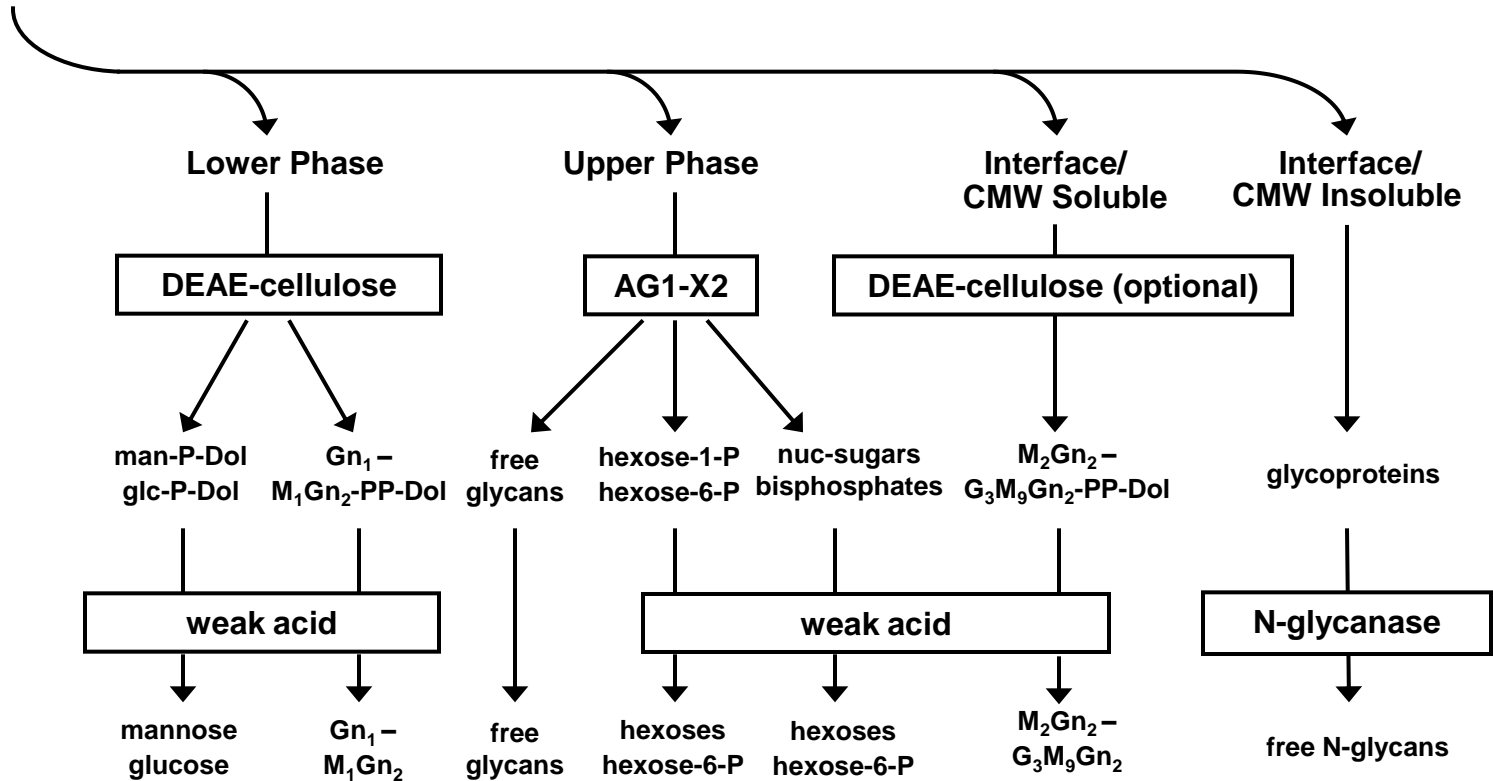
## **L. Figure Legends**

*Figure 1. Overview of FACE Analysis Of The Dolichol Pathway and Related Saccharides.* Details for the different fractions and their preparation are given in the protocol.

*Figure 2. Flowchart of LLO extraction from methanolic supernatants.* Initial samples are always prepared as methanolic suspensions. However, it is important to avoid the seemingly obvious step of simply drying the entire suspension, because this often results in a dry, tough, residual material that is refractory to later extraction. The key features of this protocol are that the initial methanol-insoluble material is not dried ("moist pellet"), and that a three-phase separation is used with an upper phase, lower phase, and interfacial layer. The tubes labeled M (methanol suspension), P (pellet), U (upper phase), and L (lower phase) are explained in detail in the protocol. The initial methanol supernatant in tube M is not discarded because it contains significant amounts of LLOs and other saccharides. The flowchart shows how these are recovered, and added back to the methanol-insoluble material in tube P.

*Figure 3. Work-up of lipid-linked oligosaccharides.* This flowchart shows the conversion of LLOs to LLO glycans suitable for labeling with fluorophore. It also presents the option of DEAE-cellulose fractionation for removing traces of sugar-P-dolichols and glycogen fragments (sections E and F).

# Methanolic Homogenate / Three-Phase Separation



AMAC labeling/  
monosaccharide gel:

+

+

+

+

ANDS labeling/  
oligosaccharide gel:

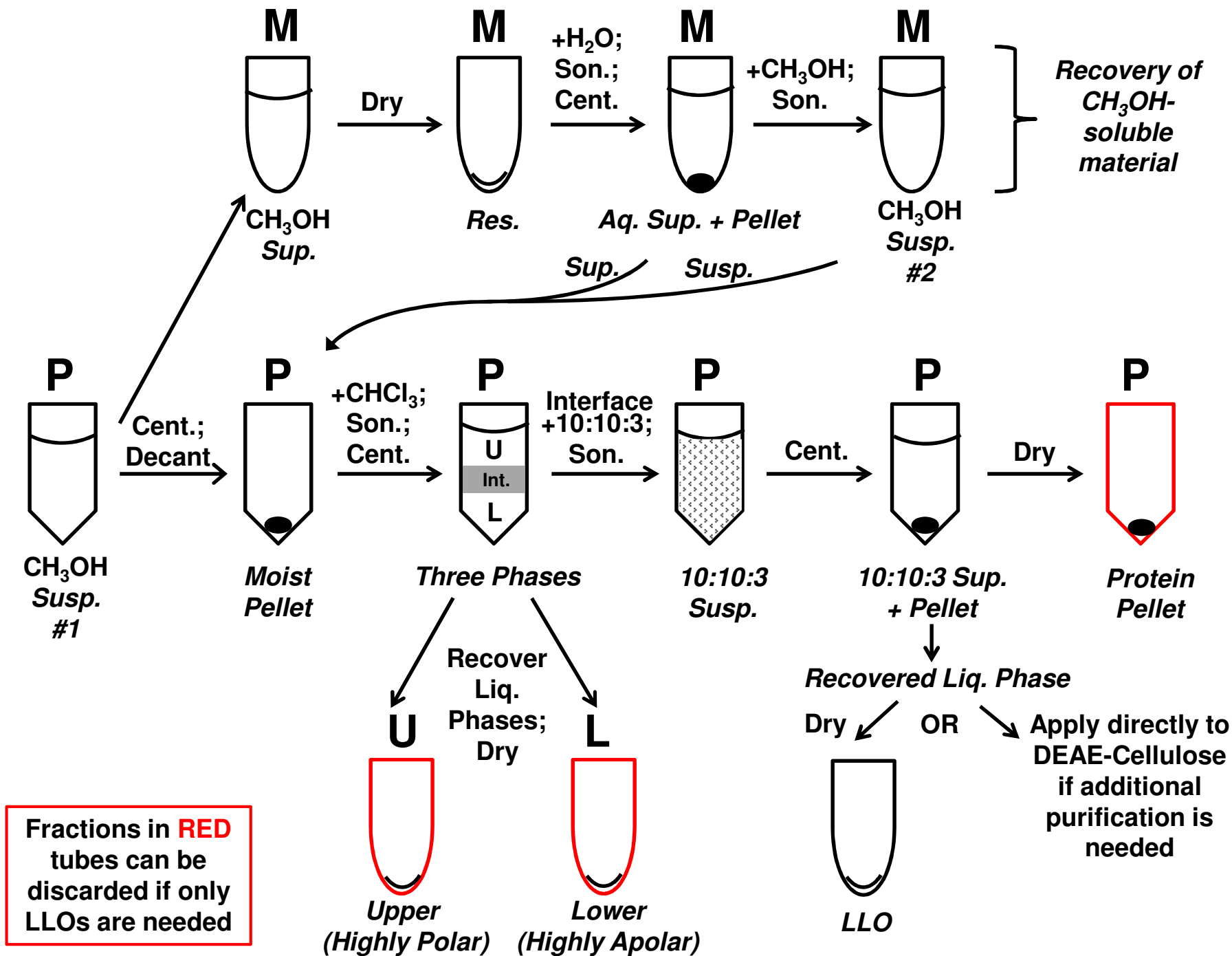
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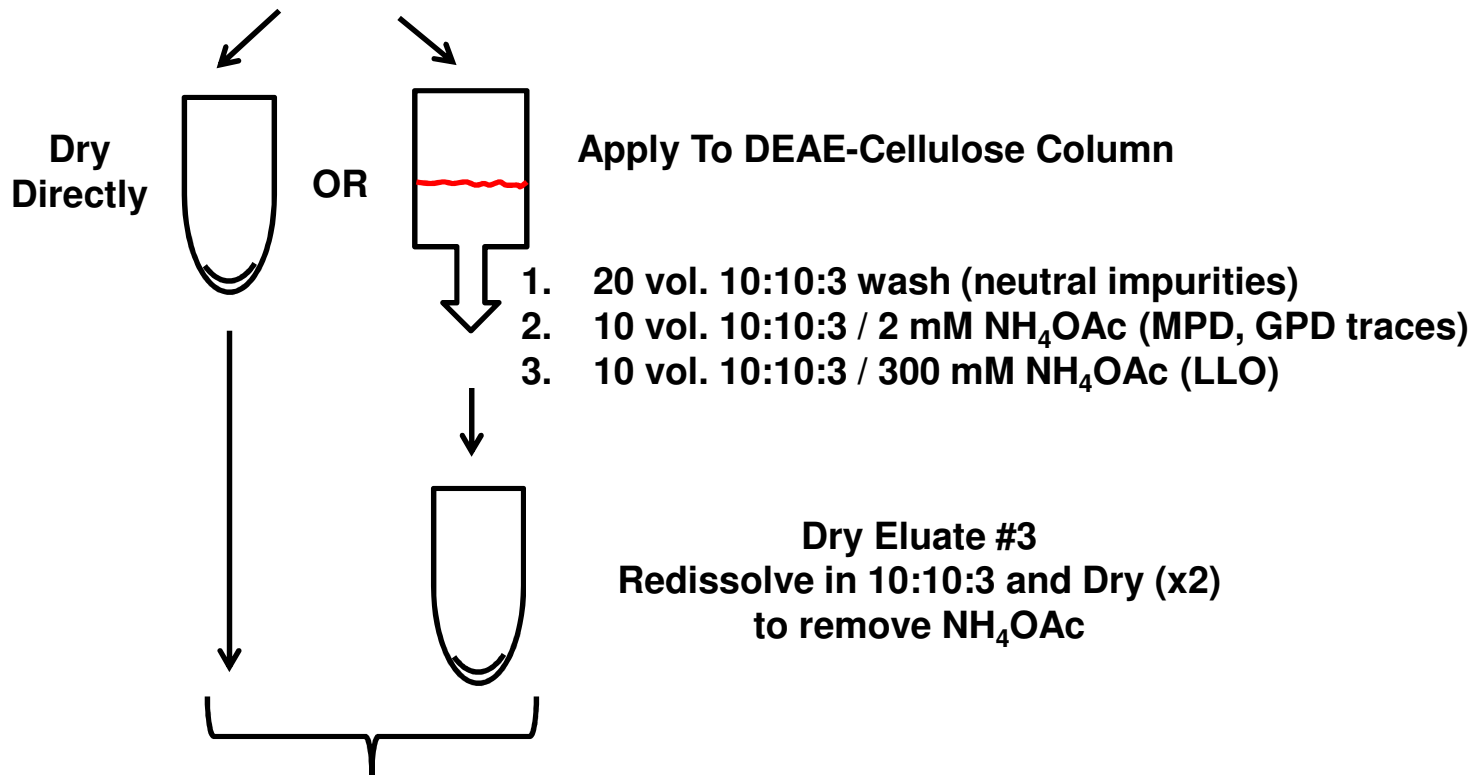
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**Recovered 10:10:3 Liq. Phase (LLO)**



**Hydrolysis: 0.1 N HCl/isopropanol/50 °C/1 hr.**

**Dry**

**Partition: *n*-butanol/water**  
**Collect lower water phase**

**Dry**

***LLO-derived Glycans***