

## Extraction of Plant RNA

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### Summary

Optimal sampling procedures for sampling plant tissue for RNA extractions are outlined in this chapter. To extract RNA, kits supplied from biotechnology companies are appropriate, but some procedures will not work with particular plant tissues. Two alternative methods are supplied for troublesome material. Basic methods to check RNA quantity and, more particularly, RNA quality before use are supplied.

**Key Words:** *Arabidopsis*; fruit; guanidine; plant; plant tissue sampling CTAB; polysaccharides; RNA.

### 1. Introduction

Plants are diverse, and individual species and organs or tissues of plants can behave differently during extraction of RNA (and DNA) for use in molecular studies. Hence, a range of extraction methods has also been devised, depending on the tissue or genotype being extracted. Problems encountered include the presence of large quantities of polysaccharides; high levels of RNases; various different kinds of phenolics, including tannins; low concentrations of nucleic acids (high water content); tissue, such as lignin (wood), that is difficult to break up; and so on. In addition, sampling techniques can have an effect on yield and lack of degradation, recognizing also that most tissues extracted are generally composed of a range of cell types and, hence, functions. In some instances, kits obtained from biotechnology supply companies are sufficient to perform the task, but, in other instances, especially with a new plant or tissue that has not had RNA extracted from it before, methods may need to be modified to suit the particular characteristics of that material. There is no simple indication that a tissue will be difficult. Depending on the use of the RNA extracted, further purification of messenger RNA (mRNA) may be required. In

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general, commercial kits will perform this job more than adequately, and any of a range of methods of quantification will be successful. The biggest problem encountered in RNA extraction usually originates from the initial sampling and extraction protocols, and from personal technique and care taken. Three extraction protocols will, therefore, be outlined that have had widespread success in plant RNA extractions in our laboratory.

## 2. Materials

### 2.1. Sampling

1. Liquid nitrogen (and container). **Caution:** wear protective clothing and gear.
2. Polystyrene box and/or second liquid nitrogen-proof container.
3. Sharp knife, scalpel, razor blade, tweezers, cork borer, metal needle/probe, and flame source.
4. Eppendorf tubes, tinfoil, and plastic bottles of various sizes.
5. Analytical balance.
6. Plant material.
7.  $-80^{\circ}\text{C}$  freezer or liquid nitrogen storage container and/or dry ice.

### 2.2. Arabidopsis Extractions: Trizol™ or Guanidine Isothiocyanate-Based Method (see Note 1)

1. Trizol reagent (Invitrogen). Refer to the manufacturer's instructions and guidelines for stability and storage, and handle with eye and glove protection.
2. Chloroform.
3. Isopropyl alcohol.
4. 75% ethanol in RNase-free water.
5. RNase-free water (made by adding 0.01% DEPC [v/v], standing or stirring overnight, then autoclaving; or made by using Barnstead™ Ultrapure RNase-free water).
6. 0.1 M NaOH-washed and UV-treated plasticware, oven-baked sterile glassware, sterilized Eppendorf tubes, or clean sterile Falcon tubes (conical bottom).
7. Liquid nitrogen and mortar and pestle.
8. Benchtop centrifuges (refrigerated or access to cold room).
9. mRNA purification kit (Amersham Biosciences, now GE).

### 2.3. Extractions From Problem Tissues: CTAB Method (see Notes 1 and 2)

1. Sterile Falcon tubes (conical bottom, 25 or 50 mL).
2. Oakridge tubes (round bottom, sterile, and RNase-free).
3. Mira-Cloth® (Calbiochem).
4. Liquid nitrogen and mortar and pestle.
5. Benchtop centrifuge, vortex machine, refrigerator, and freezer.
6. RNase-free water in a baked storage bottle (in an oven at  $>150^{\circ}\text{C}$ , for  $>4$  h).
7. Chloroform:isoamyl alcohol (24:1).
8. 12 M LiCl (use RNase-free water and autoclave or filter through a Nalgene™ 50-mm kit with 0.2- $\mu\text{m}$  pore size).

9. Extraction buffer: 2% hexadecyl trimethyl-ammonium bromide (CTAB); 2% polyvinylpyrrolidone K30, 100 mM Tris-HCl, pH 8.0, 25 mM EDTA, sodium form, pH 8.0; 2 M NaCl, 0.5 g/L spermidine, and 2%  $\beta$ -mercaptoethanol. Use RNase-free water for dissolving, and autoclave before using.
10. Sodium dodecyl sulfate-Tris-HCl-EDTA (SSTE) buffer: 1 M NaCl; 0.5% sodium dodecyl sulfate (SDS), 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, sodium form, pH 8.0. Use RNase-free water and autoclave before using.

#### **2.4. Extractions From Problem Tissues: Non-CTAB-Based or Non-Guanidine-Based Method (see Notes 1 and 2)**

1. Eppendorf and/or Falcon tubes (sterile and RNase-free).
2. Liquid nitrogen and mortar and pestle.
3. Polytron™ homogenizer.
4. Oakridge tubes (sterile and RNase-free) and Corex tubes (sterile and RNase-free).
5. Benchtop centrifuge, vortex machine, refrigerator, and freezer.
6. RNase-free water in a baked storage bottle (in an oven at  $>150^{\circ}\text{C}$ , for  $\geq 4$  h).
7. Preheated ( $65^{\circ}\text{C}$ ) lysis buffer: 150 mM Tris-HCl, 50 mM EDTA, 4% SDS, pH 7.5 titrated with boric acid, 1%  $\beta$ -mercaptoethanol, and 1% w/w polyvinylpolypyrrolidone (PVPP). Use RNase-free water and autoclave before use.
8. 5 M potassium acetate; use RNase-free water and autoclave or filter as in **Subheading 2.3., item 8**.
9. Cold absolute ethanol.
10. Chloroform:isoamyl alcohol (24:1).
11. Tris-HCl-equilibrated phenol, pH 8.0. Keep phenol in dark bottles in cold room (or  $-20^{\circ}\text{C}$ ); do not use old phenol that has been opened for a long time and is discolored. Make the Tris-HCl buffer RNase-free by adding DEPC to make buffer in a baked (or sterile) bottle, do not autoclave buffer; otherwise, filter as in **Subheading 2.3., item 8**. Equilibrate by melting 500 mL phenol at  $65^{\circ}\text{C}$  and adding 100 mL of RNase-free water, mixing, and leaving to partition overnight (can last for 4–6 wk). Discard the top, aqueous phase. Repeat two more times, but with 0.5 M Tris-HCl, pH 8.5, the first time, and 0.1 M Tris-HCl, pH 8.5, the second time. Phenol can now be used (some buffer can be left on top, but prevent carrying it over while pipetting). Store in the dark at  $4^{\circ}\text{C}$  for up to 2 to 3 wk.
12. 12 M LiCl (use RNase-free water and autoclave or filter as in **Subheading 2.3., item 8**).

#### **2.5. mRNA Extraction**

Use a kit from a biotechnology supplier. We have found the Amersham Biosciences (now GE) kit to work well, but other kits work equally well.

#### **2.6. Quantification, Degradation, and Storage (see Note 3)**

1. RNase-free water.
2. Two UV-capable glass spectrophotometer cuvetts.

3. 10X stock Tris-base–boric acid–EDTA buffer: 108 g Tris-base, 55 g boric acid, and 40 mL of 0.5 M EDTA, pH 8.0, in 1 L deionized water.
4. 37% formaldehyde.
5. Ultrapure™ agarose (Invitrogen).
6. 10X MOPS buffer: 0.2 M MOPS (3-[N-morpholino] propanesulfonic acid, 50 mM Na acetate, and 10 mM EDTA, as in **Subheading 2.6., item 3**).
7. Loading buffer (store in aliquots at –20°C): 0.75 mL deionized formamide, 0.15 mL of 10X MOPS buffer (autoclaved or filtered), 0.24 mL formaldehyde, 0.1 mL RNase-free water, 0.1 mL glycerol (autoclaved), and 10% w/v bromophenol blue dye. Add 3 µL ethidium bromide to 300 µL loading buffer before using.
8. Ethidium bromide as a 10% solution (**Caution:** ethidium bromide is toxic, handle with gloves; see **Note 4**).
9. RNase-free electrophoresis gel boxes, beds, and combs.
10. –80°C freezer.
11. Agilent™ chip.

### 3. Methods

Extraction of excellent quality plant RNA starts with good practice tissue sampling and storage. To reflect mRNA present in a snapshot moment in the growing intact plant, tissues need to be treated in a manner very similar to that for analysis of metabolic intermediates or active enzymes. Partial or complete degradation of mRNA can occur because of tissue sampling and storing techniques. Successful extraction may require alternative techniques, and we outline:

1. A standard method now used for *Arabidopsis* (the model plant in which the genome has been fully sequenced), tomato, maize, tobacco, and other commonly researched plants.
2. Two methods for use in more difficult tissues in which guanidine-based methods result in zero yield—the CTAB (1) and the hot phenol/chloroform methods (2)—which have had much greater success in tissues with low yields and/or high polysaccharide, secondary product, or RNase contents.

The need for care and for use of RNase-free solutions and equipment, including during quantification and storage, is common across all methods.

#### 3.1. Sampling

Ample liquid nitrogen supply is essential. In general, tissue from a plant is sampled by plucking and covering immediately with liquid nitrogen in a polystyrene container, such as those in which chemicals are dispatched on dry ice by laboratory suppliers. For example, with leaves, whole leaves, rapidly hand-shredded leaves, or cork borer discs of leaves can be sampled and killed in a short time, equilibrating to liquid nitrogen temperatures within less than 1 min. It is essential to have a minimal time between removal from the plant and

immersion in liquid nitrogen, to minimize the expression of new mRNAs because of tissue wounding or detachment from the plant. Some mRNA has been shown to be upregulated within 5 min of tissue detachment from a plant. Other tissues are more bulky, e.g., fruit or tubers; these tissues take longer to equilibrate to liquid nitrogen temperatures if immersed whole. Bulky tissues hold more heat, and exchange is slower with liquid nitrogen. This leads to tissue damage (altering osmoticum leading to leaky cells) and degradation of the mRNA present, because RNases gain access to the mRNA. Hence, for bulky tissues, it is better to rapidly remove the tissue from the plant and to subsample quickly (preferably a minute between detachment and immersion of subsamples in liquid nitrogen). Slicing and/or dicing with cork borers is very effective. Take care to sample the tissue in which you are interested in a representative manner. Tissue samples can be added directly to preweighed Eppendorf tubes or storage containers or to homemade tinfoil pouches immersed in the larger liquid nitrogen container. If using Eppendorf tubes, prepare the tubes with a small hole (heat a needle over a flame and pierce the lid) to prevent explosions because of the remnants of liquid nitrogen inside the sealed tube when the lids are closed. This can also be done with other containers, or else the container can be drained before placing the lid on the container. The amount of tissue can be calculated if the containers or tubes are preweighed, then weighed again after the tissue has been killed and the liquid nitrogen evaporated from the container. Care needs to be taken, however, that the tissue does not thaw during weighing.

After the tissue has been sampled, store the sample in a liquid nitrogen storage container or a  $-80^{\circ}\text{C}$  freezer. Take care not to remove tissue or allow it to reach subzero temperatures, by keeping the tissue in liquid nitrogen as much as is practical during subsampling and weighing before grinding (if not performed before storage) to extract the mRNA. Repeated removal from storage and subsampling of tissues has often led to reduced quality of mRNA. To extract the RNA, grind the weighed material in a mortar and pestle, under liquid nitrogen, to a fine powder and transfer the powder to the extraction buffer. **Caution:** do not ever let the plant tissue thaw after killing the tissue in liquid nitrogen and before complete mixing in extraction buffers after grinding the tissue to a powder.

The amount of tissue required to achieve acceptable yields of RNA varies according to the material. Tissues with a high water content require higher amounts of tissue to be extracted. For example, 2 g *Arabidopsis* leaf tissue yields approx 60 to 200  $\mu\text{g}$  RNA (Trizol method); and 5 to 8 g fruit tissue (high water) yields approx 400  $\mu\text{g}$  RNA (non-CTAB/non-guanidine-based method).

### 3.2. *Arabidopsis* Extractions

1. Grind approx 0.1 g tissue in liquid nitrogen.
2. Add 1 mL of Trizol reagent to the ground powder (see **Note 5**).

3. Transfer into Eppendorf tubes.
4. Centrifuge at 12,000g for 5 min at 2 to 8°C.
5. Remove supernatant to new Eppendorf tube.
6. Add 200  $\mu$ L of chloroform and shake vigorously by hand for approx 15 s.
7. Let stand at room temperature (~20–25°C) for 3 min.
8. Centrifuge at 12,000g for 15 min at 2 to 8°C.
9. Carefully transfer the upper aqueous phase to a new Eppendorf tube (ensure no interface debris is transferred, *see* **Note 1**).
10. Add 0.5 mL of isopropyl alcohol. Mix.
11. Let stand at room temperature for 10 min.
12. Centrifuge at 12,000g for 10 min, at 2 to 8°C.
13. Carefully discard supernatant (tip Eppendorf with the pellet position angled up and away from you and pipet out the supernatant). The pellet may be slightly glassy and transparent or may not be clearly visible at all.
14. Add 1 mL of 75% ethanol.
15. Vortex briefly and centrifuge at 12,000g for 5 min at 2 to 8°C.
16. Discard the supernatant as in **step 13** and allow pellet to air-dry for 10 min.
17. Dissolve the pellet in 20  $\mu$ L of RNase-free water by very gently sucking the liquid up and down with a pipet.
18. Quantify the RNA, check the purity and degradation, and either store at –20 or –80°C until used, or extract the mRNA using commercial kits (*see* **Subheadings 3.4.** and **3.5.**).

### 3.3. *Extractions From Problem Tissues*

#### 3.3.1. *CTAB-Based Method*

1. Pipet 15 mL of extraction buffer (minus  $\beta$ -mercaptoethanol) into an RNase-free Falcon tube and add 300  $\mu$ L of  $\beta$ -mercaptoethanol. Warm in a water bath to 65°C (*see* **Note 6**).
2. Grind the tissue in liquid nitrogen and add the tissue gradually to the heated buffer so that no powder coagulates (freezes into a lump) and, therefore, thaws before mixing fully with the buffer. Vortex after each small addition to ensure that the powder is fully dispersed and thawing in the heated buffer.
3. Leave the sample sitting at room temperature while processing the next samples.
4. Mix the samples using the Polytron homogenizer for approx 1 min at full speed until the sample foams close to the top of the tube. Wash the Polytron homogenizer with distilled water after each sample.
5. Add an equal volume of chloroform:isoamyl alcohol, mix (vortex), transfer to an RNase-free Oakridge tube, balance the samples with buffer, and centrifuge (Sorvall SS34 rotor; 11,984g) for 10 min at room temperature to separate the phases.
6. Filter the upper aqueous phase through an autoclaved Mira-Cloth into a new RNase-free Oakridge tube (or carefully pipet off the top aqueous phase, ensuring no transfer of any interface material to a new tube). **Caution:** it is better to leave some aqueous phase behind than to transfer contaminants.

7. Add an equal volume of chloroform:isoamyl alcohol, mix, and centrifuge as in **step 5** to separate the phases.
8. Remove the top aqueous phase to an RNase-free Falcon tube and estimate the volume to the nearest milliliter. Add an appropriate volume of LiCl solution to give a final concentration of 2 M LiCl (1 volume of 4 M, 0.5 volumes of 6 M, 0.33 volumes of 8 M, 0.25 volumes of 10 M, or 0.2 volumes of 12 M).
9. Leave at 5°C (refrigerator) overnight.
10. Centrifuge at 4°C and 11,984g for 20 min (Sorvall SS34 rotor).
11. Pour off the supernatant, and invert the tubes to drain onto a tissue.
12. Preheat SSTE buffer to 65°C. Dissolve the pellet in 200 µL of heated SSTE, and transfer to a 1.5-mL, RNase-free Eppendorf tube.
13. If the SDS in the SSTE buffer precipitates (a white cloudiness), place the Eppendorf with the sample in a heating block (37°C) until it has dissolved again before continuing.
14. Add an equal volume of chloroform:isoamyl alcohol. Vortex immediately before adding and immediately after to mix completely.
15. Add 2 volumes of absolute ethanol to precipitate the RNA (>30 min at -70°C, or >2 h at -20°C).
16. Centrifuge the tube in a microcentrifuge in a cold room (or in a temperature-controlled microcentrifuge) for 20 min at maximum speed.
17. Discard the supernatant, allow the pellet to air-dry, and resuspend in 20 µL RNase-free water, as in **Subheading 3.2., step 17**.

### 3.3.2. Non-CTAB-Based or Non-Guanidine-Based Method (see **Note 2**)

1. Very slowly add 5 g of ground powder to 15 mL of preheated lysis buffer containing PVPP and freshly added β-mercaptoethanol, and vortex between additions (do not allow powder to thaw or form lumps).
2. Homogenize the suspension using the Polytron homogenizer at maximum speed for 20 s, or until the froth reaches the top of the tube.
3. Add 0.1 volumes (1.5 mL) of 5 M potassium acetate and 0.25 volumes (4 mL) of cold absolute ethanol to the tube and vortex for 30 s.
4. Put 1 volume of chloroform:isoamyl alcohol into each of two Oakridge tubes and put half of the homogenate into each tube, vortex, and centrifuge at 2000g for 10 min at room temperature.
5. Remove the top aqueous phase to an RNase-free Falcon tube and add 10 mL of buffered phenol and 10 mL of chloroform:isoamyl alcohol.
6. Vortex to mix, and centrifuge at 2000g for 10 min to separate the phases.
7. Repeat **steps 5 and 6**.
8. Remove the top aqueous phase to an Oakridge tube and add one-third volume of 12 M LiCl. Incubate overnight at -20°C.
9. Centrifuge at 20,000g for 20 min to precipitate the pellet.
10. Pour off the supernatant and resuspend the pellet (by vortexing) in 10 mL of 3 M LiCl (12 M LiCl diluted with RNase-free water). Centrifuge at 20,000g for 20 min.

11. Pour off the supernatant, and resuspend the pellet (by vortexing) in 2 mL of RNase-free water, transfer to a 30-mL Corex tube.
12. Add 180  $\mu$ L of 5 M potassium acetate and 6 mL of cold absolute ethanol. Cover with Parafilm and leave at  $-20^{\circ}\text{C}$  for 1 h.
13. Centrifuge at 12,000g for 10 min.
14. Pour off the supernatant and dry pellet in air for 10 min.
15. Dissolve the pellet in 200  $\mu$ L of sterile RNase-free water. Store at  $-20^{\circ}\text{C}$  until use, with or without aliquoting into different tubes.

### 3.4. mRNA Extraction

Use a manufactured kit. We find the Amersham Biosciences kits (now GE) effective.

### 3.5. Quantification, Degradation, and Storage (3)

1. To quantify and assess the degree of purity, take a 2.5- $\mu$ L aliquot (or larger or smaller) and dilute with 1 mL of water. Scan in a scanning spectrophotometer from 190 nm to 320 nm. Alternatively, read in a spectrophotometer at 260 and 280 nm. Discard the 1 mL sample—it will now be degraded.
2. Calculate the concentration of RNA by the formula:  $\text{OD}_{260} \times \text{dilution factor}/25$ ;  $1 \times \text{OD}_{260} = 40 \mu\text{g/mL RNA}$  (*see Note 7*).
3. To assess whether extracted RNA is degraded and to confirm the quantification, run an aliquot on an agarose gel. Place between 1 and 2.5  $\mu$ L of RNA in an Eppendorf tube and add 10  $\mu$ L loading buffer.
4. To prepare the gel apparatus, soak the apparatus for at least 1 h in water plus SDS (~10%) to denature any RNases. Rinse in RNase-free water.
5. Prepare a 1% formamide agarose gel. For a 30-mL gel, take 3 mL of 10X MOPS buffer, add 25.3 mL RNase-free water and 0.3 g agarose; heat in a microwave oven for 35 s (**Caution:** do not close the container; *see Note 4*), and add 1.7 mL of 37% formaldehyde.
6. Pour the solution from **step 5** into the gel apparatus and wait until set (*see Note 4*). Remove the combs. Add 200 mL of 1X MOPS running buffer to cover the gel and wells.
7. Pre-equilibrate gel by running at 80 V for 10 min.
8. Add 2 volumes of RNA loading buffer to 1 volume of sample. Heat at  $65^{\circ}\text{C}$  for 10 min. Rinse wells with buffer, and load the RNA samples into lanes. Load one lane with 5  $\mu$ L or the recommended quantity of an RNA standard. Run the gel for approx 1.5 h at 80 V. Visualize under UV light. Wear UV-protective goggles.
9. RNA degradation (or contamination) can be detected by:
  - a. A blob at the running edge end of the gel—the RNA is totally degraded.
  - b. A smear with indistinct bands present (this can also mean there is a lot of polysaccharide in the sample).
  - c. The two main ribosomal bands are equal in intensity, or the lower band is higher than the upper band.



- d. There is a bright band at the top of the gel near the loading wells indicating the presence of DNA in the sample. Depending on the use of the RNA, this may not be a problem. It can be removed by digesting with an RNase-free DNase.
10. RNA can, alternatively, be both quantified and assessed for degradation using an Agilent Chip and Agilent Technologies 2100 Bioanalyzer. The samples are run on a prefabricated gel associated with the chip, including a specific RNA ladder. The chip is scanned and the ladder is used to quantify the RNA in each sample, which can also be examined visually for degradation of RNA.

#### 4. Notes

1. Generally, the method used by *Arabidopsis* researchers is adequate for a wide range of plant tissues. However, many plant tissues also contain other compounds that interfere with the extractions. In particular, some tissues (e.g., algae, some fruits, some leaves, and woody material) have high concentrations of polysaccharides, derived either from the plant cell wall or present as mucilages. These generally entrap the RNA during extraction and, if they are not removed in the first steps and partitioned into a discarded phase, they will remain through the rest of the extraction. Hence, it is important not to take any debris or interface material during the chloroform partitioning. Heat is a good way of removing polysaccharides, by making them more soluble. Many plant tissues also have high levels of RNases and, generally, the best way to remove these RNases is to increase the SDS present in the extraction buffer. We have gone as high as 8% for a fruit that also had high polysaccharide content. The result of insufficient SDS is partially or fully degraded RNA. The PVPP helps to bind polyphenolics, which can also be a problem in some tissues that have high levels of polyphenolics. With very low RNA/high water-containing tissue, more material to extract in a given volume of buffer is usually required. Otherwise, there is insufficient RNA present to partition properly during purification.
2. For reasons we do not understand fully, both CTAB and guanidine can cause precipitation problems or other mixing problems when extracting some tissues. We have not found a way to predict this occurrence (other than the presence of polysaccharides).
3. To minimize the presence of RNases, it is important to keep equipment aside for use only with RNA extractions. Pipet tips and Eppendorf tubes should be used only for RNA work and not mixed. Gloves should be used at all times. Keep one set of gel electrophoresis equipment for use just with RNA. Keep solutions RNase free by not using the solutions in other procedures. Always use RNase-free water for solutions. The solid chemicals are NOT RNase free, therefore, buffers should be autoclaved. Tris-HCl buffers cannot be autoclaved.
4. RNA gel matrices can be prepared in advance by dissolving the agarose in buffer in a Sorvall or similar bottle (able to be autoclaved). Melt the aliquot and add formaldehyde and continue as indicated. Take care to fill the bottles only partially full (e.g., 500 mL in a 1 L bottle). This can be stored with the lid on until use. If you have a microwave oven, loosen the lid so that air can escape and heat in a microwave oven until melted. Remove with care (it will be hot) and pour out whatever is

required for the gel. Leave the remainder to resolidify in the bottle and store again. Take care when handling ethidium bromide, it is a mutagen and toxic, and gloves should always be used and surfaces wiped down after use.

5. Do not let ground (or intact) plant tissue thaw without being in the presence of extraction buffer. Small amounts of material can thaw when taking samples in and out of the freezer, weighing out aliquots, or during grinding in liquid nitrogen. It can be particularly important to ensure that the tissue does not form a lump, where the outside is in contact with the buffer, but the inside is thawing directly. When adding tissue to a hot extract, add only a little at a time, using a spoon or spatula that has also been precooled in liquid nitrogen.
6. Always add  $\beta$ -mercaptoethanol fresh on the day of extraction. It will become ineffective in solutions within 12 to 24 h.
7. A ratio of approx 1.8 to 2.0 ( $A_{260}/A_{280}$  nm) means that the RNA is sufficiently pure and without polysaccharide contamination for use in most applications and is soluble. A lower ratio generally means polysaccharide contamination and/or insolubility. A high reading at 240 nm also suggests polysaccharide contamination.

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