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Ethanol Precipitation

Introduction

Ethanol precipitation is a widely used technique to purify or concentrate nucleic acids. This is accomplished by adding salt and ethanol to a solution containing DNA or RNA. In the presence of salt (in particular, monovalent cations such as sodium ions (Na+)), ethanol efficiently precipitates nucleic acids. The purified precipitate can be collected by centrifugation, and then suspended in a volume of choice. See Fig. 1.

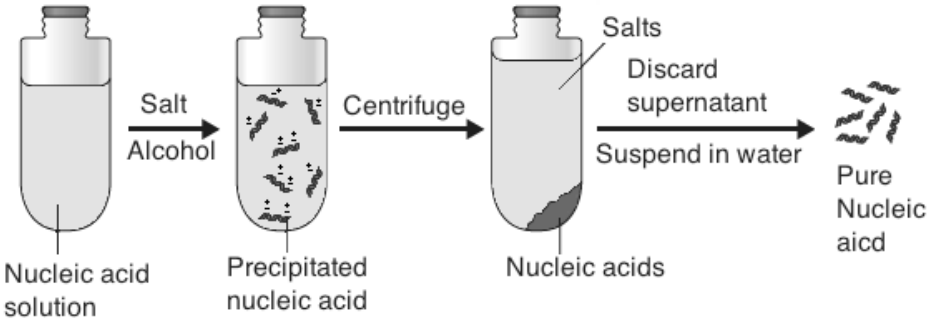

Figure 1. Schematic overview of an ethanol precipitation of nucleic acids.

Principles of Precipitation

Most molecules carry no net charge, but some possess an electric dipole or multipole. This occurs when there is unequal sharing of electric charge between atoms within a molecule. For example, in hydrogen chloride (HCl), the chlorine atom pulls the hydrogen's electron toward itself, creating a permanent dipole. This occurs because chlorine is more *electronegative* than hydrogen is (electronegativity is a measure of an atom's tendency to attract electrons toward itself; on a scale from 0 to 4, the higher an element's electronegativity number, the greater it attracts electrons toward itself[1]). Molecules with permanent dipoles are referred to as *polar* molecules (Israelachvili 2011).

A solute dissolves best in a solvent that is most similar in chemical structure to itself (bonds between a solute particle and solvent molecule substitute for the bonds between the solute particle itself)[2]. With respect to solvation, whether or not a solute and a solvent are similar in chemical structure to each other depends primarily on each substance's polarity. Typically, polar solutes will *only* dissolve in polar solvents, and non-polar solutes will *only* dissolve in non-polar solvents (which is to say that, a non-polar solute *will not* dissolve in a polar solvent)

[1] The 0-4 electronegativity scale was introduced by Linus Pauling in 1932 (Pauling 1932).
[2] *Like dissolves like*. This is a guiding generalization; there are exceptions to it.

(McMurry 2003). Accordingly, a very polar solute such as urea is very soluble in highly polar water, less soluble in somewhat polar methanol, and almost insoluble in non-polar solvents such as chloroform and ether (O'Neil 2006).

Sodium acetate (CH_3COONa, ) is a carboxylate salt commonly used in ethanol precipitations; at 0.3 M, pH 5.2, it is used in most routine precipitations of DNA and RNA (Maniatis, Fritsch and Sambrook 1982). Carboxylate salts are composed of a carboxylate anion ($RC(=O)O^-$) and a positively charged metal ion. Carboxylate anions are polar[3] (because oxygen is more electronegative than carbon is, and the difference between the two atom's electronegativities is large enough to make the C=O bond moderately polar), and therefore carboxylate anions dissolve in polar substances, such as water. Sodium cations are also soluble in water (they attract the electronegative oxygen atom in water molecules). In water, at room temperature, water molecules surround and separate the carboxylate ions by forming *solvation shells* (often called *hydration shells* when the solvent is water) around the ions. As a result, in water, at room temperature, sodium acetate completely dissociates into an acetate anion and a sodium cation (Eq. 1) (Chang 2005):

$$CH_3COONa_{(s)} \xrightarrow{H_2O_{(l)}} CH_3COO^-_{(aq)} + Na^+_{(aq)} \quad (1)$$

$$CH_3COO^-_{(aq)} + H_2O_{(l)} \leftrightarrow CH_3COOH_{(aq)} + OH^-_{(aq)}$$

Nucleic acids are polymers of nucleotides. A nucleotide is composed of a nucleobase (a nitrogenous base), a five-carbon sugar (either ribose or 2-deoxyribose, for RNA or DNA, respectively), and a phosphate group. Nucleotides within a nucleic acid polymer are joined together by the formation of phosphodiester bonds; the oxygen on the 5' carbon of one nucleotide is connected to the oxygen on the 3' carbon of its neighboring nucleotide. This chain of phosphodiester bonds is often referred to as the *backbone chain*.

Figure 2. DNA surrounded by water molecules in solution, forming primary and secondary hydration shells. Image from **(Chuprina, et al. 1991)**.

Nucleic acids are polar. The oxygen atoms and the nitrogen atoms in the backbone are what make nucleic acids polar (the oxygen and nitrogen atoms act as hydrogen-bond acceptors,

[3] It is important not to conflate 'anionic' with 'polar', since it is possible that an ion be an anion but be non-polar. For example, if the center of gravity of an electron cloud of a monatomic ion coincides with the position of its nucleus, then such an ion would be non-polar (Haaland, 2008).

and the various protons act as hydrogen-bond donors). Nucleic acids are therefore also soluble in water (20). Unlike salt compounds, nucleic acids do not dissociate in water (because the intramolecular forces linking nucleotides together are stronger than the intermolecular forces between nucleic acids and water). Nevertheless, water molecules form solvation shells through dipole-dipole interactions with nucleic acids, separating different polymers of nucleic acids from each other, effectively dissolving the molecules in solution (Fig. 2). In order for sodium cations to readily interact with the negatively charged phosphate groups, these hydration shells must be depleted

The *dielectric constant* (ε) of a solvent refers to its relative permittivity (Smith 2000). The *relative permittivity* of a solvent is expressed as the ratio of the permittivity of that solvent to the permittivity of that solvent in a vacuum, which, effectively, is a) a measure of the ability of a solute within that solvent to resist that solvent's electric field (in other words, a measure of that solvent's ability to insulate charges within it from each other), and b) the solvent's polarity (Smith 2000, Collins English Dictionary: Complete and Unabridged 2003). In low-permittivity solvents, ions of opposite charges easily form ion-pairs (Izutsu 2003).

The dielectric constant of an ethanol-aqueous solvent at room temperature can be estimated by the expression in Eq. (2):

$$\varepsilon_T = \frac{V_i \varepsilon_i}{V_T} \quad (2)$$

where V_i and ε_i are the volume and dielectric constant of solvent *i*, respectively; and where V_T is the total volume of solution (Khalil and Al-Resayes 2012). Other alcohol-aqueous dielectric constants may need to be empirically estimated, using, for example, a dielectric constant apparatus.

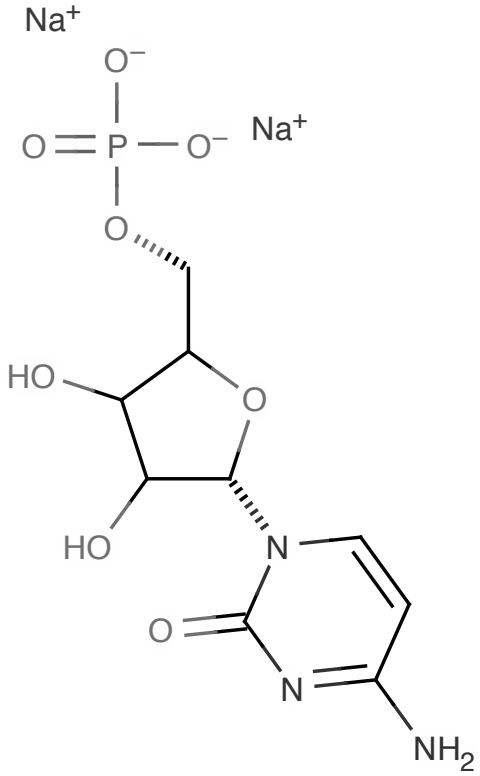

Figure 3. Sodium cations neutralizing a nucleotide.

Interactions between two charges within a solvent are governed by Coulomb's law (Eq. 3). Although Coulomb's law is strictly *only* valid for point charges, it reliably predicts trends with respect to ion interactions within a solvent. Coulomb's law describes the attraction or repulsion between two charges q_1 and q_2 at a distance *r*. It is affected by the dielectric constant of a solvent, with ε in the denominator. k_e is a proportionality constant.

$$F = \frac{q_1 q_2}{4\pi\varepsilon_0 \varepsilon_r r^2} = k_e \frac{q_1 q_2}{\varepsilon_r r^2} = 8.9875 \times 10^9 \frac{q_1 q_2}{\varepsilon_r r^2} \text{ newtons} \quad (3)$$

Ethanol is the most commonly used alcohol component in precipitations of DNA and RNA (Maniatis, Fritsch, & Sambrook, 1982). Due to the structural differences between ethanol and water, ethanol has a much lower

dielectric constant than water does (24 vs 80, respectively). By lowering the dielectric constant of a solution containing nucleic acids and monovalent cations, the Coulomb force of attraction increases between the cations and the negatively charged nucleic acid backbone (that is, the resistance from the solvent's electric field sufficiently diminishes to permit efficient interaction; the solvation shells surrounding the solute's charges depletes) (Fig. 4). When the cations and negatively charged nucleic acid backbone interact, nucleic acids are neutralized, therefore no longer dissolve in water and precipitate out of solution (Fig. 3). Also, ethanol induces conformational changes to the nucleic acid structure; the repulsive forces between the negatively charged phosphates with a nucleic acid polymer are so diminished that inter-helical interactions are possible, allowing the nucleic acids to aggregate (Piškur and Rupprecht 1995, Eickbush and Moudrianakis 1978).

Practical Considerations

Choice of alcohol

Ethanol is the most widely used alcohol for nucleic acid precipitations. However, isopropanol (isopropyl alcohol) can also be used. Isopropanol has a lower dielectric constant than ethanol does (20 vs 24, respectively); this is because, isopropanol is less polar than ethanol is (isopropanol (C_3H_8O) has one more carbon atom than does ethanol (C_2H_6O); consequently, the alcohol group (-OH), which is the polar portion of the molecule, has less of a net effect on isopropanol than it does on ethanol; as the size of the molecule increases, the influence of the -OH group diminishes and the properties of the molecule are dominated by the essentially nonpolar C-H bonds). Consequently, half the volume of isopropanol is required to precipitate at the same efficiency as a given volume of ethanol (Fig. 4). This is particularly helpful when working with large volumes of samples. However, because isopropanol is less polar than ethanol, more salts are less soluble in isopropanol than ethanol (and consequently, more salt might co-precipitate with the nucleic acids). Also, isopropanol is less volatile than ethanol (as the number of carbons increases, the vapor pressure (an indication of a liquid's evaporation rate) decreases, and the alcohol becomes less volatile), and so may require more time to air-dry the nucleic acid pellet than would be required when using ethanol.

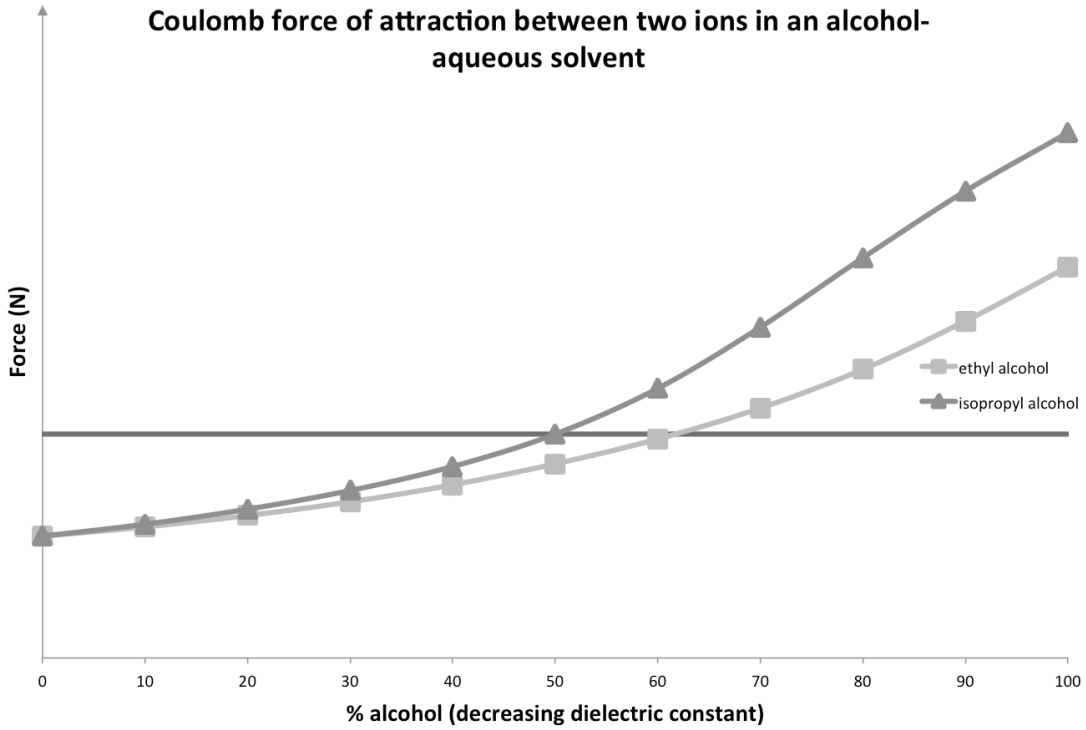

Figure 4. Absolute force of attraction between two opposing ions in either an alcohol or an isopropanol-aqueous solvent. The force of attraction was calculated using Eq. 3 assuming equally opposing charges and constant distance. As can be seen, roughly 2x the volume of ethanol is required to obtain the same force of attraction between two opposing ions as is 1x the volume of isopropanol. Values from **(Akerlof 1932)**.

Choice of salt

Salts (in particular, monovalent cations) are critical for effective precipitation of nucleic acids. In some situations, one salt is preferred over another. Consult Table 1 for advice.

TABLE 1 | Salts.

SALT	ADVANTAGES/DISADVANTAGES
Ammonium acetate	Reduces co-precipitation of dNTPS or oligosaccharides; good for use after digestion of agarose gels; do not use if downstream reactions includes phosphorylation (polynucleotide kinase is inhibited by ammonium ions) (Maniatis, Fritsch and Sambrook 1982).
Potassium acetate	**<PLACE_HOLDER>**
Lithium chloride	Does not efficiently precipitate DNA, protein or carbohydrates (Barlow, et al. 1963). Loss of tRNAs, 5S RNAs, snRNAs, and other RNAs < 250-300 nt noted (Cathala, et al. 1983).
Sodium chloride	Use if sample contains SDS (SDS remains soluble in 70% ethanol) (Maniatis, Fritsch and Sambrook 1982).
Sodium acetate	Most commonly used salt for most routine precipitations (Maniatis, Fritsch and Sambrook 1982).

Choice of carrier

Carriers (or co-precipitants) are substances can be used during alcohol precipitations to facilitate recovery of target nucleic acids. They are insoluble in ethanol or isopropanol solutions, and they form a precipitate that helps to trap nucleic acids by bulk. During centrifugation, carriers form a visible pellet, which aids in removing the supernatant without perturbing the nucleic acid pellet. Precipitations with carriers from diluted solutions of nucleic acids are also quantitatively more efficient than precipitations without carriers.

TABLE 2 | Carriers.

CARRIER	FINAL CONCENTRATION	ADVANTAGES/DISADVANTAGES
Yeast tRNA	10–20 µg⁄mL	Inexpensive, biologically active material. Inhibits downstream reactions catalyzed by polynucleotide kinase or terminal transferase (terminal transferase requires a free 3'-OH terminus to act, which yeast tRNA also possess) (Michelson and Orkin 1982). Inhibits tailing (Michelson and Orkin 1982). Inhibits template-dependent cDNA synthesis; gives rise to template-independent, low molecular weight product when random hexamers used to prime reverse transcription; cDNA synthesis not inhibited, however, when primed by oligo(dT)-T7 primer, nor does it interfere with in vitro transcription by T7 RNA polymerase (Wang, et al. 2002).
Salmon sperm	10–20 µg⁄mL	**<PLACE_HOLDER>**
Glycogen	50–150 µg⁄mL	Glycogen is a purified polysaccharide. It is an inert carrier, free of host DNA/RNA. Glycogen does not inhibit later restriction endonucleases up to 30 mg/ml, T4 DNA ligase activity at up to 7 mg/ml, nor nucleic acid hybridization reactions (Tracy 1981). Glycogen may, however, interfere with nucleic acid:protein interactions (Gaillard and Strauss 1990), and may inhibit reverse

		transcription of large templates in a concentration dependent manner (Baugh, et al. 2001).
Linear polyacrylamide	10–20 µg/mL	Inert, neutral carrier efficient at precipitation of picogram amounts of nucleic acids with ethanol; very short fragments will not co-precipitate (≤ 20 bp) (Gaillard and Strauss 1990). Does not inhibit DNA:protein interactions (Strauss and Varshavsky 1984). Does not inhibit cloning, enzyme reactions (including polynucleotide kinase reactions and ligation by T4 DNA ligase), or electrophoresis (Aruffo and Seed 1987).

A note on temperature

As the temperature of an alcohol-aqueous solution decreases, its dielectric constant increases. Therefore, in principle, at increasingly chilled temperatures, precipitation efficiency should diminish. Moreover, as the temperature decreases, the viscosity of the solution increases, which retards the movement of the nucleic acid aggregate, especially if the aggregate is small (Zeugin and Hartley 1985). In addition, since solubility decreases at lower temperatures, more salts will begin to co-precipitate with the nucleic acids at lower temperatures. Therefore, incubation at temperatures below 0°C is counter-productive and not recommended.

Protocol for Precipitating RNA from Animal Cells or Tissues

Materials

Reagents

- Isopropanol alcohol
- Sodium acetate (pH 5.2, 3.0 M)
- Glycogen (optional)

Miscellaneous

You will need access to a fume hood, vortexer, micropipettes, chilled microcentrifuge, and all standard equipment of a biochemistry-molecular biology laboratory. ▲**CRITICAL** Maximum care should be taken to not contaminate samples with RNases. For this reason, use pipettes reserved for RNA work to prevent cross-contamination with RNases from shared equipment. Always wear disposable gloves, as skin cells as well as bacteria and molds can contaminate samples and can be sources of RNases. Use either disposable, sterile plasticware or nondisposable glassware or plasticware that is RNase-free. For this, glassware should be cleaned with a detergent, thoroughly rinsed, and oven baked at 240 °C for at least 4 h. Since autoclaving alone will not fully inactivate many RNases, glassware can be treated with

diethylpyrocarbonate (DEPC). Fill glassware with 0.1% DEPC (0.1% in water), incubate for 12 h at 37 °C, and then autoclave or heat to 100 °C for 15 min to eliminate residual DEPC. Plasticware can be soaked for 10 min in 0.5 M NaOH, 1 mM EDTA followed by RNase-free water. Chloroform-resistant plasticware can be rinsed with chloroform to inactivate RNases (Qiagen n.d.). **! CAUTION** Isopropanol can cause severe eye, respiratory tract, or moderate skin irritation (Fisher Scientific 1999). Ingested, it can cause adverse reproductive and fetal effects, and may cause central nervous system depression; it may also produce liver, kidney, or heart damage. Wash hands thoroughly after handling, and use only in a well-ventilated area (Fisher Scientific 1999). **! WARNING** Isopropanol is a flammable liquid and vapor (Fisher Scientific 1999).

Setup

Before beginning, clean the workstation (pipettor shaft, benchtop) with a surface decontamination solution that destroys RNases (such as RNase-zap); and chill the microcentrifuge to 4 °C.

Procedure

1| Use option A if starting from *Protocol for Isolation of RNA from Animal Cells or Tissues* or option B otherwise.

▲**CRITICAL** Procedure is done at room temperature (15-25 °C) unless otherwise indicated. This minimizes co-precipitation of salt.

(A) Proceed to step 2.

(B) Adjust the salt concentration, if necessary, with sodium acetate (pH 5.2, 3.0M(to a final concentration of 0.3 M.

◆ **TIP** Add a carrier if working with small amounts of RNA.

Precipitation

2| Add 1x the volume of isopropanol to the aqueous RNA solution. Mix well.

▲**CRITICAL** Mix gently; violent mixing may shear the RNA.

▲**CRITICAL** When mixing and shaking, make sure that the caps are tightly closed!

3| Incubate the samples for 1 hr on ice.

■ **PAUSE POINT** You can stop at this point, store your samples at –20 °C, and complete the procedure later.

4| Centrifuge at 12,000 x g for 30 min at 4°C (chilled centrifugation prevents overheating of samples and more firmly fixes the pellet to the bottom of the tube). After centrifugation, the RNA precipitate, often invisible before centrifugation, should form a gel-like pellet (especially if a carrier was used). Discard the supernatant.

◆ **TIP** Orient the hinge of the micro-centrifuge tube outward to assist in locating the nucleic acid pellet, which will then be located on the same side of the hinge.

▲**CRITICAL** Care should be taken not to perturb the pellet. Perturbation of pellet might decrease overall RNA yield.

RNA wash

5| Resuspend the RNA pellet with 0.5–10 ml (depending on the starting volume) of 75% ethanol and gently mix.

■ **PAUSE POINT** You can stop at this point, store your samples at either at 4 °C for up to 1 week or –20 °C, and complete the procedure later.

6| Centrifuge at 12,000 x g for 15 min at 4°C. After centrifugation, heat the centrifuge to room temperature (15–25°C).

7| Incubate samples for 15 min at room temperature to dissolve co-precipitated salts.

8| Centrifuge at 12,000 x g for 5 min at 4°C. Discard the supernatant.

▲**CRITICAL** Extra care should be taken when discarding the supernatant. Pellet may more easily dislodge this time than previously.

9| Repeat steps 6-8.

10| Air-dry the RNA pellet for 5–10 min at room temperature.

▲**CRITICAL** Do not over-dry the pellet or use a vacuum centrifuge, as this will greatly decrease its solubility.

RNA solubilization

11| Dissolve the pellet in RNase-free water.

12| Incubate RNA for 15 min at room temperature (15–25°C) to ensure complete solubilization.

■ **PAUSE POINT** You can store the sample or proceed to quantification by spectrophotometry or fluorescence. You can also check the quality by agarose gel or capillary electrophoresis.

▲**CRITICAL** RNA stored in RNase-free water should be stored at –80 °C when not in use.

● **TIMING**

Ethanol precipitation: about 2 h.

? TROUBLESHOOTING

Troubleshooting is discussed in **Table 3**.

TABLE 3 | Troubleshooting table.

PROBLEM	POSSIBLE REASON	SOLUTION
Little or no RNA in eluate	a) RNA failed to precipitate	Ensure the precipitate is centrifuged at the proper speed for 30 min. Recover RNA by centrifuging for longer and at higher speeds. Try another isopropanol batch.
	b) RNA pellet was lost	Isopropanol pellets may be difficult to see or loosely attached to the side of the tube, so remove alcohol phases carefully. Mark the outside of the tube before centrifugation, or ensure hinge of tube faces outward.

	c) RNA was poorly redissolved	Check that RNA is completely redissolved. Be sure to wash any RNA off the walls of the tube
RNA difficult to dissolve	a) pellet was over dried	Air-dry only (do not use a vacuum). Attempt recovery by warming the solution slightly, and by allowing more time for dissolving.
	b) residual alcohol in pellet	Ensure that pellets are washed with 70% ethanol to remove traces of isopropanol. Redissolve RNA by warming the solution slightly, and allowing more time for redissolving. Increase volume of RNAase-free water used for redissolving if necessary
	c) too much salt in pellet	Ensure that isopropanol is at room temperature for precipitation, and wash the pellet twice with room temperature 75% ethanol. Recover RNA by increasing the volume of buffer used for redissolving.
	d) improper pH	Nuclease-Free water has a pH value of between 5.0 and 8.0.
	e) suspension volume too low	Increase resuspension volume if the solution is highly viscous.
Contaminated/poor-quality RNA	a) genomic DNA in the eluate	Re-purify using phenol-chloroform. Or, treat with DNase.
	b) nuclease contamination	Check buffers for nuclease contamination and replace if necessary. Use new RNase-free glass- and plasticware, and wear gloves. Treat bench and all working materials with RNase solution.
Poor downstream	a) too much salt	Ensure that isopropanol is at

performance		room temperature for precipitation, and wash the pellet twice with room temperature 75% ethanol. Precipitate the RNA again to remove the salt.
	b) residual protein	Remove with phenol-chloroform extraction.

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