



Evaluation of Yeast Inoculated in Parallel to the Autochthonous Microbiota in Food Samples

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Abstract

Fermentation is one of the oldest and most economical methods of producing and preserving food. For a greater standardization of the fermentative processes, new cultures are selected to reduce the fermentation time, increase the microbiological quality of the food, and alter and standardize the sensory attributes. Therefore, starter cultures are a practice performed to improve quality and add value to food and beverage fermented. The combination of traditional and molecular techniques is widely used to monitor and quantify the yeast starter population. This chapter will cover some techniques used to evaluate and quantify the inoculation strain and the microbiota involved during the food and beverage fermentation.

Key words Starter yeasts, qPCR, PFGE, Fermented food, MALDI-TOF

1 Introduction

Fermentation is one of the oldest and most economical methods of producing and preserving food and beverage. The preparation of these fermented foods and beverages was in an artisan way and without any knowledge of the microorganisms' role (bacteria, yeast, and filaments fungi) involved [1]. Methods for the fermentation of meat [2], coffee beans [3], cocoa beans [4–7], wine [8, 9], yogurt [10], kefir [11–13], cheese [14], alcoholic beverage [15, 16], Kombucha [17, 18] have been described.

Starter cultures are live microorganisms that develop through the fermentation of a particular substrate present in the medium, bringing some benefits to the product generated, such as adding organoleptic characteristics, better product stability, reducing processing time, and others. The *Saccharomyces cerevisiae* is an example of yeast commonly used as a starter culture, and these strains are employed in main industrial and laboratory processes; different commercial strains of *S. cerevisiae* were used and showed differences

in the production of various fermented products as wine [8], cocoa [19], coffee [20], sugar cane spirit (cachaça) [21, 22] and others.

However, several other species non-*Saccharomyces* are also used. In wine, the yeasts *Wickerhamomyces anomalus*, *Torulaspota delbrueckii*, *Meyerozyma guilliermondii*, *Kazachstania aerobia*, and *K. servazzii* can be inoculated [9]. Giopardini and Zullo [23] used the *Candida diddensiae*, *C. adriatica*, and *W. anomalus* species to improve the production in the fermented foods of table olives. *Zygosaccharomyces rouxii* and *M. guilliermondii* are used as a starter culture for soy sauce fermentation [24].

Several studies using selected yeasts isolated from coffee have been successfully carried out. The inoculation of a starter culture was shown to improve coffee flavor and aroma, reduce processing time and drying time, and increase the product's economic value [25, 26]. *S. cerevisiae* and *T. delbrueckii*, isolated from natural coffee fermentative, improved the beverage quality, and some attributes have been found through it, such as caramel, chocolate, herbaceous materials, yellow fruits, and almonds [25]. The authors used both qPCR and DGGE to analyze quantitatively and qualitative yeast populations.

S. cerevisiae, *T. delbrueckii*, *Pichia kluyveri*, *Hanseniaspora uvarum* were inoculated to enhance cocoa fermentation and improved chocolate taste [5, 6, 19]. The methods for yeast enumeration and identification were plate counting, DGGE, qPCR. In some food fermentation, yeast is inoculated as a monoculture, while in others as a mixed cocktail containing yeasts and bacteria like, for example, the Kefir [13], Kombucha [18], Cocoa [4], nondairy beverage [27], among others.

The food industries routinely use starter culture; therefore, a great deal of research is needed to study how the starter culture develops in food, whether it is present throughout the fermentation process, and in what quantity. Different methods can be used, always trying to evaluate the yeasts inoculated in the food to characterize its behavior in the fermentation process.

The methods described in the following sections are used to evaluate and monitor yeast strains in the laboratory and industrial processes. Some of them are used in a unique way or the combination of more than one method. The currently available and validated methods for the determination of yeasts in foods include 3M™ Petrifilm™ Rapid Yeast and Mold Count (RYM) [28, 29], slide culture technique [30], pulse field gel electrophoresis (PFGE) [31, 32], quantitative polymerase chain reaction (qPCR) (Table 1) [34, 35], and matrix-assisted desorption ionization-time of flight mass spectrometry (MALDI-TOF) (Fig. 1) [46–48]. This chapter will cover the use of starter cultures in fermented foods and techniques to assess the wild and inoculum population during the fermentation process.

Table 1
Specific primer used for yeast

| Target yeast group | Primer | Sequence (5'—3') | Size (bp) | References |
|-----------------------------------|------------|---|-----------|------------|
| <i>Universal yeast</i> | YEASTF | GAGTCGAGTTGTTTGGGAATGC TCTCTTTCCAAAGTTCTTTTCATC TTT | 124 | [33] |
| <i>Brettanomyces bruxellensis</i> | DB RUXF | GGATGGGTGCACCTGGTTTACAC GAAGGGCCACA TTCACGAACCCCG | 79 | [36] |
| <i>Kluyveromyces marxianus</i> | * | TCCTCGACAGTAATGATAA AGCACTCAATTCATCGTA | 140 | [37] |
| <i>Debaromyces anomalus</i> | * | GAGCAGACTGAGAAGTTC CGACCATAGAAGAGTGAG | 100 | [37] |
| <i>Kazachstania turicensis</i> | * | GTTGCATGGCAATCAAAA CGAAGACGCTCAAGAATA | 101 | [37] |
| <i>Saccharomyces cerevisiae</i> | * | CGACAACAAATTGCTGAA CTCTCGAACATAACTCTGTA | 147 | [37] |
| <i>Pichia kluyveri</i> | PK-5fw | AGTCTCGGGTTAGACGT GCTTTTCATCTTTCCTTCACA | 169 | [38] |
| <i>Hanseniaspora uvarum</i> | HU-5fw | GGCGAGGATACCTTTTCTCTG GAGGCGAGTGCATGCAA | 172 | [38] |
| <i>S. cerevisiae</i> | SC-5fw | AGGAGTGCGGTTCTTTGTAAAG TGAAATGCGAGATTCCCCT | 215 | [38] |
| <i>P. anomala</i> | Anom | GTAAAAACCTTTAACCAATA AAATGACGCTCAAACA | nd | [39] |
| <i>P. guilliermondii</i> | Guill | CAAAACCACATTTAATTATTT AAATGACGCTCAAACA | nd | [39] |
| <i>P. kluyverii</i> | Kluy | CACCAAACACCTAAAAT AAATGACGCTCAAACA | nd | [39] |
| <i>Candida albicans</i> | Calb-F | CYGGCTCUGTCTATGITYC GTCTARGCTGGCAGTATCG | 411 | [40] |
| <i>C. glabrata</i> | Cgla-F | CYGGCTCUGTCTATGITYC TAACACTCTACACCGAGGCG | 398 | [40] |
| <i>Clavispora lusitaniae</i> | Clus-F | CCTGCGGGAHGTAAATTG UACGCCAGCGTCCTAGAAT | 442 | [40] |
| <i>Issatchenkia orientalis</i> | Iori-F | CAGGUGGAGTCTGTGTGGA TCTGGCCCTGGCTATAACACC | 416 | [40] |
| <i>Trichosporon asahii</i> | Tasa-F | AATCCCCTGCTTGATACGAC GRGRAGTCACATTCTAC | 319 | [40] |
| <i>Torulasporea delbrueckii</i> | Tods L2 | CAAAGTCATCCAAGCCAGC TTCTCAAACAATCATGTTTGGTAG | nd | [41] |

(continued)

Table 1
(continued)

| Target yeast group | Primer | Sequence (5'—3') | Size (bp) | References |
|----------------------------------|---------|--|-----------|------------|
| <i>I. orientalis</i> | Isa 1 | GTTTGAGCGTCGTTTCCATC AGCTCCGACGCTCTTTACAC | nd | [41] |
| <i>Metschnikowia pulcherrima</i> | MPL3 | CTCTCAAACCTCCGGTTTG GATATGCTTAAGTTCAGCGGG | nd | [41] |
| <i>Kazachstania slooffiae</i> | KSact-f | CAAACCGCTGCCCAATCTTC GCTTCAAGACCCAAGACGGA | 131 | [42] |
| <i>Zygosaccharomyces bailii</i> | ZBF | CATGGTGTTTTTCGCGCC CGTCCGCCACGAAGTGGTAGA | 122 | [43] |
| <i>Brettanomyces species</i> | BRET | G TTCACACAATCCCCTCGATCAAC TGCCA ACTGCCGAATGTTCTC | 108 | [44] |
| <i>Yarrowia species</i> | YAL | ACGCATCTGATCCCTACCAAGG CATCCTGTCGCTCTTCCAGGTT | 106 | [44] |
| <i>Meyerozyma guilliermondii</i> | MeF | GAGATCAGACTCGATATTTTGTGAG GTCTAGGCAGGCAGCATCAAC | 156 | [45] |

Asterisk primer name not set, nd not described

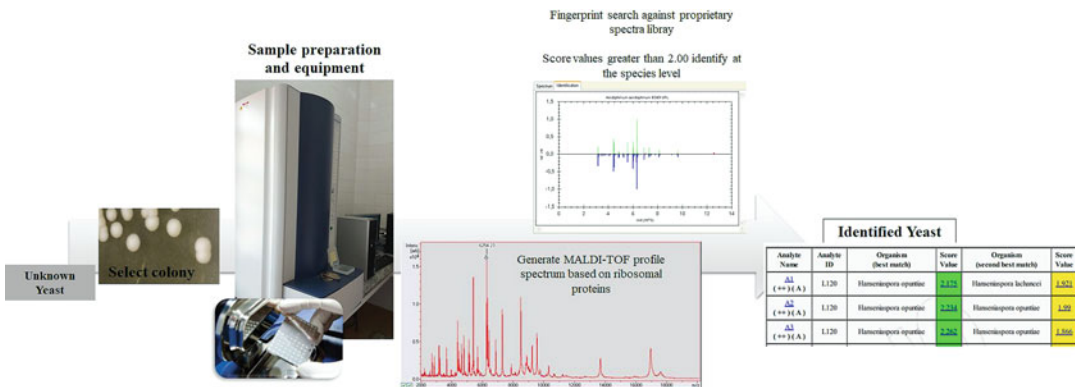


Fig. 1 Steps for identification by MALDI-TOF

2 Materials

The following materials are needed to evaluate yeast starter according to the technique used.

2.1 Cell Viability

1. Methylene blue (1 g).
2. Distilled water (10 mL).
3. Sodium citrate, dihydrate (2 g).
4. Neubauer chamber.

2.2 3M™ Petrifilm™ Rapid Yeast and Mold Count

1. 0.1% peptone water sterile: Dissolve 0.1 g/L of peptone in water and proceed with sterilization. This solution is used for samples suspension cells.
2. 3M™ Petrifilm™ Rapid Yeast and Mold Count (RYM).
3. Pipette.
4. Incubator (28 °C).

2.3 Slide Culture Technique

1. YPD agar (g/L): Yeast extract (10), peptone (20), glucose (20), agar (20).
2. Sterile petri dishes.
3. Sterile dissecting knife.
4. Sterile microscopy slide.
5. Sterile coverslip.
6. Wetting chamber.
7. Calcofluor White dye (5.0 mg/mL).
8. Microscopy.

2.4 Pulse Field Gel Electrophoresis (PFGE)

1. Lysing enzymes.
2. CPES buffer.
3. CPE buffer.
4. Proteinase K.
5. Ethylenediamine tetraacetic acid (EDTA).
6. TE buffer.
7. Agarose for PFGE.
8. TAFE buffer.

2.5 Quantitative Polymerase Chain Reaction (qPCR)

1. Rotor for qPCR analysis.
2. Rotor-Gene PCR SYBR Green Mix Master 2× (kit contains taq polymerase, dNTP, buffer).
3. Species-specific primer (forward and reverse).
4. Talc-free gloves.

5. Pipettes and tips of 0.1, 10, 100 μL .
6. 200- μL microtubes.
7. Quantified template DNA (extracted DNA from the inoculum and sample DNA separately).

2.6 Matrix-Assisted Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF)

1. Ultrapure water.
2. Formic acid.
3. Ethanol.
4. Acetonitrile.
5. Trifluoroacetic acid (TFA).
6. Alpha-Cyano-4-hydroxycinnamic acid (CHCA).
7. Eppendorf, pipette and tip.

3 Methods

Many industrial processes need to determine the proportion of living cells in yeast cell material through microscopic observation with methylene blue dye [49]. The counting of viable cells is done in a Neubauer chamber (*see* Chapter 11).

3.1 3M™ Petrifilm™ Rapid Yeast and Mold Count (RYM)

1. Aseptically prepare a 1:10 dilution of each test portion (*see* **Note 1**).
2. Prepare tenfold serial dilutions in 0.1% peptone water (*see* **Note 2**).
3. Place the 3M Petrifilm RYM Plate on a flat, level surface.
4. Lift the top of the film and dispense 1 mL of each dilution onto the center of the bottom film of each plate.
5. Roll the film down onto the sample.
6. Place the 3M Petrifilm Flat Spreader on the center of the plate of the spreader to distribute the sample evenly (*see* **Note 3**).
7. Remove the spreader and leave the plate undisturbed for at least 1 min to permit the gel to form.
8. Incubate the 3M Petrifilm RYM Count Plates at 28 °C in a horizontal position with the clear side up in stacks of no more than 40.
9. Enumerate plates after 48 h of incubation (*see* **Note 4**).
10. Analyze colonies morphology (*see* **Note 5**).
11. The circular growth area is approximately 30 cm², plates containing greater than 150 colonies can be either estimated (*see* **Note 6**).
12. Food samples may occasionally show interference on the 3M Petrifilm RYM Count Plates (*see* **Note 7**).

13. If required, colonies may be isolated for further identification by direct microscopy or biochemical analysis, lift the top film, and pick the gel's colony.

3.2 Slide Culture Technique

1. Add the components of YPD agar to sterile distilled/deionized water and autoclave for 15 min at 121 °C.
2. Cool to 45–50 °C.
3. Add the Calcofluor White dye (*see Note 8*).
4. Pour into sterile petri dishes (*see Note 9*).
5. Cut the YPD agar block (~20 × 20 mm) using a sterile dissecting knife.
6. Placed in a sterile microscopy slide.
7. Make the respective dilutions of the yeast suspension (*see Chapter 8*).
8. Inoculate 20 µL of cell suspensions over the YPD block (*see Note 10*).
9. Covered the cells with a 24 × 24 mm sterile coverslip.
10. Placed in a wetting chamber, containing 100 µL of sterile.
11. Incubation at 25 °C (16–24 h) (*see Note 11*).
12. Observed under a light microscope (micro-colonies and single cells) (*see Note 12*).

3.3 PFGE Analysis

1. Grow yeast for 48 h.
2. Transfer some colonies using sterile tips (the tip of the colony tip) to microtubes containing 80 µL of a Lysing-enzymes solution (*see Note 13*).
3. Mix gently with a tip.
4. Prepare the plug agarose: 75 mg of agarose to 6.25 mL of CPE buffer (*see Note 14*).
5. Mix 80 µL of the plug agarose in the microtubes containing the cells with the enzyme (*see Note 15*).
6. Mix gently, avoiding the formation of bubbles, and quickly apply the mixture to the wells (*see Note 16*).
7. Then transfer the plugs to microtubes (identified) containing 0.5 mL of CPE buffer.
8. Incubate 4 h at room temperature.
9. Afterwar, remove CPE buffer solution from the microtubes and add 0.5 mL of solution 3 with proteinase K (*see Note 17*).
10. Keep overnight in a water bath at 50 °C.
11. Prepare a solution of EDTA (186.1 g EDTA (disodium ethylenediaminetetraacetate•2H₂O) and 800 mL distilled water (*see Note 18*).

12. The next day, if the run is not processed, remove solution 3 with proteinase K solution and add 1 mL of 0.5 M EDTA.
13. Store in a refrigerator.
14. Following the run, start washing the plugs with 0.5 mL TE buffer (Tris-HCl 10 mM, EDTA 1 mM pH 8.0): 3 times at 50 °C for 20 min and 4 or 5 times at room temperature for 15 min.
15. Preparation of the gel: (1) Weigh 1.1% agarose and dilute in 170 mL of 1× TAFE buffer (*see Note 19*). Reserve about 5 mL of agarose to weld the wells; (2) After solidifying, remove the comb and place the plugs in the wells. Solder the wells with the remaining agarose in a 65 °C bath; (3) Then place the gel in the tub (CHEF) containing 0.5× TAFE buffer and program the device.
16. Race conditions: *Block 1*—All chromosomes migrate together, release the plug for the gel (initial pulse = 5 s, press second and third buttons simultaneously; final pulse = 5 s, press third and fourth buttons simultaneously; running time = 1 h, run time marks in h; volts/cm = 6 (volts); and chain do not move); *Block 2*—The smallest chromosomes migrate (initial pulse = 60 s; final pulse = 60 s; running time = 8 h; and volts/cm = 6); *Block 3*—The largest chromosomes migrate (initial pulse = 100 s; final pulse = 100 s; running time = 12 h; and volts/cm = 6).

3.4 qPCR Analysis

1. Place the equipment block in the freezer the previous day to keep the samples refrigerated during the assembly of the reaction.
2. Reactions prepared with a final volume of 20–25 µL, and the calculation of the number of reactions (*see Note 20*).
3. Reaction preparation: Each reaction was composed of 12.5 µL of Rotor-Gene PCR SYBR Green Mix Master 2×, 0.8 µM of each primer (forward and reverse), 1 µL of DNA template (standardized at 50 ng), and the volume is made up to 25 µL with ultra-pure water. Example of the number of reactions:

Samples + 6 standard curve points × triplicate = 33 reactions

- (a) 1 reaction = 12.5 µL of Rotor-Gene.
- (b) 33 reactions = X .
- (c) $X = 412.5$ µL of Rotor-Gene (*see Note 21*).
4. Standard curve: For standard curves, yeast species should be grown on YPD agar at 28 °C for 24 h. The cells are counted using a Neubauer chamber. DNA is extracted using the QIAamp DNA Mini Kit and serially diluted (1:10) from 10^8 to 10^3 cells/mL (*see Note 22*).

5. After the ready mix, distribute 24 μL in Eppendorf for analysis and then add 1 μL of template DNA to each Eppendorf.
6. Take the samples for analysis on the equipment.

3.5 MALDI-TOF Analysis

1. Preparation of the matrix: weigh 0.01 g in 1 mL of organic solution (*see Note 23*). Weigh the CHCA (0.01 g in 1 mL) in the Eppendorf, add the organic solution, vortex, and centrifuge for 1 min at 12,000 rpm (keep the matrix in the dark, refrigerated for up to 15 days).
2. Isolated should be growing for 18–21 h.
3. Transfer a small amount of yeast colony with a sterile toothpick to the Eppendorf.
4. Add 6 μL of 2.5% formic acid (2.5 mL of formic acid for 7.5 mL ultra-pure water).
5. Mix with a vortex mixer for 1 min.
6. Place 0.6 μL of the suspension obtained earlier (**step 4**) on the MALDI-TOF plate (specific to the equipment) (*see Note 24*).
7. Add 1 μL of the matrix to each well and mix carefully not to mix the samples.
8. Wait for the plate to dry completely before placing it in the equipment for analysis.

4 Notes

1. Dairy products: Pipet 11 mL or weigh 11 g of sample into 99 mL sterile 0.1% peptone water. Shake 25 times to homogenize. All other foods: Weigh out 25 g of a sample from test portion into a sterile stomacher bag and dilute with 225 mL of 0.1% peptone water; shake at high speed to homogenize.
2. Use appropriate sterile diluents: Butterfield's phosphate buffer (ISO 5541-1), Buffered Peptone Water (ISO), 0.1% peptone water, peptone salt diluent, saline solution (0.85–0.90%), bisulfite-free Lethen broth, or distilled water. Do not use diluents containing citrate, bisulfite, or thiosulfate with 3M Petrifilm RYM Plates; they can inhibit growth. If citrate buffer is indicated in the standard procedure, substitute with 0.1% peptone water, warmed to 40–45 °C.
3. Spread the inoculum over the entire 3M Petrifilm RYM Count Plate growth area before the gel is formed. Do not slide the spreader across the film.
4. To enhance interpretation is allowed for an additional 12 h of incubation time. If a 60-h time-point for interpretation is not convenient, extending the incubation time to 72 h is an acceptable alternative.

5. Yeast colonies appear raised and small with defined edges. Colonies may appear pink/tan to blue/green in color. Mold colonies appear flat with a dark center and diffused edges. Colonies may appear blue/green to variable upon prolonged incubation.
6. Estimation can only be done by counting the number of colonies in one or more representative squares and determining the average number per square. The average number can be multiplied by 30 to determine the estimated count per plate. If a more accurate count is required, the sample will need to be retested at higher dilutions; report the final results in colony-forming units/gram (UFC/g).
7. Example: (a) Food with a uniform blue background color (usually seen in organisms used in cultivated products). These should not be counted (b) intense blue stains (usually seen with spices or granulated products).
8. Calcofluor White dye from a sterile water stock solution of 5.0 mg/mL at a final concentration of 2.5 μ g/mL.
9. Pour 12.5 mL of YPD agar into a sterile 90-mm petri dishes and allow to solidify. The medium should not be too thin to guarantee the availability of nutrients and reduce the possibility of dehydration, important overtime incubation times (16–24 h). On the other hand, it should not be very thick to allow focusing with 100 \times objective
10. Cell density must be high enough to facilitate counting of micro-colonies without overlap, after a long incubation time (16–24 h).
11. A minimum incubation time of 16 h is necessary to assess the viability of the yeast accurately, and the incubation should not exceed 18 h; longer incubation times may result in underestimating the percentage of viable cells.
12. Observe cells by phase-contrast or by epifluorescence microscopy using an epifluorescence microscope; the cells that gave rise to a micro-colony (four cells or more) are deemed as viable. Single (unbudded), double, or triple cells are considered as non-viable.
13. 7 mg of the enzyme (lysing enzyme) for 1 mL of CPES buffer (citric acid 0.210, Na₂HPO₄ 0.426, EDTA-Na₂ 0.186, sorbitol 5.630, and dithiothreitol 0.020 g per 25 mL). The CPES buffer must be filtered and stored in the refrigerator.
14. CPE buffer (for 200 mL add citric acid 1.68 g, Na₂HPO₄ 3.41 g, and EDTA-Na₂ 1.49 g). The buffer must be filtered and stored in the refrigerator. Prepare in a falcon tube and melt agarose in a microwave bath. The falcon tube goes into a Becker with water.

15. Before using the holder, make sure it has gone through the cleaning process (cleaned with 10% hydrogen peroxide and rinsed with sterile distilled water). After applying the plugs, you can put them in the refrigerator for half an hour to solidify more quickly.
16. Agarose should be added at a temperature of 65 °C, so before adding it, keep it in a water bath at this temperature.
17. Solution 3 (for 200 mL add tris 0.24 g, Lauryl sodium sulfate 2.0 g, EDTA-Na₂ 33.5 g) Adjust pH to 9.0 with NaOH. The buffer must be filtered and stored in the refrigerator. Before use, take time before dissolving the SDS.
18. For the preparation of a solution of EDTA: Stir the EDTA into distilled water, adjust the pH to 8.0 with [NaOH solution](#), if you use solid NaOH pellets, you will need 18–20 g of NaOH. Add the last of the NaOH slowly so that you do not overshoot the pH. You may wish to switch from solid NaOH to a solution toward the end for more precise control. The EDTA slowly goes into the solution as the pH nears 8.0. Dilute the solution to 1 L with distilled water and filter the solution through a 0.5-micron filter. Dispense into containers as needed and sterilize in an autoclave.
19. Buffer TAFE 20× (g/L Tris-base 24, EDTA-Na₂ 2.9, glacial acetic acid 5 mL). Autoclave and store in the refrigerator. Dilute in distilled water when using. Comments: Leave the tub already cooling with water (3 L) with a hose passing in a cold bath at approximately 3 °C since the previous day and add the buffer just before the race to cool down too.
20. The calculation of the number of reactions to be made must be carried out carefully. To prepare the mix, calculate the number of reactions and multiply by the volume of the reagent. Always run the standard curve with the samples and perform the entire analysis in triplicate.
21. Mix all reagents (except for template DNA) in a larger Eppendorf and then distribute the volume in the rest of the Eppendorf.
22. The highest cell concentration of the standard curve should be two to three log/cell more than the inoculated. Each point on the calibration curve is measured in triplicate.
23. Organic solution: 33.3% ethanol, 33.3% acetonitrile, 33.3% TFA 10% for 20 mL organic solution. Then you must measure 6.66 mL of ethanol, 6.66 mL of acetonitrile, and 0.66 mL of TFA and make up to 6 mL.
24. Place the yeast solution carefully in each well of the plate, perform the analysis in triplicate, and do not let the samples mix; after adding all the samples, wait for the solution to evaporate.

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