Please cite this article as: Cravener and Mitchell, (2020). *Candida albicans* Culture, Cell Harvesting, and Total RNA Extraction,*Bio-protocol* 10 (21): e3803. DOI: 10.21769/BioProtoc.3803.

bio-protocol www.bio-protocol.org/e3803 Bio-protocol 10(21): e3803. DOI:10.21769/BioProtoc.3803

Candida albicans Culture, Cell Harvesting, and Total RNA Extraction

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[Abstract] Transcriptional analysis has become a cornerstone of biological research, and with the advent of cheaper and more efficient sequencing technology over the last decade, there exists a need for high-yield and efficient RNA extraction techniques. Fungi such as the human pathogen *Candida albicans* present a unique obstacle to RNA purification in the form of the tough cell wall made up of many different components such as chitin that are resistant to many common mammalian or bacterial cell lysis methods. Typical *in vitro C. albicans* cell harvesting methods can be time consuming and expensive if many samples are being processed with multiple opportunities for product loss or sample variation. Harvesting cells via vacuum filtration rather than centrifugation cuts down on time before the cells are frozen and therefore the available time for the RNA expression profile to change. Vacuum filtration is preferred for *C. albicans* for two main reasons: cell lysis is faster on non-pelleted cells due to increased exposed surface area, and filamentous cells are difficult to pellet in the first place unlike yeast or bacterial cells. Using mechanical cell lysis, by way of zirconia/silica beads, cuts down on time for processing as well as overall cost compared to enzymatic treatments. Overall, this method is a fast, efficient, and high-yield way to extract total RNA from *in vitro* cultures of *C. albicans*.

Keywords*: Candida albicans*, RNA extraction, Cell harvest, Fungal transcription, RNA isolation

[Background] The need for fast, reproducible, and efficient RNA extraction techniques has grown significantly over the last several years due to the steady increase in use of RNA sequencing and other expression analysis techniques that have become more affordable and faster with improvements in sequencing technology. There are many different kits and protocols out there from various companies and labs that attempt to meet this need. However, methods that are built specifically for one type of fungi may not be usable for another, and kit platforms can often fall short by way of being too broad in their application. Here we describe a cell culture, harvest, and RNA extraction method for the pathogenic fungus, *Candida albicans*, that utilizes a combination of techniques to give both high yield and high-quality RNA in a consistent and efficient manner.

One of the main attributes unique to this approach is the harvest of cells via vacuum filtration rather than by centrifugation. Centrifugation of a 25 ml culture of filamentous cells, as is used in this protocol, must be done over a period of 5 min in order to pellet the cells enough to aspirate the growth media. This additional time before freezing the cells and halting cellular processes opens the door for unwanted transcriptional changes. Previous studies have shown that yeast cells can alter their expression profiles well within the 5 min that is needed to spin cells down (Dikicioglu *et al.*, 2011). It is then of critical importance to shorten the time between the incubation/growth period and when the cells are frozen, and

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vacuum filtration serves this purpose well. It can take anywhere from 5-10 min between incubation and freezing when using centrifugation, but that time decreases to 1-2 min using vacuum filtration with the most time-consuming step being transporting the samples from the bench to the freezer. Not only does this saved time cut down on transcriptional variation, but it also allows for more samples to be processed in the same amount of time increasing overall throughput for this method.

The second variation in this this approach that differs from other methods is the use of zirconia/silica beads in combination with a lysis buffer for cell disruption as opposed to enzymatic or lysis buffer only methods. Mechanical cell disruption via bead beating has been shown to significantly increase RNA yields in *C. albicans*, compared to vertical vortexing without beads in lysis buffer alone (Rodríguez and Vaneechoutte, 2019). Zirconia/silica beads have a higher density than the typical glass beads used in bacterial (3.7 g/cm^3 and 2.5 g/cm^3 respectively) which increases their efficiency at rupturing the tough fungal cell walls. An additional benefit of mechanical cell disruption is that enzymatic methods such as zymolyase digestion have been shown previously to alter RNA expression profiles of the sample being assayed by activating stress response pathways thereby confounding the downstream analysis (Suzuki and Iwahashi, 2013).

Overall, by utilizing mechanical cell disruption and vacuum filtration combined with the commercially available Qiagen RNEasy MiniKit, this technique represents a fast, and efficient method for cell harvesting and RNA extraction saving time and giving more consistent and reliable transcriptional data.

Materials and Reagents

1. 15 ml polypropylene culture tubes (VWR, catalog number: 82050-274, item #187262)
   Note: Can be from any source.
2. 25 ml serological pipettes (VWR, catalog number: 89130-912)
   Note: Can be from any source.
3. 50 ml conical screw cap tube (Fisher Scientific, catalog number: 0553913)
   Note: Can be from any source.
4. 1.5 ml screw cap tubes (Fisher Scientific, catalog number: 1415-8700)
   Note: Can be from any source.
5. RNase-free 1.5 ml microcentrifuge tubes (Fisher Scientific, catalog number: 14-666-319)
   Note: Can be from any source.
6. 250 ml 0.1 µm PES membrane vacuum filtration unit (Fisher Scientific, catalog number: 09741201)
   Note: Can be from any source.
7. Cuvettes PS Semi-micro (VWR, catalog number 9700-586)
   Note: This is dependent upon the method of measuring the OD_{600} of cell cultures to be used.
8. Uvette 220-1600 nm (Eppendorf, catalog number: 952010051)
   Note: This is dependent upon the method of quantifying purified RNA.
9. 0.5 mm zirconia/silica disruption beads (Research Products International, catalog number: 9834)

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Follow manufacturer instructions to sterilize and eliminate nucleic acid contamination and store at -20 °C

10. MF-Millipore™ 0.45 µm, 47 mm diameter gridded filter membrane (Merk Millipore Ltd., MF-Millipore™, catalog number: HAWG04700)
11. Relevant *C. albicans* strains (SC5314 and an SC5314 derived *efg1Δ::HIS1* mutant strain constructed by the authors were used in this example)
12. Yeast extract (BD, Bacto™, catalog number: 212750)
13. Peptone (BD, Bacto™, catalog number: 211677)
14. Dextrose (Sigma Life Science, catalog number: D9434-1KG)
15. RPMI 1640 (Sigma-Aldrich, catalog number: R4130-10L), store at 4 °C
16. Fetal Bovine Serum Premium (Atlanta Biologicals, R&D Systems, catalog number: S11150H), store at -20 °C
17. Phenol:chloroform:isoamyl alcohol (Sigma Life Science, catalog number: 77617-100ml), store at 4 °C
18. Qiagen RNEasy Mini Kit (Qiagen, catalog number: 74104)
19. NaOH salt (Sigma-Aldrich, catalog number: 221465-500G)
    Note: Can be from any source.
20. 100% pure Ethanol (Sigma-Aldrich, catalog number: E7023-1L)
    Note: Can be from any source.
21. Deionized H_2O (any source)
22. β-mercaptoethanol (Sigma-Aldrich, catalog number: 444203-250mL)
    Note: Can be from any source.
23. YPD liquid growth media (see Recipes)
24. 10 N NaOH (see Recipes)
25. RPMI + 10% FBS (see Recipes)

Equipment

1. Glass 125 ml Erlenmeyer flasks (Fisher Scientific, catalog number: FB501125)
   Note: Can be from any source.
2. 30 °C incubator (any source)
3. Rotator drum for overnight cultures (New Brunswick Scientific, model: TC-7, catalog number: M1053-4004)
   Note: This is out of production but can be substituted for a similar rotator that can reach 60-70 rpm.
4. Eppendorf BioPhotometer® D30 (Eppendorf, catalog number: 6133000010)
   Note: This can be substituted for another spectrophotometer with similar capabilities.
5. Shaking incubator 37 °C (New Brunswick Scientific, Eppendorf, model: I-26, catalog number: M1324-0000)

Note: This can be substituted for another shaking incubator with similar capabilities.

6. Microcentrifuge (Thermo Scientific, model: Sorvall Legend Micro 21, catalog number: 75002436)
   Note: This can be substituted for another microcentrifuge that can spin at ≥ 17,000 x g.
7. 1-2 L sidearm Erlenmeyer flask (any source)
8. MiniBeadBeater-16 (BioSpec Products, model: 607)
   Note: The authors have not attempted this protocol with another type of bead beater, however a similar horizontal bead beater with similar specifications would most likely yield similar results.
9. Millipore 47 mm glass base and stopper (Millipore, catalog number: XX1014702)
10. 50 ml conical tube rack (any source)
11. -80 °C and -20 °C freezer (any source)
12. 4 °C refrigerator or cold room (any source)
13. Standard benchtop vortexer (any source)
14. Forceps

Procedure

Note: This procedure is written with volumes and quantities for two strains, mutant and Wild Type (WT), with three replicates each for a total of six independent samples.

A. Cell culture and harvesting
   1. Inoculate WT and mutant strains in 5 ml liquid YPD and incubate overnight at 30 °C with 60 rpm rotation.
   2. Pre-warm 200 ml RPMI + 10% FBS and empty sterile 125 ml Erlenmeyer flasks overnight (O/N) 37 °C, or for a minimum of 2 h prior to Step A3 with more time for larger volumes.
   3. Aliquot 25 ml pre-warmed RPMI + 10% FBS into each of the 6 pre-warmed 125 ml flasks and return to 37 °C incubator to stabilize temperature at least 90 min before first inoculation.
   4. Vortex O/N WT culture thoroughly, measure OD600 using Eppendorf BioPhotometer and Cuvettes PS Semi-micro, and inoculate first pre-warmed flask of RPMI + 10% FBS to a final OD600 of 0.2 from the O/N WT culture.
   5. Immediately transfer to I-26 air shaker incubator for 4 h at 37 °C and 225 rpm rotation. Replace O/C in 30 °C incubation.
   6. Wait 8-10 min between inoculation of replicates.
      Note: This gap is to allow for adequate time for cell harvesting between replicates. Time can be shortened or lengthened as needed to allow for working speed of person performing protocol.
   7. Repeat Steps A4-A6 for WT replicates 2 and 3 as well as mutant replicates 1-3.
   8. This leaves roughly 3 h until the first culture is ready to harvest. Take this opportunity to set up cell harvesting equipment.
      a. Connect 1-2 L sidearm flask to a vacuum line and trap and mount the Millipore 47 mm glass base and stopper for vacuum filtration in the top of the flask.
      b. Turn on vacuum and wash filter base 2x with dH_2O and 2x with 70% EtOH to eliminate

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debris and allow filter base to dry before applying filter membrane.

c. Chill 50 ml conical tube rack at -80 °C.

d. Pre-chill and label 6 50 ml conical screw-cap tubes in ice.

9. Just before 4-hour timepoint apply MF-Millipore™ 0.45 µm, 47 mm diameter gridded filter membrane to filter base grid side up using sterile flat-blade forceps and turn on vacuum (Figure 1).

Figure 1. Cell harvest filtration setup. Millipore 47 mm glass base and stopper inserted into sidearm flask and connected to vacuum line. Wash glass base thoroughly with water and 70% ethanol with vacuum running and let dry. Place MF-Millipore™ 0.45 µm, 47 mm diameter gridded filter membrane onto glass base. In-line trap and filter are suggested to avoid carryover of waste.

10. At 4 h, remove the WT replicate 1 flask from the shaker and swirl gently to dislodge cells stuck to the sides of the flask.
11. Quickly, use a 25 ml serological pipette to transfer entire culture to filter membrane as fast as the vacuum will allow without overflowing the filter membrane.
12. As soon as the liquid has been pulled off of the cells, transfer filter membrane to bottom of a pre-chilled 50 ml conical tube using sterile flat-blade forceps, and place immediately in -80 °C freezer.
13. Clean the filter base as before with dH_2O and 70% EtOH.
14. Repeat Steps A9-A13 for each of the remaining WT and mutant replicates.
    Note: These steps are time sensitive. Shortening the time between when the cells are incubating to when they are frozen at -80 °C is critical for accurate expression data.
15. Incubate cells at -80 °C for at least 1 h to ensure they are frozen before proceeding to RNA extraction.

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Note: Cells are stable at this stage for several days to weeks, so if many samples are needed, cell culturing and harvesting can be split up over multiple days prior to RNA extraction.

B. RNA extraction and quantification

1. Aliquot 300 µl 0.5 mm zirconia/silica disruption beads and 600 µl 25:24:1 phenol:chloroform:isoamyl alcohol to 1.5ml screw cap tubes and chill at 4 °C for at least 1 h (1 tube per cell culture replicate). Prepare fresh 600 µl RLT (Qiagen RNeasy Mini Kit) + 1% β-mercaptoethanol (BME) per sample and pre-chill in ice. Pre-chill 2 ml sterile dH_2O per sample on ice. Pre-chill 1.5 ml microcentrifuge tubes (1 per sample) on ice.
   Note: Steps B2-B10 must be done at 4 °C. Keep materials on ice or work in a cold room.
2. Remove 50 ml conical tube containing filter membrane and cells from -80 °C freezer and thaw on ice for 10 min.
3. Wash cells off of filter membrane with 900 µl of chilled sterile dH_2O and vortex at top speed for 30 s. Transfer suspension to chilled 1.5 ml microcentrifuge tube and place on ice.
4. Wash filter a second time with an additional 900 µl of chilled sterile dH_2O and vortex at top speed for 30 s. Transfer suspension to the same chilled 1.5 ml microcentrifuge tube and place on ice.
5. Repeat Steps B3 and B4 for all samples using a new tube for each sample.
6. Centrifuge in standard desktop microcentrifuge at max speed (≥ 17,100 *x g*) for 30 s and discard supernatant.
7. Add 600 µl prepared RLT + 1% BME solution and resuspend by vortexing at max speed.
8. Transfer 600 µl of cell suspension to chilled screwcap tubes with zirconia beads and phenol:chloroform:isoamyl alcohol.
9. Bead beat for 3 min at 4 °C using the BioSpec MiniBeadBeater-16 Model 607.
10. Centrifuge cells at max speed for 8 min at 4 °C in standard desktop microcentrifuge.
11. Transfer 550 µl of the aqueous layer to new RNase-free 1.5 ml microcentrifuge tube and add 550 µl 70% EtOH and mix by inverting 6 times.
    Note: If there is not 550 µl of aqueous solution, measure and transfer to the new tube and add an equal volume of 70% EtOH.
12. Transfer up to 700 µl of sample to RNeasy spin column and centrifuge for 15 s at ≥ 8,000 *x g*. Discard flow-through. Reload column with remaining sample and spin again discarding flow-through.
    Note: All supplies referred to in Steps B12-B17 are provided in the Qiagen RNeasy Mini Kit Protocol for the Purification of Total RNA from Yeast. Steps B12-B16 of this protocol are the same as steps 2-5 of the Qiagen RNeasy Mini Kit protocol for Purification of Total RNA from Yeast.
13. Add 700 µl Buffer RW1 to column and spin for 15 s at ≥ 8,000 *x g*. Discard flow-through.
14. Add 500 µl Buffer RPE (with Ethanol added) to column and spin for 15 s at ≥ 8,000 *x g*. Discard flow-through.
15. Repeat Step B14.

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16. Transfer spin column to a new collection tube and spin for 1 min at ≥ 8,000 *x g*. Discard flow-through.
17. Transfer spin column to RNase-free 1.5 ml microcentrifuge tube (from kit). Add 40 µl RNase-free water to the column membrane and centrifuge for 1 min at ≥ 8,000 *x g*.
18. Take 40µl eluate from Step B17 and re-apply to the spin column membrane and spin again for 1 min at ≥ 8,000 *x g*.
    Note: This is not strictly necessary but will increase overall concentration.
19. Freeze samples immediately at -80 °C for storage.
20. To quantify RNA and assess purity, prepare 1:500 dilution of total RNA and measure OD_{260} using Eppendorf BioPhotometer and Uvette 220-1,600 nm cuvettes.
    Notes:
    a. *Average yield is typically over 2 µg/µl up to around 6 µg/µl. 260/280 and 260/230 values typically range between 2.0-2.2 and 2.5-2.5 respectively.*
    b. *Dilution and use of Uvettes may not be necessary depending on equipment available.*

Notes

1. This protocol is written to accommodate 6 samples (2 strains with 3 replicates each) but is scalable given that it can be stretched over multiple days while the harvested cells are frozen. It can also be adapted for whatever media conditions outside of RPMI + 10% FBS. However, with the addition of more replicates and strains it is recommended that a single large batch of media is made, if possible, ahead of time to eliminate batch effects on the transcriptional profile of the strains. For example, here we call for 200 ml of RPMI + 10% FBS for 6 samples which allows for 50ml extra. If 60 samples were needed instead, 1.5 L of RPMI + 10% FBS should be made up in one large batch then aliquoted into smaller units for use over several days.
2. This protocol does not include a DNase treatment. This means that the final product may have some DNA contamination. The Qiagen RNeasy Mini Kit spin columns prevent most DNA carry-over by design, but residual DNA may remain. If DNA contamination must be avoided for downstream applications, DNase treatment may be done on the spin column between Steps B12 and B13 or after final elution, and a protocol for this can be found in the Qiagen RNeasy Mini Kit protocol.

Recipes

1. YPD liquid growth media
    a. Dissolve 20 g dextrose, 20 g peptone, and 10 g yeast extract with 500 ml dH_2O
    b. Bring volume to 1 L with dH_2O
    c. Autoclave to sterilize
2. 10 N NaOH

a. Dissolve 40 g NaOH in 50 ml dH_2O
b. Bring volume up to 100 ml with dH_2O

3. RPMI+10% FBS
   a. Dissolve 3.28 g RPMI1640 powder in 180 ml dH_2O
   b. Adjust pH to 7.4 using 10 N NaOH (approximately 180 µl)
   c. Sterilize RPMI solution by vacuum filtration
   d. Thaw Fetal Bovine Serum Premium (FBS) in 37 °C water bath
      Note: The FBS manufacturer recommends avoiding repeated freeze thaw cycles, so plan accordingly.
   e. Shake gently to resuspend any solids
   f. Add 20 ml thawed FBS to 200 ml RPMI solution and mix with gentle shaking to avoid foaming
   g. Store at 4 °C for up to a month

Acknowledgments

Work on *C. albicans* RNA in this lab is supported by NIH grants 1R21AI144878-01 and 1R01AI146103 (PI: Mitchell). This protocol evolved from protocols in use in the Mitchell lab since 1988.

Competing interests

The author declare that no competing interests exist.

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