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Purification of Wild Type eEF2 from pelleted yeast

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Clark Fritsch[1]

[1]University of Pennsylvania

Clark Fritsch
University of Pennsylvania

COMMENTS 0

ABSTRACT

The purpose of this protocol is to describe how to purify endogenous eEF2 from Saccharomyces cerevisiae (budding yeast). The protocol can be completed in two days, but three days should be budgeted to complete the protocol. When possible, all steps of the protocol should be carried out at 4 C to prevent protein degradation.

PROTOCOL CITATION

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Yeast Culture

1 To begin, you will want to inoculate a single colony of wild-type or modified yeast in 25 mL of YAPD medium and grow for 16 hours at 30 C. By the time that the incubation is complete, the cells should be in stationary phase and be at a high enough cell number to allow you to re-inoculate the yeast into a larger culture volume.

2 After the initiation 16 hour incubation, re-inoculate the yeast so that the optical density (OD) of the culture is 0.1 - 0.2. For large scale purifications of eEF2, it is advisable to begin with at least 6 liters of culture, as this will give you enough starting material to work with for downstream experiments. You can of course use less starting material, but using less than 2 liters of culture will make it difficult to obtain sufficient amounts of eEF2 for any experiments that you want to do later on.

3 Grow the culture for at least 16 hours and then measure the OD and record it below:

Starting OD for Yeast Culture:

Final OD for Yeast Culture:

4 Transfer the culture to appropriately sized centrifuge tubes and pellet the cells by centrifuging at 3,000 rcf for 10 minutes. After centrifuging the cells, pour off the supernatant and resuspend the cells in milliQ water. Because of the large volume of your culture, it will be necessary to split the cells into several centrifuge tubes and pellet them separately at first. It is best to use this opportunity to gather as many of the cells as possible in one centrifuge tube. Continue to pellet your cells and wash with milliQ water until you have all of your cells pelleted in one centrifuge tube.

5 Once you have pelleted your cells, you can either freeze them in liquid nitrogen and store at -80 C for future use or you can proceed to the cell lysis step of the protocol. Regardless, record the weight of your cell pellet below:

Weight of Empty Tube:

Weight of Tube + Cell Pellet:

Weight of Cell Pellet:

Cell Lysis, Centrifugation and Dialysis

6 If you have frozen your cells, you will need to thaw them before proceeding with the protocol. If you have freshly prepared your cell pellet, then you can continue with the protocol uninterrupted.

7 Resuspend your cell pellet in **Buffer S-300**. The volume that you resuspend your pellet in may vary depending on the size of your pellet. However, it is wise to use as little buffer as possible to prevent the volume of your lysate from getting too large. You can start off by resuspending your pellet in ~80 mL of **S-300 buffer**. Regardless, when you are resuspending your pellet, you need to make sure that the pellet is fully resuspended and that there are no clumps present in your sample.

8 Once your have resuspended your pellet, you will lyse your cells as described in the *"Protocol for Using the*

Microfluidizer Lysis Apparatus" document. Note that the volume of your lysate will increase because you have to wash the microfluidizer apparatus with additional **S-300 buffer** following lysis to retrieve all of your lysate from the sample chamber of the machine. Assuming that you start with an initial volume of ~80 - 100 mL for your cell suspension, you should expect to get ~150 mL of lysate following lysis with the microfluidizer.

Volume of Crude Cell Lysate:

9 Note that following lysis your lysate should become slightly darker in color. Continue on by titrating the pH of your lysate to a pH of 7.0 using **1 M Tris (not titrated)**. You can obtain the pH of the lysate by using a normal pH meter. The lysate is acidic (pH = ~6.0). You should pH your lysate while stirring. It is very important to get the pH of your lysate to 7.0. Don't go too high, though, as this will prevent binding to the cation exchange column.

Initial pH of Crude Cell Lysate:

Final pH of Crude Cell Lysate:

Volume of **1 M Tris (not titrated)** Used:

10 Continue by pelleting the cell debris by centrifuging at 13K rpm with a FAS14C rotor at 4 C for 15 minutes. Then centrifuge again at 15K rpm with an SS34 rotor at 4 C for 15 minutes. Do not proceed if the lysate looks creamy. The perform a final spin using a Ti45 rotor at 45000 rpm for 1 hour at 4 C. Note that from this spin you will get a big pellet and a top phase with lipids. Avoid transferring any of this when pooling supernatants. Collect cleared supernatant in a beaker on ice.

Volume of Cleared Supernatant:

11 To get eEF2 to bind on a cation exchange column, you need very low salt concentration in the protein buffer. Because of this, you need to dialyze the Cleared Supernatant against 4 L of **Buffer S** for 3 hours and then against 4 L of
Buffer S overnight at 4 C while stirring. This should get the salt concentration below 20 mM. For the dialysis, use the SPECTRA/PRO dialysis bag with a molecular weight cutoff of 12000 - 14000 Daltons.

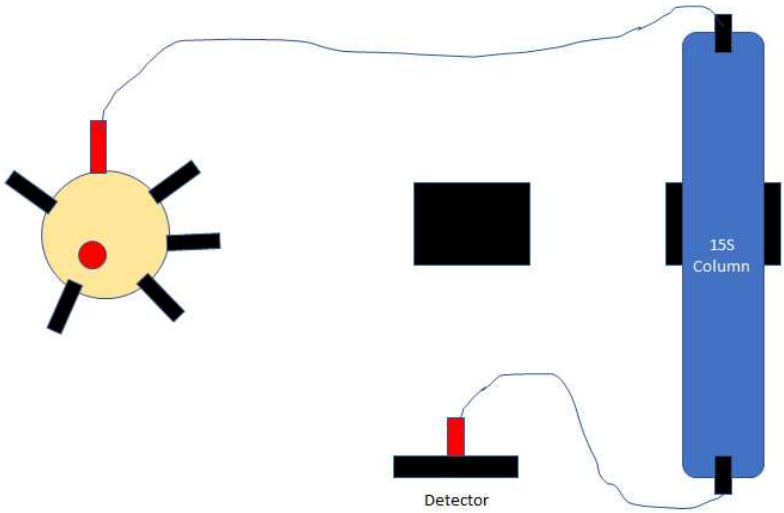
12 Following the overnight dialysis, you will notice that a good amount of protein has precipitated out of your cleared supernatant. To get rid of the precipitated protein, pool the dialyzed protein and centrifuge for 20 minutes at 30000 rcf at 4 C.

13 Then filter the supernatant twice (first through 0.45 um then 0.22 um pore size filters) so as to not clog the columns.

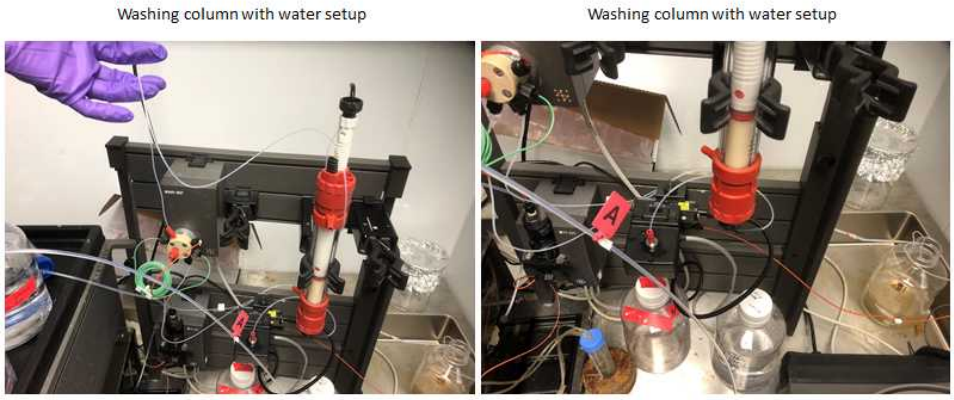
Volume of Supernatant:

Washing the Source 15S Column (GE)

14 Attach your Source 15S Column to the FPLC as illustrated in the following images:



Cartoon diagram of the connections from the Source 15S column to the FPLC.



Images of the connections between the Source 15S column and the FPLC.

15 Before loading your sample onto the Source 15S column you need to wash it several times. It is recommended that you start out by washing the column with distilled water. You can program the FPLC to do this by using the following steps (after you have turned the UV lamp of the FPLC on):

1. Insert buffer lines A and B into a container of distilled water.
2. In the FPLC program, go to the "Manual" dropdown menu and select "1. Pump".
3. Flow --> Change to 1.00 mL / min
4. Press Insert

5. Change to "Gradient" --> Target: 100% B; Length: 0.10 min

6. Press Insert

7. Manual --> Pump --> Pump Wash

8. Turn Pump A on and Pump B on

9. Press Insert

10. Manual --> Other --> End Timer

11. Set timeout to 12.0 minutes, although the longer that you wash the column with water the better.

12. Press Insert

13. Sanity Check: Flow: 1.00; Gradient: 100, 0.10; Pump Wash on, on; End_Timer Acc time, 12:0

14. Press Execute

15. Press Close

16 After you have washed the Source 15S column with distilled water, you also need to wash the column with 100%
S-300 Buffer (high salt) to get rid of any remaining junk on the column. To this as follows:

1. Insert buffer lines A and B into your container of **S-300 Buffer**.

2. In the FPLC program, go to the "Manual" dropdown menu and select "1. Pump".

3. Flow --> Change to 1.00 mL / min

4. Press Insert

5. Change to "Gradient" --> Target: 100% B; Length: 0.10 min

6. Press Insert

7. Manual --> Pump --> Pump Wash

8. Turn Pump A on and Pump B on

9. Press Insert

10. Manual --> Other --> End Timer

11. Set timeout to 12.0 minutes

12. Press Insert

13. Sanity Check: Flow: 1.00; Gradient: 100, 0.10; Pump Wash on, on; End_Timer Acc time, 12:0

14. Press Execute

15. Press Close

17 Finally, after washing the Source 15S column with **S-300 Buffer**, you want to equilibrate the column with **S-20 Buffer** (low salt) to prepare the column for your sample. Do this as follows:

1. Insert buffer lines A and B into your container of **S-20 Buffer**.

2. In the FPLC program, go to the "Manual" dropdown menu and select "1. Pump".

3. Flow --> Change to 1.00 mL / min

4. Press Insert

5. Change to "Gradient" --> Target: 100% B; Length: 0.10 min

6. Press Insert

7. Manual --> Pump --> Pump Wash

8. Turn Pump A on and Pump B on

9. Press Insert

10. Manual --> Other --> End Timer

11. Set timeout to 12.0 minutes

12. Press Insert

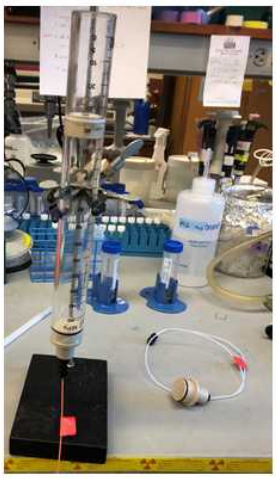
13. Sanity Check: Flow: 1.00; Gradient: 100, 0.10; Pump Wash on, on; End_Timer Acc time, 12:0

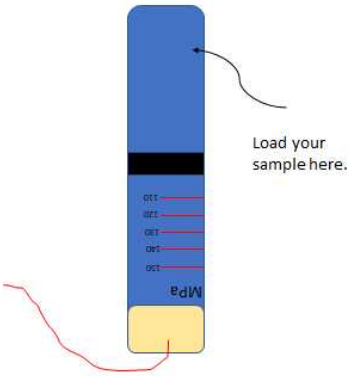
14. Press Execute

15. Press Close

Loading Protein Solution into Sample Loop and Connecting Sample L

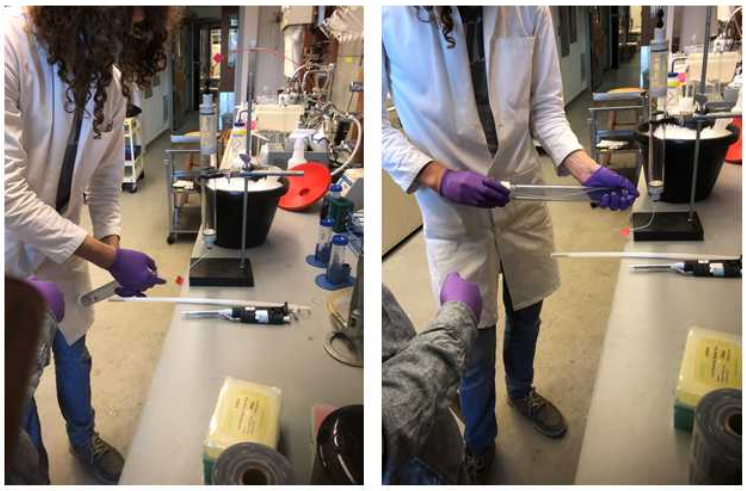
18 While you are washing your Source 15S column, you can add your sample to the sample loop as illustrated in the following images:

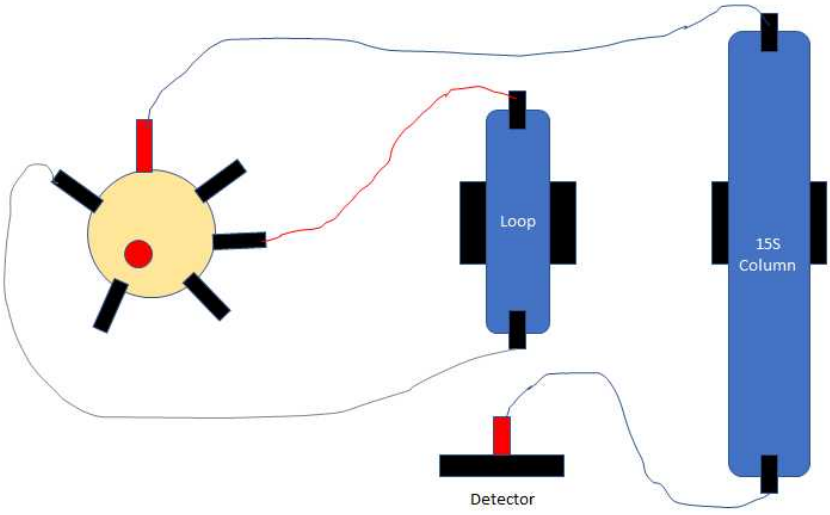




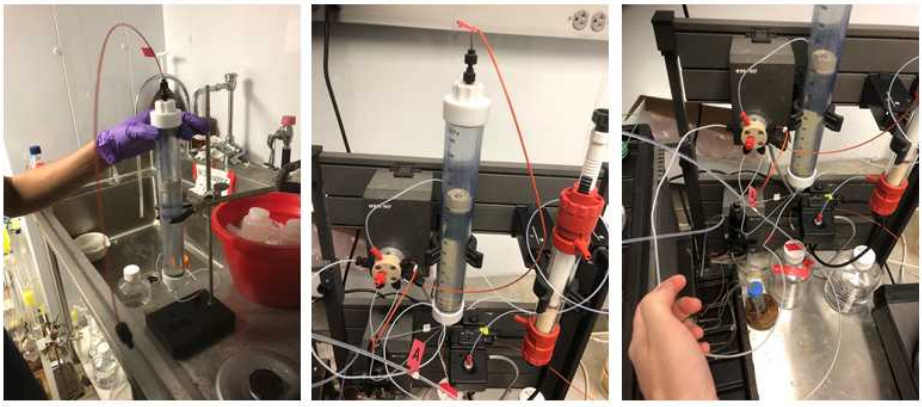
19

20





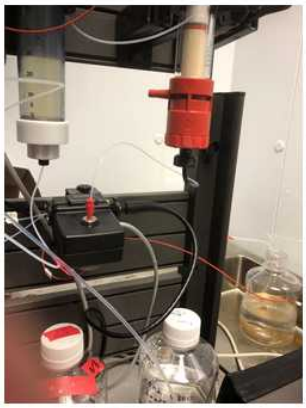
Cartoon diagram of the connections between the sample loop and the FPLC.



Orientation of sample loop with sample loaded into it before putting on the FPLC

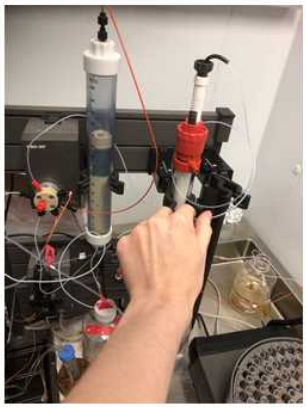
Setup with the tubes of the sample loop and Source 15S column in their proper positions

Setup with the tubes of the sample loop and Source 15S column in their proper positions



Setup with the tubes of the sample loop and Source 15S column in their proper positions

Setup with the tubes of the sample loop and Source 15S column in their proper positions



Setup with the tubes of the sample loop and Source 15S column in their proper positions

Loading Sample onto Source 15S Column

21 Now that you have equilibrated the Source 15S column with the **S-20 Buffer** and have positioned your sample loop onto the FPLC, you are ready to load your sample onto the column. You can do this by setting the FPLC program as follows:

1. Manual --> Flow --> Flowrate: 5.0 mL / min
2. Press Insert
3. Flow Path --> Injection Valve --> Position: Inject
4. Press Insert
5. Other --> End_Timer --> Timeout: 60 minutes
6. Press Insert
7. Execute

NOTE: We used the above settings for the FPLC program while the top part of the sample loading loop was still being filled with **S-20 Buffer**. After the top of the sample loading loop was filled, we switched the flow rate from 5.0 mL / min to 2.0 mL / min for the remainder of the sample loading step. If the pressure for the column does not go above 1.5 MPa and stays at about a pressure of 1.0 MPa, then you can leave the flow rate at 5.0 mL / min.

NOTE: When the sample is close to being finished loading, make sure that you don't inject any bubbles from the sample loop into the column. If you have to leave a bit of sample in the sample loop to prevent bubbles getting into the column, then that is fine. Just stop the program prematurely.

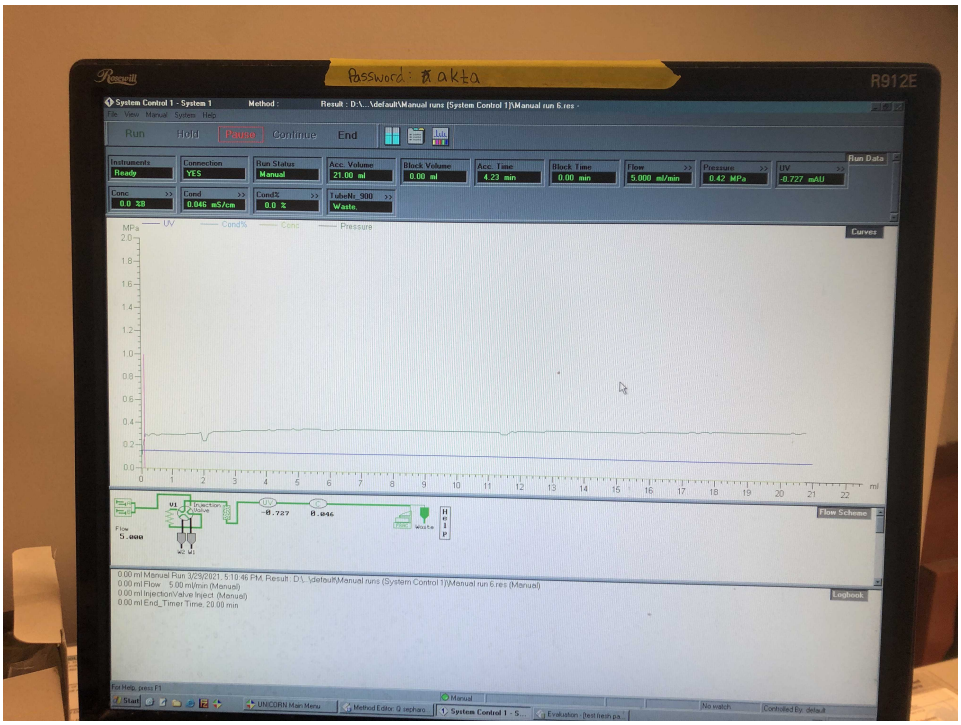


Image of what the UV signal tends to look like while loading the Source 15S column before your actual sample has reached the column.

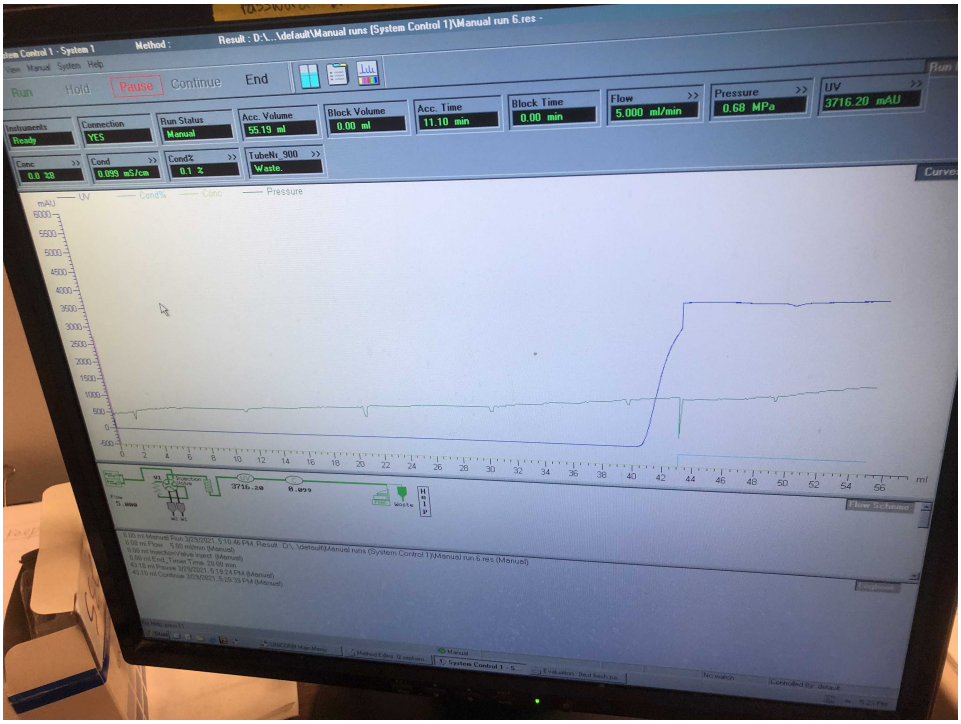


Image of what the UV signal tends to look like while loading the Source 15S column after your sample has reached the column and non-binding material is passing through the column to reach the detector.

Purification of eEF2 using Source 15S Column

22 After you have loaded your sample onto the Source 15S column, you are now ready to actually purify the eEF2. Begin by inserting Buffer Line A into the **S-20 Buffer** and Buffer Line B into the **S-300 Buffer**.

23 Then prepare the carousel for the FPLC by inserting new tubes into the carousel and adding PMSF so that the final concentration of PMSF in each of your fractions is 0.1%. This should help minimize degradation of your sample if you are not able to immediately proceed from the Source 15S column to the Source 15Q column.

24 Unlike for the washing steps, we already have a pre-made program for the purification of eEF2 using the Source 15S column. The program is called "**Source 15S 10 mL ver03**". Find this program after entering the FPLC program, click it, and then click "okay". Make sure to save the results in a separate directory so that you can access them later on if necessary.

25 After opening the program, you can then run it by clicking "Start". Make sure that the sample is going into the carousel properly and make sure to add new tubes to the carousel before starting.

26 Once you have finished fractionating your sample, wash the column first with distilled water and then with 20% ethanol using the same washing steps as described above. Then cap the tubes on either end of the column and store it in a safe place.