

Yeast Vacuole Isolation and Fusion Assay

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This protocol describes the isolation and fusion of vacuoles from yeast. It has been derived from the original method as published by Albert Hass:

Methods in Cell Science **17**: 283-294 (December 1995)

Contents

Section I	Vacuole Isolation	Page 2
Section II	The Fusion Assay	Page 7
Section III	Reagent Preparation	Page 9
Section IV	Pure Components	Page 15
Section V	Vacuole Isolation Worksheet	Page 16
Section VI	Fusion Unit Calculations	Page 17

Section I

Yeast Vacuole Isolation

Reagents

- 1M Tris-Cl pH 9.4
- 1 M DTT
- 0.2% YPD (specifically YP with 0.2% dextrose)
- Dextran
- Oxalyticase
- 1M Potassium Phosphate pH 7.5
- 1M Pipes KOH pH 6.8
- 4M Sorbitol
- 0% Ficoll /PS Buffer
- 4% Ficoll /PS Buffer
- 8% Ficoll /PS Buffer
- 15% Ficoll /PS Buffer

Gear

- High-Speed Centrifuge with SW41 or 40 Rotor and
- Low-speed Centrifuge with rotors to handle 500ml) and
- 38 ml oak ridge tubes
- 30 °C Waterbath
- Spectrophotometer (Beckman DU-500 used here)

Preculture

On the morning before the isolation, inoculate 20 ml of YPD in 50 ml culture flasks with single colony of BJ 3505 and DKY 6281. Grow the cultures at 30°C with shaking at 225 RPM. Towards the end of the day, the cultures should reach an OD 600 nm of about 0.4. At this point inoculate 1 liter of YPD in a 2 liter flask with enough of each culture so that at a convenient time during the following morning, the cultures will reach an OD 600 of about 0.52-0.65 OD for BJ and 0.60-0.8 for DKY. Note that the doubling time of the BJ Strain is about 110 min. and about 90 min. for the DKY strain. Note: For 15 hours, seed BJ at 1.4 ODml / liter and for DKY use 0.52 ODml / liter. Incubate for the appropriate time at 30°C with shaking at 225 RPM.

Vacuole isolation Procedure

Step 1 Harvest the cultures

Harvest the yeast cultures between 0.52-0.65 OD for BJ and 0.6-0.8 for DKY. Centrifuge the cultures in 500 ml bottles at 3,000 x g (5,000 RPM in Beckman JA-10 bottles) for 5 min. at 24°C. Decant the supernatant and add the other half-liter of culture. Recentrifuge as before for a maximum of 900 OD ml. per tube. Calculate the actual total OD ml for each strain on the **Vacuole Preparation Worksheet** (included).

Step 2 Wash the cultures

Resuspend the cultures in 50 ml. of **Wash Buffer** (see **Reagent Preparation**). Gently vortex the bottle to resuspend the culture and incubate the bottle in a 30°C waterbath for 10 minutes. Swirl occasionally. Centrifuge at 3,000 xg for 3 min. at 24°C to recover the cultures. Dump the supernatant.

Step 3 Spheroplasting the yeast by treatment with oxalyticase

Resuspend the yeast pellet in 15 ml (per liter) of **Spheroplasting Buffer** (see **Reagent Preparation**) with gentle vortexing. Add the appropriate amount of **Oxalyticase Solution** (see **Vacuole Preparation Worksheet**) and swirl to mix. Incubate the tubes at 30°C for 30 min. with occasional swirling. Prepare the **Dextran Solution** (see **Reagent Preparation**)

Step 4 Treatment with Dextran

Centrifuge the spheroplasted yeast cultures by transferring the lysate to the pre-chilled oak ridge tubes with a 25 ml glass pipette. Centrifuge for 2 min. at approx. 1,000 xg (3,000 RPM for Beckman JA 20) at 4°C. Alternately, centrifuge the cultures for 2 min. at 2500 RPM and turn the speed up for an additional 2 min. at 3,500 RPM. Carefully aspirate ALL of the sup't. Note: the pellet is loose and cannot be decanted. Resuspend the pellet in 2.5 ml of 15% ficoll using vigorous swirling and avoid making bubbles. This can take several minutes. A little of the pellet will always be recalcitrant, leave it. Alternately, vortex the tubes on a low setting for 2 x 15 second intervals. Add the appropriate amount of Dextran solution (see **Vacuole Preparation Worksheet**). Incubate on ice for 2 minutes with occasional swirling. Transfer the tubes to the 30°C waterbath and incubate for 3 min. Swirl occasionally and then put back on ice.

Step 5 Floating the Vacuoles

The vacuoles are floated on a four-tiered gradient of ficoll. Begin the gradient by pipeting up to 6 ml. of the lysate/15% ficoll solution in the bottom of the high-speed centrifuge tube. Overlay this layer with 2.5 ml. of 8% ficoll And then with 2.5-3.0 ml. of 4% ficoll to about 1 cm from the rim of the tube. Now fill the SW 40 (or SW41) tube with 0% ficoll (PS Buffer) to within 3 mm of the top of the tube. The volume should be about 2 ml. Centrifuge the gradients at 175,000-182,000 xg (32,000 for the Beckman SW41) for 90 minutes at 4°C.

Step 6 Harvesting the Vacuoles

Recover the vacuoles from the interface of 0% and 4% layers of the gradient with a P200. Withdraw in 200 ul batches. Place in a Falcon 2063 tube or standard 1.5 ul microfugte tube on ice. Note: avoid diluting the vacuoles at this step.

Step 7 Quantitate the Vacuoles

Quatitate the vacuoles by standard Bradford assay on 5 ul of the suspension. Dilute the vacuoles to 0.3 mg/ml or alternately add to the assay as a more concentrated solution in order to leave more room for inhibitors and other components. The yield of vacuoles typically ranges from 0.5 to 1.2 ug of vacuole protein for each OD ml of starting cells.

Step 8 Freezing the Vacuoles

The vacuoles can be frozen for later use. Add glycerol from a 50% glycerol (in PS buffer) solution to a final concentration of 10%. Vacuoles can be frozen at concentrations of up to 0.6 mg/ml (highest tested) with the additional volume being PS buffer. Add the vacuoles dropwise to liquid nitrogen to form “BB’s” and store at –80°C. These vacuole BB’s are conveniently stored in wide mouth Nalgene containers and handled with small ceramic spoons (Fisher). Vacuoles stored in this way have retained activity for up to 2 years (maximum tested).

Section II

The Fusion Assay

Reagents

10X Salt
ATP Regenerating System
Pure Components
Developer Solution
Stop Solution

Gear

27°C Waterbath
30°C Waterbath
Spectrophotometer

Procedure

The fusion reactions are carried out in standard microfuge tubes. All tubes and solutions are kept on ice during the set-up of the assay. The final volume of the reaction is usually 30 ul however it can range between 28 and 38 ul. Master mixes of various components are routinely used. Often, the “10X salt’ solution, ATP regenerating system and various “pure components” are added this way. A 20 X salt solution can also be used. The vacuoles are added at a final concentration of 3 ug of each type per reaction. Preparations of yeast cytosol and protease inhibitor cocktail as described in the original publication are not necessary. Reaction volumes are equalized with PS buffer. Thus a typical reaction contains:

10X Salt	3 ul
ATP Regenerating System	3 ul
BJ Vacuoles (0.3 mg/ml)	10 ul
DKY Vacuoles (0.3 mg/ml)	10 ul
Pure components, inhibitors, PS Buffer, etc.	0-12 ul

The tubes are mixed gently and incubated in a 27°C waterbath for 90 min. At the end of the assay, add 470 ul of **Developer Solution** at timed intervals, vortex gently and incubate in a 30°C waterbath for 5 min. Next add 500 ul of **Stop Solution** at timed intervals, vortex gently and then read the AB 400 nm of the reaction. Finally calculate the units of fusion activity (see section VI).

Section III

Reagent Preparation

1M Tris-HCl pH 9.4 500 ml

60.5 g Tris-Base ICN819638
Add 400 ml dH₂O
Adjust PH to 9.4 with HCl
Bring to 500 ml. with dH₂O
Store at Room Temp.

1M DTT 100 ml

15.4g DTT Sigma D0632
Dissolved in dH₂O and brought to 100 ml
Aliquote at 1 and 3 ml and store at -20°C

0.2% YPD

YPD from Diffco
Prepared according to manufacturer's specs.

1M Pipes-KOH pH 6.8 500 ml

151 grams Pipes Sigma P6757
Add 300 ml dH₂O
PH with 10M KOH
 Note: Add a couple of ml at a time and then dropwise to finish.
 The solution will appear milky until the pH is close to 6.8
Bring to 500 ml with dH₂O
Note: This stuff can go bad so aliquote at 20 ml and store at -20°C

4M Sorbital 500 ml

364 g Sigma S1876
Add 200 ml dH₂O and mix with stir rod until it is a paste.
Heating in a microware will facilitate this process
Use magnetic stirrer until the solution is clear
Adjust the volume to 500 ml with dH₂O
Store at room temp.

PS Buffer (0% ficoll) 1 liter

Add 1M Pipes pH 6.8 to 20 mM final. 20 ml
Add 4M Sorbitol to 200 mM final. 50 ml

15% Ficoll 300 ml

45 g Pharmacia 17-0400-02
200 ml dH₂O
Mix with stir rod until it is a paste and then.
Use magnetic stirrer to get a clear solution.
Add 1M Pipes-KOH pH 6.8 to 10 mM final. 3 ml
Add 4M Sorbitol to 200 mM final. 15 ml
Bring to 300 ml final volume with dH₂O.
Store at 4°C.

8% Ficoll

Prepare from 0% and 15% ficoll

0% Ficoll	15% Ficoll	Total Volume
18.4	21	39.4
36.8	42	78.8
73.6	84	157

4% Ficoll

Prepare by mixing equal volume of 8% and 4% ficoll.
Store at 4°C

1M Potassium Phosphate pH 7.5 100 ml

1M K₂HPO₄ 17.4 g / 100 ml dH₂O ~80.0 ml
1M KH₂PO₄ 13.6 g / 100 ml dH₂O ~19.8 ml
Mix to pH 7.5
Store at room temp.

Dextran Solution

Pharmacia 17-0350-01

Prepare by floating 25 mg of dextran powder on 1.0 ml of PS buffer. After about ten minutes, vortex gently into solution. This reagent can be stored at 4°C for up to one month.

The following buffers and reagents need to be prepared **fresh** on the day of isolation.

Wash Buffer

Prepare 50 ml per liter of cells

Item	Volume per 100 ml
1M Tris-Hcl pH 9.4	10 ml
1M DTT	1.0 ml
DH ₂ O	89 ml

Spheroplasting Buffer

Prepare 15 ml per liter of cells +1 extra for making the Oxyliticase solution.

Component	1.0 liter	3.0 liter	5.0 liter	7.0 liter
1M KPi	0.75	2.25	3.75	5.25
0.2% YPD	12	36	60	84
4M Sorbitol	2.25	6.75	11.25	15.75

Oxalyticase

Several types and preparations of oxalyticase have been used successfully in this protocol. The first three are no longer available.

- 1) The original, and most expensive type was made by the former Enzogenetics of Corvallis OR. It was used from 1995 to 2001. To use this material, dissolve it in **spheroplasting buffer** at 10 mg/ml and add it to cultures of BJ at 1.5 mg per OD liter of cells and to DKY at 0.8 mg/OD liter of cells.
- 2) The second type is Yeast Lytic Enzyme manufactured by ICN (#360944) and it is prepared in **spheroplasting buffer** at a concentration of 50 mg/ml. This method was used from 2001 to 2003. Add it to cultures of BJ at 70 mg/OD liter and DKY at 35 mg/OD liter.
- 3) The third type is the second type repurified as per the **Oxalyticase Repurification Protocol** contained in section VI. This method was used from 2003-2006
- 4) The fourth and current oxalyticase is manufactured by Seikagaku Corporation of Japan and is purchased from Cape Cod Associates. Zymolyase 20T (Cat # 120491) is repurified exactly as oxalyticase type 3.

Reagents for the Fusion Assay

10X Salt

[Reaction]	[Final]	Item	[Stock]	Volume / 100 ml
125 mM	1.25 M	KCL	3 M	4.17 ml
5 mM	50 mM	MgCl ₂	1 M	0.5 ml
20 mM	20 mM	Pipes-KOH pH 6.8	1 M	200 ul
200 mM	200 mM	Sorbitol	4 M	500 ul

ATP Regenerating System

Mix the following on ice in a 50 ml Falcon tube

8 ml ddi water
200 ul 1M Pipes-KOH pH 6.8
500 ul 4M Sorbitol
100 ul 1M MgCl₂

Step 1) In Falcon 2059 tubes weigh out separately:

60 mg ATP
100 mg creatin kinase
0.95 creatin phosphate

Step 2) Dissolve each component separately in 1, 2, and 3 ml respectively of above buffer keepin all tubes on ice.

Step 3) Add solutions to the remainder of the above buffer in the folowing order:

Kinase, phosphatase and finally the ATP

Step 4) Check the pH. It is usually about 6.0 and should be adjusted to pH 6.8 using approx. 140 ul off 1M KOH

- Step 5) Rinse the 2059 tubes with 900 ul of ddi water and add to the ATP solution to bring the final volume to 10 ml.
- Step 6) Aliquote into 100 ul aliquotes on an ice block using a repeat pipetter but only filling the pipetter 25%. Freeze aliquotes in liquid N₂ and store at -80°C.

Developer Solution	(bulk solution)	Vol. Per 100ml
1M Tris-HCL	pH 8.5	25.0 ml
1M MgCl ₂		1.0 ml
20% Triton-X	100	2.0 ml
H ₂ O		71.0 ml

Just before use complete the Developer solution by adding 100 ul of 100 mM P-Nitrophenol to 10 ml of the Developer Solution. 100 mM P-Nitrophenol (Sigma N-3254) in H₂O is prepared in advance, aliquoted and stored at -20°C.

Stop Solution

1M Glycine-KOH pH 11.5

Section IV

Pure Components

Sec18

LMA1

Vam7

Calmodulin

Coenzyme A 600 uM stock. Use 0.5 ul per 30 ul reaction

HMA

Section V

Vacuole Isolation Worksheet

Pre-Cultures:

Start Time: ___:___ am/pm (for 16 hours)

BJ 3505 (112min/ doubling) DKY 6281 (89min/ doubling)

BJ: $OD_{600} = \underline{\hspace{1cm}}$ Need 0.72 OD*ml per liter of YPD

DKY: $OD_{600} = \underline{\hspace{1cm}}$ Need 0.19 OD*ml per liter of YPD

Harvest:

Time: ___:___ am/pm

BJ: $OD_{600} = \underline{\hspace{1cm}}$ x $\underline{\hspace{1cm}}$ ml = $\underline{\hspace{1cm}}$ OD*ml

DKY: $OD_{600} = \underline{\hspace{1cm}}$ x $\underline{\hspace{1cm}}$ ml = $\underline{\hspace{1cm}}$ OD*ml

Oxalyticase Solution:

(ICN lyticase repurified)

BJ: $\underline{\hspace{1cm}}$ OD*ml x 0.71 ul / ODml oxalyticase = $\underline{\hspace{1cm}}$ ml

DKY: $\underline{\hspace{1cm}}$ OD*ml x 0.51 ul / ODml oxalyticase = $\underline{\hspace{1cm}}$ ml

Dextran Solution:

(25mg/ml solution in 0% Ficoll / PS)

BJ: $\underline{\hspace{1cm}}$ OD*ml x 0.19 ul / OD ml

DKY: $\underline{\hspace{1cm}}$ OD*ml x 0.09 ul / OD ml

Vacuole Yield:

BJ: concentration = $\underline{\hspace{1cm}}$ ug/ul x $\underline{\hspace{1cm}}$ μ l = $\underline{\hspace{1cm}}$ μ g Total; or $\underline{\hspace{1cm}}$ μ g / OD*ml

DKY: concentration = $\underline{\hspace{1cm}}$ ug/ul x $\underline{\hspace{1cm}}$ μ l = $\underline{\hspace{1cm}}$ μ g Total; or $\underline{\hspace{1cm}}$ μ g / OD*ml

Section VI

Fusion Unit Calculations

1 Unit of Fusion Activity is the amount needed to produce AB400nm equal to 1 uMol of pNitrophenol (pNP) in 1.0 min. from 1 ug of vacuole protein. This definition has been changed and now is per **1nM** of pNP. Thus.....

$$1 \text{ Unit} = 1 \text{ nM pNP} / \text{min} / \text{ug vacuole}$$

We have created a standard curve of pNP concentrations and measured their AB400nm. This curve can be used to convert AB400nm to Units of Vacuole Fusion.

Reagents

PS Buffer
pNitrophenol (Sigma #104-8)
Developer Solution
Stop Solution

Proceed

- 1) Make pNP stock solution. 1Mol pNP = 139g, 50 uMols = 6.95 mg
Dissolve in 1.0 ml of H₂O.
- 2) Dilute 1:10 with PS buffer. Now we have 5 uMols in 1.0 ml. which is also 0.15 uMols in 30 ul. Use this as serial dilution #1. make serial dilutions (1:2) of 50 ul up to #8.
- 3) Add 30 ul of each dilution to tubes 1-8
- 4) Add 470 ul of developer solution
- 5) Add 500 ul of stop solution
- 6) Read AB400nm
- 7) Plot OD vs uMol pNP

nMols pNP	AB 400 nm
0.0	0
2.	.049
4.6	.087
9.0	.175
18	.337
37	.601
75	1.0

$$\text{AB 400} = y = 0.042533 + 13.491 X \quad R = 0.99347$$

$$\frac{y - 0.042533}{13.5} = X = \text{nMols pNP}$$

1 unit of Fusion = 1 nMol pNP / ug Vacuole Protein / minute

the fusion assay uses 3 ug o Vacuoles and develops for 5 minutes so....
divide the number of nMols by 15 to get Units of vacuole fusion.

ie. an AB400 of 0.33 od = 0.021 nMols/15 = 1.4 units