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Indicators of Candida Contamination in the scFv Yeast Display Library and Methods for its Control

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To the recipients of the PNNL's naïve human scFv yeast surface display library:

The Affinity Reagent group at Battelle Laboratories (PNNL) has recently confirmed that some, if not all, of the aliquots of the scFv yeast surface display library (described in Feldhaus *et. al.*, *Nature Biotechnology* (2003)) are contaminated with *Candida parapsilosis*. This is a very low level contamination, present at 1 contaminant per 1x10e6 - 1x10e7 *Saccharyomyces cerivisiae* (EBY100). However, the *Candida* grow faster than the EBY100 and with repeated "outgrowths", selections, and FACS sorting the *Candida* can overwhelm the culture making a selection difficult.

We wish to thank Chris Szent-Gyoryi at Carnegie Mellon University for first bringing the contaminant problem to our attention and for his work in characterizing the contaminant. We also wish to thank Nathalie Scholler at Fred Hutchinson Research Center for developing a method for reducing the contaminant during selections.

This document outlines how to identify if your library is contaminated and how to reduce its presence during selections.

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Background

The making of the scFv library was a multi-lab task and involved the mixing of several small libraries to create one large library. When the scFv library was complete our lab at PNNL performed numerous successful selections using a variety of antigens (Feldhaus *et. al.*, *Nature Biotechnology* (2003)). During these selections there were only a few instances of contaminated cultures but in these cases the contaminants were bacterial or protozoan. There was not indication of contamination with *C.parapsilosis.*

In January of 2003 our group prepared a large batch of the scFv library such that aliquots could be sent to other labs. A successful selection was performed with one of these aliquots with no indication of contamination. After the initial QC very few selections were performed with these library aliquots as they were reserved for distribution in our library kits.

We first became aware of the contaminant in early 2005 when one of the library recipients contacted us with the concern that his library was contaminated with another yeast. A new aliquot of the library was sent to the recipient and once again the yeast contaminant was found. At this time we were uncertain if this was an isolated incident or possibly an indication that all of the scFv aliquots were contaminated.

We have since identified a low level of *C.parapsilosis* contaminant in several vials of the scFv library. At this time it is unclear if all aliquots are contaminated. We have only had two recipients (out of 87) raise concerns about contamination issues.

Indicators of Contamination:

1. **Cultures growing to a high OD_{600}**. In our lab EBY100 in SD-CAA media will tend to saturate between an OD600 of 5-7. Depending on growth conditions we have seen an OD_{600} as high as 9. If your culture routinely exceeds ODs greater than 10, then the culture may be contaminated. In addition, in our lab when we induce a culture in SGR-CAA starting at an OD600=0.5 and incubate overnight at 20 C we usually see approximately 0.5-1.5 doublings. Seldom do see anything above this. If your induced cultures exceed 2 doublings overnight, then it may be contaminated. *C. parapsilosis* grows much faster than EBY100 in SGR-CAA media at 20 and 25 C (see Table 1).

Table 1: Growth Characteristics of EBY100 vs *C. Parapsilosis*

Yeast	Medium	Temperature (C)	Doubling Time (Hrs)
EBY100*	SD-CAA	30	2.0
EBY100*	SGR-CAA	30	3.4
EBY100*	SGR-CAA	20	6.9
C. parapsilosis	SD-CAA	30	1.5
C. parapsilosis	SGR-CAA	30	1.4
C. parapsilosis	SGR-CAA	20	3.1

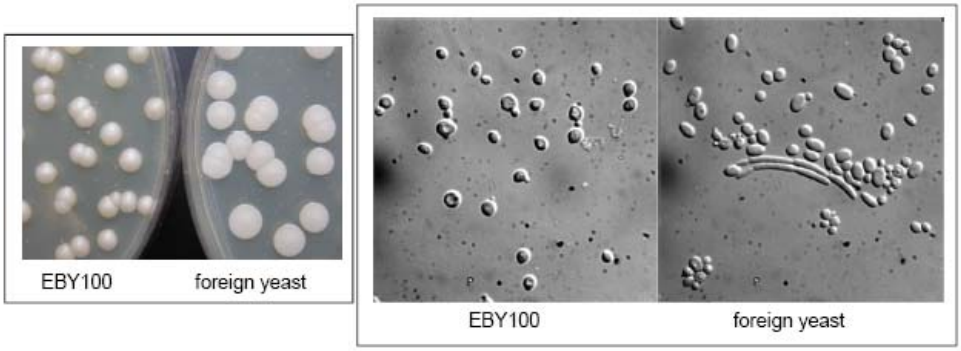
*Pure culture of an individual clone. All cultures were inoculated with starters grown at 30 C in like media, and grown under aerated conditions using baffled flasks with vigorous agitation. (Data provided by Chris Szent-Gyoryi, Carnegie Mellon University)

Note: To familiarize yourself with the growth characteristics of EBY100, we recommend that you grow a pure culture of EBY100 (provided in the scFv kit) under the conditions that you intend to grow the scFv library. Use similar flasks (baffled vs. non-baffled) and similar shaking speeds and take OD600 readings throughout the growth cycle to determine when the cultures are reaching log phase and stationary phase. Once this is established limit growth conditions such that the cultures will not be grown past saturation.

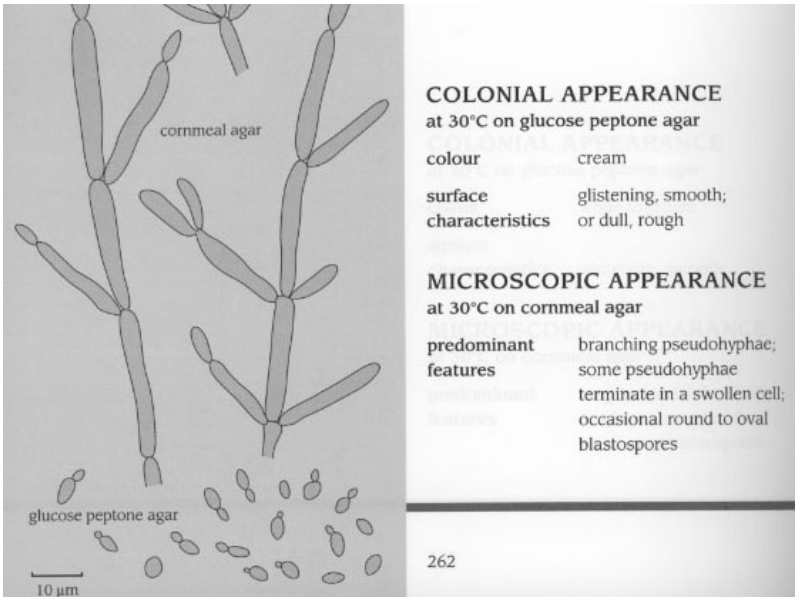
2. **Loss of myc expression during selection process**. *C.parapsilosis* does not express the c-myc tag. The selection process should be enriching for myc positive clones. If you find that during the selection process that your myc positive population is decreasing (from 25% to ~7-10%), then your cultures may be contaminated.

3. **Cell and Colony Morphology.** Using cell and colony morphology can be helpful in identifying a Candida contamination, but is not always reliable. The large cells shown in the figure and photo below are usually only seen when the cultures are grown on agar plates. (see examples below)

Figure 1: Colony and Cell Morphology



*Micrographs provided by Chris Szent-Gyoryi



*source unknown

Positive Identification of *C. parapsilosis*

1. **Growth on SM-CAA plates.** This is a simple and quick to way to distinguish EBY100/pPNL6 from *C. parapsilosis*, but only if the culture is >10% *C. parapsilosis*. SM(maltose)-CAA plates can be easily prepared by following the recipe for SD-CAA plates listed in the scFv Users Manual and **SUBSTITUTING** maltose (2%) for dextrose. *C. parapsilosis* grows very well on these plates whereas EBY100 grows very slowly. Plate a liquid culture that may be contaminated on SM-CAA plates such that individual colonies can be distinguished (300-500 cfu). Incubate plate at 30 C for 24 hours. If the culture was actively growing, the *C.parapsilosis* colonies will usually be visible at 24 hrs and EBY100 will NOT. Mark the colonies (on the bottom of the plate) that grow up after 24 hrs and continue to let plates incubate for several days. Even after several days EBY100 colonies will be quite small.

2. **Yeast Identification Kit**: Biomerieux Vitek sells a yeast identification kit called API 20 C Yeast ID (Fisher Cat.# B20210RRP) that is fairly easy to use. Results take about 48 hours. Plate out culture that may be contaminated and incubate on SD-CAA or SM-CAA. Look for colonies similar to what is shown in the photos and test individual colonies.

3. **PCR Analysis** (refer to *Single primer pair for PCR identification of Candida parapsilosis group I isolates, Pontieri, et.al. J.Med. Microbiol.-vol. 50(2001), 441-448*): Our group has had success with this method using purified genomic DNA from pure cultures of *C. parapsilosis*. However, we have not attempted to perform colony PCR nor have we perfected a technique to identify the contaminant when it is in low abundance (1 in $1x10^6$ cells)

Minimizing the Contaminant

1. **Avoid Prolonged Growth**
   Our best advice is to avoid conditions that favor the growth of *C.parapsilosis*.
   - Do not grow the library past saturation.
   - Limit growth times in SGR-CAA.
   - If you cannot use the culture for several days, it is best to make a glycerol stock as the contaminant will grow even at high densities and low temperatures.

2. **Pre-sort c-myc+ Population**
   If you notice that during the selection the c-myc population is decreasing significantly (<10%) perform a "myc+ pre-sort". To do this, stain your sample with α-c-myc and a secondary reagent (goat α-mouse alexa-fluor-488) and then sort the c-myc+ population. In many instances we have pre-sorted a library that was 10-12% myc + and increased the myc+ population to ~60-70%. The newly sorted population can then be stained the same day with antigen and sorted for antigen binding yeast.

   Note – You should first rule out other causes of poor myc-expression (e.g. induced at wrong temperature or induced in wrong media) before doing a pre-sort.

3. **Treatment with Ketoconazole and Intraconazole**
   Nathalie Scholler at the Fred Hutchinson Research Center has effectively used the anti-fungal drugs ketoconazole (#K1003, Sigma) and intraconazole (#I6657, Sigma) to control the growth of *C.parapsilosis*. These drugs affect both EBY100 and *C.*

parapsilosis but are more detrimental to the latter. The drugs will not completely eradicate the contaminant but can be used to prevent the contaminant from over taking the culture. They recommend using 0.25 ug/mL of ketoconazole during growth for analysis purposes and 1 ug/mL of intraconazole during induction prior to sorting. Listed below are the results of a study to investigate the effects of these drugs on the viability of EBY100.

Cell Viability Test
(performed by Nathalie Scholler and Jennifer Gross at Fred Hutchinson Research Center)

Three individual colonies of EBY100 were grown in SD+CAA + Trp overnight. From these cultures 20 ul were used to inoculate the same media containing either no drug, 0.25 µg/ml of KETO or 1 µg/ml of ITC. These cultures were grown overnight. Cultures were then diluted and 10 ul were plated onto SD+CAA +Trp plates in duplicate. After 48 hours colonies were counted to determine the titer of the treated cultures.

Results:

No Treatment:	6.3 x 10e6 cells/ml
KETO:	4.3 x 10e6 cells/ml
ITC:	3.5 x 10e6 cells/ml

Conclusions: Drug treatment has a minimal effect on the viability of EBY100. Given that the cells were grown several generations overnight, a 2-fold reduction in cell number represents a very low level of drug toxicity, either via killing and/or slowing of growth rate.

Note: Our group at Battelle PNNL has not tested these drugs so we can only recommend what has been listed above.

References

Michael J. Feldhaus, Robert W. Siegel, Lee K. Opresko, James R. Coleman, Jane M. Weaver Feldhaus, Yik A. Yeung, Jennifer R. Cochran, Peter Heinzelman, David Colby, Jeffrey Swers, Christilyn Graff, H. Steven Wiley, K. Dane Wittrup. "Flow-cytometric isolation of human antibodies from a nonimmune Saccharomyces cerevisiae surface display library." (2003) *Nature Biotechnology* 21:163 – 170.

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