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Methodology for preservation of yeast-bound single chain fragment variable antibody affinity reagents

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Abstract

Readily accessible affinity reagents are critical to the validation of biomarkers and to the development of new diagnostic tests. As alternatives to monoclonal antibodies, yeast-bound single chain fragment variable antibody (yeast-scFv) can be rapidly selected from yeast display libraries. An important characteristic for any diagnostic reagent is its stability or ability to store it. A lyophilization procedure that has extended the shelf life of yeast-scFv by a factor of 10-fold relative to previous reports is reported. Real time stability for three yeast-scFv clones to three distinct *Entamoeba histolytica* potential diagnostic antigen targets for one year at room temperature as well as at 37°C and 45°C. Retention of full binding activity and specificity for their cognate antigen is shown by flow cytometry. Lyophilization can easily be carried out in batches and in single-use vials.

Graphical abstract

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1. Introduction

Binding molecules with specific avidity properties to desired biomarkers are critical to most aspects of biomedical research, biosensors and diagnostics. To date most affinity reagents are monoclonal antibodies generated via mouse hydridoma technology. However, there are increasing numbers of *in vitro* approaches to generate similar binder molecules including small binder molecule generation through protein engineering, and selection of binders from large libraries of affinity molecules through techniques such as phage display, synthetic peptide libraries, or yeast display (Bradbury et al. 2011, McCafferty and Schofield, 2015). Yeast display of single chain fragment variable (scFv) antibodies is used to generate binders specific to biomarkers of interest (Feldhaus et al. 2003) with applications for diagnostics (Venkatesh et al. 2015). The ability to use flow cytometry for selection, as well as for the verification of the functionality for the selected yeast displayed scFvs, makes this a very attractive approach to identify binders with the desired affinity properties (Feldhaus et al. 2003; Gray et al. 2010) (Figure 1A).

Recently, it was shown that yeast-displayed scFv can be used in assays that support biosensor platforms while still associated with yeast cell walls, either on whole cells or on cell wall fragments (Grewal et al. 2014; Wang et al. 2014).

This technical note describes, a robust methodology and associated formulation that enables stabilization of yeast-scFv for significantly longer time periods than the 30 days reported previously (Gray et al. 2012). The methodology is illustrated for multiple yeast-scFv clones specific to recombinant proteins of the enteric parasite *Entamoeba histolytica*, the causative agent for intestinal amoebiasis in many countries in the developing world. The recombinant proteins were made for parasite specific proteins that had previously been identified in stool samples (Ehrenkaufer et al 2007, Ali et al 2012). The clones were rapidly selected by screening a nonimmune library of scFv displayed on the surface of yeast cells as described (Gray et al. 2010; Gray et al. 2012).

2.0 Materials and Methods

2.1 Growth of yeast-scFv

A single yeast-scFv clone grown on a SD + CAA agar plate was transferred to 2ml of SD + CAA broth and grown overnight while shaking at 30°C. The next day, two ml of overnight culture was diluted into 38 ml of SD+CAA and grown overnight at 30°C while shaking. On the following day, yeast-scFv were diluted into SG/R + CAA induction media and allowed to grow overnight at 30°C while shaking. Finally on the 4th day, the yeast-scFv were lyophilized.

2.2 Lyophilization of yeast-scFv

Following growth in induction media and confirmation of antigen-specific binding by flow cytometry as described below, yeast-scFv were centrifuged for 5 minutes at 1000×g. The pelleted yeast were resuspended in formulation media, which consisted of 10% Dextran + 5% Monosodium Glutamate (MSG) and was sterilized by filtration. The yeast-scFv, at 3×10^7 in 1.2ml of formulation, were aliquoted into 5 ml borosilicate glass vials (Kimble Chase

#66013-122) and were loosely capped with gray butyl rubber stoppers (Kimble Chase # 66010-865). Lyophilization was performed in a Millrock Laboratory Freeze Dryer (Millrock Technology, Kingston, New York, USA). The formulations in the vials were rapidly frozen to -60°C, with the vacuum set at 100mTorr and held for 4 hours, followed by primary drying at -10°C to 25°C over a period of 48 hours. Secondary drying was conducted at 25°C for one hour. After freeze drying, the vials were filled with nitrogen gas and stoppered. Finally, samples were removed from the lyophilizer, and each vial was crimped with a tear-off style aluminum seal (Kimble Chase # 66010-798).

2.3 Purification of proteins 350, 030 and 14-3-3

Proteins 350 and 780 were constructed and expressed as previously reported (Gray et al. 2012). Proteins 030 and 14-3-3 were obtained from the Seattle Structural Genomics Center for Infectious Disease (SSGCID). All four antigens were biotinylated using either the Pierce EZ-Link NHS-Peg 4-Biotin or the Sulfo-NHS-LC-Biotin kit (Thermo Scientific, Rockford, IL). Biotinylation was confirmed by Western blots probed with 0.4 ug/mL HRP-conjugated streptavidin (Sigma Aldrich, St. Louis, MO).

2.4 Yeast-scFv staining for flow cytometry

Typically, 3×10^7 lyophilized cells in a single vial were rehydrated in 1 ml of PBS, vortexed and incubated at room temperature for ten minutes. The cells were washed three times with yeast wash buffer (PBS + 0.5%, pH 7.4) and resuspended in 1 ml of yeast wash buffer. Fifty microliters of each suspension was pipetted into a 96 well round bottom plate. To confirm expression of scFv, each clone was incubated with anti-c-Myc-FITC for 30 minutes at room temperature. After washing, the yeast-scFv clones were stained with 100nM of biotinylated antigen, washed and then labeled by the addition of Streptavidin, R-Phycoerythrin Conjugate (SAPE) for 30 minutes in contrast to the previously published protocol where the latter two steps are performed concurrently (Feldhaus et al. 2003). Yeast-scFvs were analyzed by flow cytometry for scFv expression (x-axis; FITC Fluorescence) and antigen binding (y-axis; PE Fluorescence). All secondary reagents for flow cytometry were acquired through Molecular Probes (Invitrogen, Carlsbad, CA)

3.0 Results

3.1 *Entamoeba histolytica* proteins

Four *E. histolytica* proteins were used as the ligand-specific epitope in binding studies. Depending on size, whole sequences or selected fragments were expressed as recombinant proteins and ranged in size from 17 to 30 kDa (Table 1). Antigens 350 and 780 are chromodomain-containing proteins whose mRNA transcripts are upregulated in *Entamoeba histolytica* encystation (Ehrenkaufer et al. 2007, Ali et al. 2012). Antigens 030, 780 and 14-3-3 were detected by mass spectrometry in *Entamoeba-histolytica* positive stool samples (Ali et al. 2012). Using the methodology described previously (Gray et al. 2012), three yeast-scFv clones were selected to perform the stability studies. The clones used were 350-E2, 030 C and 14-3-3 D, selected for specific binding to the *E. histolytica* antigens 350, 030 and 14-3-3 respectively (see Table 1).

3.2 Optimal formulation for lyophilization of yeast-scFv

Among the desired characteristics for freeze-dried products is an intact cake, which exhibits strength and uniformity in color, indicating adequate dryness and porosity. Typical combinations of sugars including sucrose plus trehalase, sucrose plus glycine, and sucrose plus mannitol in various ratios, as well as formulations typically used in freeze drying yeast such as combinations of skim milk plus trehalase, and skim milk plus dextran plus sodium glutamate resulted in poor quality lyophilized yeast-scFv cakes, characterized as pancake like, foamy, transparent, or airy with holes in the cake without any structure. Binding studies were continuously performed to verify functionality of the lyophilized products. Binding improved with “cake” quality. Yeast from lyophilization conditions resulting in poor “cake” products had poor binding characteristics (Figure 1B and 1C). The optimal formulation resulting in regular cake formation consisted of 10% Dextran + 5% monosodium glutamate, and a final starting yeast density of 3×10^7 yeast scFv/ml. These conditions were used for the stability studies.

3.3 Functionality and stability of lyophilized yeast-scFv

Functionality of the yeast-scFv clones was demonstrated by incubation of the cells with biotinylated cognate (target) antigen for retention of binding activity and non-cognate (non-target) antigen for specificity, followed by a second stain with streptavidin conjugated to phycoerythrin. Confirmation of scFv expression was demonstrated by staining with anti-c-Myc-FITC (scFv is expressed as a fusion to the Myc tag). (Figure 1A) The proportion of functionally active yeast scFv cells was determined as those with scFv expression (FITC stained) and binding to antigen (PE stained) (Gray et al, 2012). Binding studies were performed before and immediately after lyophilization (Table 2). Binding to cognate antigen pre- and post-lyophilization was comparable, indicating that there was no loss in binding due to the formulation or the lyophilization cycle. In addition, non-cognate antigens did not bind. Each of the three clones selected for this study demonstrated retention of specific binding functionality to anti-c-myc-FITC and cognate antigen post lyophilization.

A real-time stability study for 1 year for all three clones lyophilized in vials and stored at 25°C, 37°C and 45°C was performed. The results are presented in Figure 2. These data demonstrate that the three clones, yeast-scFv 350E2, 030C, and 14-3-3D maintained specific binding to the cognate antigen for at least 12 months. The viability of the stabilized yeast-scFv cells was assessed. While the yeast-scFv were viable prior to lyophilization, upon hydration post-lyophilization viability decreased from approximately 3×10^7 viable yeast/ml to 1×10^4 viable yeast/ml across all three clones.

4.0 Conclusion

A key constraint on the development and availability of a diagnostic test is the stability of the reagents. A previous report of whole-cell yeast-scFv reagents (Gray et al. 2012) described simple lyophilization and storage procedures that resulted in limited stability (30 days). Utilizing this lyophilization protocol and this formulation, the lyophilized yeast-scFvs in this study have demonstrated an extended shelf life both in real-time for one year at 25°C, 37°C and 45°C stability data. The yeast-scFv cells were mostly non-viable following

lyophilization and rehydration. Given that antigen binding activity can change significantly when yeast grow and divide, their non-viable state may help promote stability.

Recently, lyophilized yeast-scFv produced as described here were shipped at ambient temperature from the United States to Australia and used to generate “nanoyeast-scFv” reagents in developing diagnostic assays for the detection of *Entamoeba histolytica* antigens, including electrochemical and surface-enhanced Raman scattering (SERS) bioassay platforms (Grewal et al. 2014; Wang et al. 2014). While stability data is shown for 3 distinct yeast-scFv clones, the protocol is robust to all yeast-bound scFv tested so far (unpublished data). Thus the protocol and formulation appears to be universally applicable to this class of biologics (yeast-scFv) increasing their utility both for research purposes and diagnostic product development.

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Highlights

1. A method to stabilize yeast-bound single chain fragment variable antibody is shared
2. Real-time stability results at 45°C for one year are shown
3. The protocol allows easy sharing and storage of new affinity reagents

Figure 1.

Lyophilization of yeast bound single chain variable fragment (yeast scFv). A. schematic of the biologic capture reagent: the scFv heavy and light chains (V_H and V_L) are double tagged (HA and myc tags) and present as a-agglutinin Aga2p fusion proteins bound to Aga1p subunit on the surface of the yeast cell from which they are expressed. Functionality of the yeast scFv can be determined by flow cytometry by detection of doubly stained cells with: the anti-c-myc FITC labelling (for the scFv) and streptavidin, R-phycoerythrin conjugate (SAPE) against biotinylated antigen. B. “Poor” quality lyophilized product resulting from lyophilization of 3×10^7 yeast scFv/ml clone 350-E2 in 18.7% sucrose + 1.87% mannitol. C. “Good” quality lyophilized product resulting from lyophilization of 3×10^7 yeast scFv/ml clone 350-E2 in 10% Dextran + 5% MSG.

Figure 2.

Stability data for: (A) Binding of yeast-scFv 350-E2 to anti-c-myc FITC and biotinylated antigen and SAPE; (B) Binding of Yeast 030C to anti-c-myc FITC and biotinylated antigen and SAPE, and (C) Binding of Yeast 14-3-3D to anti-c-myc FITC and biotinylated antigen and SAPE. Multiple vials of yeast-scFv were lyophilized and stored in incubators at 25°C (squares), 37°C (diamonds) and 45°C (triangles). Functionality of the yeast-scFv was determined by flow cytometry to confirm availability of the scFv (with anti-c-myc FITC) and specific binding to the cognate biotinylated antigen (filled squares, diamonds and triangles) versus the non-cognate biotinylated antigen as per table 2 with SAPE (empty squares, diamonds and triangles).

Table 1

Annotated description of source target *E. histolytica* proteins and the derived recombinant antigens used to generate target specific ScFv.

Protein	NCBI Accession	Total AA	AA Expressed	MW (kDa) with Tags	Annotation	Yeast-scFv Probe
350	XP_649984.1	1247	135-271	17	Chromodomain-helicase-DNA-binding protein	350-E2
030	XP_651513.1	193	1-193	22	Rab Family GTPase	030 C
14-3-3	XP_654465.1	240	1-240	30	14-3-3 Protein 3	14-3-3 D
780	XP_653178.1	1641	470-617	19	Chromodomain-helicase-DNA-binding protein	780

Table 2

Comparison of specific binding of yeast-associated scFvs with cognate antigen before and after lyophilization. Percent of double stained cells with anti c-Myc-FITC and Biotinylated Antigen + SAPE is provided.

Clone	Pre-Lyophilization		Post Lyophilization	
	Cognate Antigen	*Non-cognate Antigen	Cognate Antigen	*Non-cognate Antigen
350-E2	42.2	1.2	41.4	0.8
030 C	33.7	3.4	38.0	0.8
14-3-3 D	29.8	4.8	39.7	2.2

* The non-cognate antigen for binding for clones 350E2, 030 C, and 14-3-3 D were demonstrated with 780 Biotinylated antigen, 14-3-3 Biotinylated antigen and 030 Biotinylated respectively