

The selection of single-domain antibody fragments (iDAbs) by intracellular antibody capture in yeast

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Introduction

Many biological functions are mediated by protein-protein interactions (PPIs) or protein-nucleic interactions that are key molecular interactions in both normal biology and disease. These can be inhibited with a number of macromolecules (macrodrugs to distinguish them from conventional drugs (1,2)) such as intracellular antibody fragments.

The use of intracellular antibodies to perturb the function of protein function *in vivo* was first shown using whole antibodies and antibody fragment comprising variable regions (V) in the form of single chain Fv (scFv) (3). Subsequently, antibody fragment variants have been described for use as intracellular antibodies, including our own use of human single domain antibodies (designated iDAbs) made of either heavy (H) chain variable regions (VH iDAbs) or light (L) chain variable regions (VL iDAbs) (4,5).

An advantage of iDAbs is the single domain has only three complementary determining regions (CDRs) allowing for easy affinity maturation, their small size (~12KD) and can, in principle, bind to antigen regions such as crevices, that scFv or whole antibody cannot reach. We have developed a method to select intracellular antibodies using yeast cell selections, called Intracellular Antibody Capture (IAC). IAC was first developed using scFv (6) and later adapted for iDAb selection (4). The advantage of the IAC method is that it is not necessary to immunise animals to obtain antibodies, which obviates the welfare issue of using animals and also avoids the problem of developing antibodies against self antigens and conserved antigens. Finally, using an *in-cell* selection means that the iDAbs are selected in reducing conditions that facilitates the selection of appropriately folded domain antibodies. We previously developed an iDAb-based IAC method (7). This was adapted to single VH iDAb libraries with CDR3 lengths of 10, 12 and 15 amino acids and for affinity maturation through sequential CDR2 and CDR1 randomization and screening (8). The current protocol online is the refinement of the methods described in Nature Protocols (7). The steps involved are summarised in Figure 1.

Reagents

- Yeast strain: L40 (MATa, leu2-3,112, his3 Δ 200, trp1 Δ 1, ade2, LYS (LexA-op)4-HIS3, URA3 (LexA-op)8-lacZ) (Invitrogen)
- Plasmids: pBTM116 and pVP16*
- Oligonucleotides (Sigma)
- Ampicillin (Merck, cat no. 171254)
- Chloramphenicol (Sigma, cat no. 857440)
- 3-amino-1,2,4-triazole (3-AT) (Sigma, cat no. A8056)
- Calf-intestine alkaline phosphatase (CIAP) (Roche, cat. no. 713 023)
- Culture media for bacterial and yeast growth (all from BD): Bacto agar (cat. no. 214010),

Bacto peptone (cat. no. 211677), Bacto tryptone (cat. no. 211705), Bacto yeast extract (cat. no. 211929)

- Restriction enzymes (all from New England Biolab (NEB)): NotI-HF (cat. no. R3189), Sfi I (cat. no. R01235)
- T4 ligase (concentrated, NEB, Cat. no. M0202T)
- Dimethylsulfoxide (DMSO) (Sigma, cat. no. D8418)
- Ethanol (Fisher, cat. no. E/0600DF/17)
- Glycerol (Sigma, cat. no. G9012)
- Phusion High-Fidelity DNA Polymerase (NEB, cat.no. M0530S)
- PEG 3350 (Sigma, cat. no. P3640)
- QIAquick Gel Extraction kit (Qiagen, cat. no. 28704)
- QIAquick PCR purification kit (Qiagen, cat. no. 28104)
- QIAprep Spin MiniPrep kit (Qiagen, cat. no. 27104)
- HiSpeed Plasmid Midi kit (Qiagen, cat. no. 12643)
- Tris base (Sigma, cat. no. T6066)
- Sheared salmon sperm DNA (Ambion, cat. no. AM9680)
- LiAc (Sigma, cat. no. L6883)
- EDTA (Sigma, cat. no. E1644)
- NaCl (Fisher, cat. no. S271-10)
- D-Glucose (BDH, cat. no. 101174Y)
- Yeast tRNA (10 mg ml⁻¹, Applied Biosystems, cat. no. AM7119)
- Sodium acetate 3H₂O (BDH, cat. no. 102354X)
- NEB[®]5-alpha Competent E.Coli (High Efficiency) (NEB, cat.no. C2987I)
- Amino acids for yeast dropout media (all from Sigma): L-Isoleucine (cat. no. I2752), L-Valine (cat. no. V0500), L-Adenine hemisulfate salt (cat. no. A9126), L-Arginine HCl (cat. no. A5131), L-Histidine HCl monohydrate (cat. no. H8125), L-Leucine (cat. no. L8000), L-Lysine HCl (cat. no. L5626), L-Methionine (cat. no. M9625), L-Phenylalanine (cat. no. T8625), L-Threonine (cat. no. P2126), L-Tryptophan (cat. no. T0254, L-Uracil (cat. no. U0750), L-Aspartic acid (cat. no. A9256), L-Serine(cat. no. S4500), L-Proline(cat. no. P5607)
- Yeast nitrogen base (without amino acids) (BD, cat. no. 291940)
- Ammonium sulphate (BDH, cat. no. 100333B)
- Succinic acid (Sigma, cat. no. S7501)
- Lyticase (Sigma, cat no. L2524-10KU)
- Sodium dodecyl sulfate (SDS, Sigma, cat no. L3771)
- Antibodies: anti-LexA (Abcam, cat. no. ab14553), anti-VP16 (Abcam, cat. no. ab4808)
- Phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma, cat. no. P2069)
- Sodium hydroxide (NaOH, Sigma, cat. no. S8045)

Equipment

- 15 ml Falcon tubes (Corning, cat. no. 430790)
- 50 ml Falcon tubes (Corning, cat. no. 430828)
- 225 ml Falcon conical tubes (BD, cat. no. 352075)
- Disposable inoculating loops (VWR, cat no. 612-9351)
- 0.2 µm Filter (Millipore, cat. no. SLGP033RS)
- Nunc Bio-Assay dish (245 mm Å~ 245 mm Å~ 25 mm) (Nalge-Nunc, cat. no.

240835)

- 10 and 15 cm petri plastic plates
- Bench-top centrifuge
- Centrifuge (Eppendorf 5810R or similar, able to spin 225 ml Falcon conical tubes at a setting of 2,500g; Eppendorf)
- Electroporation device (Bio-Rad Gene Pulser Xcell or similar, Bio-Rad)
- Stationary incubators at 30 and 37 °C
- Shaker incubators at 30 and 37 °C (able to shake 2.5-liter asks at a setting of 225 r.p.m.)
- Sterile Erlenmeyer asks (250, 500, 1,000 and 2,500 ml)
- Water baths equilibrated at 37, 42 and 50 °C

Procedure

Reagents Setup

For Yeast media

- 20% glucose solution (w/v): Weigh 100 g of D-glucose, add 500 ml of Milli-Q water and pass through a 0.2 µm filter. Store 4 °C for up to 3 months
- 100x adenine: Weigh 2 g of L-adenine, add 500 ml of Milli-Q water and pass through a 0.2 µm filter. Store 4 °C for up to 3 months
- YPAD medium and plates: Dissolve 10 g of yeast extract and 20 g of peptone (plus 20 g of agar if making plates) in 900 ml of Milli-Q H₂O and autoclave at 0.5 bar and 121 °C for 20 min. Cool to 55 °C and add 100 ml of filtered 20% glucose (w/v) and 10 ml 100x adenine solution (for plates, pour ~25 ml in sterile 9 cm dishes). Store at RT for up to 1 month.
- 5Å~ Yeast complete (YC) medium without amino acids (5Å~ YC w/o AA): Dissolve 2.4 g of yeast nitrogen base (lacks amino acids and ammonium sulphate), 10 g of ammonium sulphate, 20 g of succinic acid, 0.1 g of tyrosine and 12 g of NaOH in 400 ml of Milli-Q H₂O. Adjust the pH to 7.0 by adding more NaOH pellets as needed. Autoclave at 0.5 bar and 121 °C for 20 min. Store at RT for up to 1 month.
- Amino acid supplements mix minus 7 dropout (AA mix-7): Make the stock as a 100Å~ solution. Dissolve 1 g of phenylalanine, 1 g of isoleucine, 2 g of arginine, 1 g of valine, 1 g of aspartic acid, 1 g of proline, 1 g of serine and 2 g of threonine in 200 ml of Milli-Q H₂O and filter sterilize. Prepare 10 ml aliquots in 15 ml Falcon tubes and store at 4 °C for up to 6 months.
- Amino acid supplements: Stocks are made as 100Å~ solution (except Ura, which is made as a 10Å~ solution). Dissolve either 2 g of tryptophan, 2 g of lysine, 1 g of methionine, 2 g of adenine, 1 g of histidine or 2 g of leucine in 200 ml of Milli-Q H₂O and filter sterilize. Prepare 10 ml aliquots in 15 ml Falcon tubes and store at 4 °C for up to 6 months. For the Ura stock (10Å~), dissolve 1 g of uracil in 1 liter of Milli-Q H₂O and filter sterilize. Store at 4 °C for up to 6 months.
- 2 M 3-AT stock: Dissolve 168.2 g of 3-AT in 1 l of Milli-Q H₂O and filter sterilize. Store at -20°C for up to 6 months.
- YC dropout medium (100 ml): Mix 20 ml of 5Å~ YC w/o AA, 10 ml of filtered 20% (w/v) glucose solution, 1 ml of AA mix-7 and 1 ml of each amino acid supplement (except uracil) and 10 ml of 10Å~ uracil solution. Omit amino acid supplement of interest to make selective

dropout medium. Add autoclaved Milli-Q H₂O to 100 ml. Store at RT for up to 1 month.

- YC dropout plates (1 L): Add 20 g of agar to 200 ml of 5 \times YC w/o AA and 400 ml of Milli-Q H₂O in a 1 liter bottle with a magnetic stirrer. Autoclave at 0.5 bar and 121 °C for 20 min. Cool to 60 °C in a water bath. Combine 100 ml of filtered 20% (w/v) glucose solution, 10 ml of AA mix-7, 10 ml of each amino acid supplement (omit the amino acid supplements of interest to make selective dropout plates) and 100 ml of 10 \times uracil solution. Add the autoclaved agar to this mixture. If 3-AT needs to be added, do so at this point from 3-AT stock. Make up to 1 liter by adding pre-warmed (55 °C) autoclaved Milli-Q H₂O. Pour the mixture immediately into suitable sterile dishes in a sterile hood (~250 ml per dish in large square plates; ~65 ml in 14cm small round dishes or ~25 ml in 9 cm small round dishes). Leave the plates overnight at RT to solidify and dry. Replace the lid, invert the plate and store at 4 °C for up to 1 month.

Check for contaminating colonies before using stored plates.

Tip: Downscale the above recipe to make YC selection agar according to the volumes required for making up the desired size and number of plates.

For Yeast Transformation

- 20 mg ml⁻¹ salmon sperm DNA: Dissolve 1 g of salmon sperm DNA in 50 ml of 1 \times TE using a magnetic stirrer at 4 °C until completely dissolved. Sonicate on ice for 30 s using a large sonicator probe with 20–30% power to reduce the viscosity of the DNA solution. Prepare 1 ml aliquots in 1.5 ml micro tubes and store at – 20 °C indefinitely. Before use, boil the salmon sperm DNA for 10 min.
- 50% PEG solution (w/v): Weigh 50 g of PEG3350, add 100 ml Milli-Q H₂O and pass through a 0.2 μ m filter. Store at RT for up to 1 month.
- 1 M LiAc solution: Weigh 25.5 g of LiAc, add 200 ml of Milli-Q H₂O, adjust the pH to 7.5 with glacial acetic acid (glacial) (Fisher, cat. no. A35-500) Adjust to a final volume of 250 ml and autoclave at 0.5 bar and 121 °C for 20 min. Store at RT for up to 3 months.
- 10 \times TE: Weigh 1.21 g of Tris base and 0.37 g of EDTA, add 90 ml of Milli-Q H₂O, adjust the pH to 7.5 with concentrated HCl (Fisher, cat. no. A144-500).
- TE-PEG solution: Prepare fresh each time of use. Mix 10 ml of 1M LiAc, 10 ml of 10 \times TE and 80 ml of autoclaved Milli-Q H₂O.
- LiAc–TE–PEG solution: This solution should be prepared fresh at each time of use. Mix 10ml of 1 M LiAc, 10ml of 10 \times TE and 80ml of 50% PEG(w/v). For Plasmid Preparation from Yeast

- Lyticase stock solution: Dissolve 10KU of lyticase in 1 ml Milli-Q H₂O to make a working stock of 10U/ μ l. For Sub-Library Construction

- 3 M sodium acetate (NaOAc) solution: Weigh 40.8 g of sodium acetate 3H₂O, add Milli-Q H₂O to 90 ml, adjust to pH 5.2 with glacial acetic acid and add Milli-Q H₂O to a final volume of 100 ml and autoclave at 0.5 bar and 121 °C for 20 min. Store at RT for up to 6 months.

- 2 \times TY medium: Dissolve 16 g of tryptone, 10 g of yeast extract and 5 g of NaCl, in 1 liter of Milli-Q H₂O and autoclave at 0.5 bar and 121 °C for 20 min. Store at RT for up to 3 months if no contamination becomes apparent.

- TYE plates: Weigh 15 g of bacto-agar, 10 g of tryptone, 5 g of yeast extract and 8 g

of NaCl, add Milli-Q H₂O to 1 liter and autoclave at 0.5 bar and 121 °C for 20 min. Allow to cool to 55°C, add antibiotics, ampicillin, 100 µg ml⁻¹ or chloramphenicol, 15 µg ml⁻¹ (if necessary) while stirring and pour into sterile dishes. Store the plate at 4 °C for up to 3 months.

- SOC medium: Dissolve 4 g of tryptone, 1 g of yeast extract, 0.1 g of NaCl in 150 ml of Milli-Q H₂O and add 0.5 ml of 1M KCl. Adjust the pH to 7 with NaOH and the volume to 194.4 ml and autoclave at 0.5 bar and 121 °C for 20 min. Add 2 ml of 1 M filter-sterilized MgCl₂ and 3.6 ml of filter-sterilized 20% (w/v) glucose (to give a final volume of 200 ml). Store at RT for up to 3 months.

- Antibiotics: Stocks are made as 1,000 Å solutions and stored in aliquots at -20 °C for up to 6 months. Ampicillin, 100 mg ml⁻¹ in dH₂O or chloramphenicol, 15 mg ml⁻¹ in absolute ethanol.

Generating the antigen (bait) stable line – small scale yeast transformation

1. Clone the antigen (bait) cDNA as an in-frame fusion with LexA DNA-binding domain in pBTM116 vector using standard genetic cloning methods.
2. Streak L40 yeast from glycerol stock onto YPAD agar plate and leave to grow for 2-3 days at 30 °C.

Tip: Yeast will not grow sufficiently when inoculate from glycerol stock directly into liquid culture.

3. A day before yeast transformation, inoculate a single colony into 2 ml of YPAD medium and incubate overnight with shaking (~225 rpm) at 30 °C.
4. Measure the OD₆₀₀ of the overnight culture and calculate the volume required to dilute this culture to give an OD₆₀₀ between 0.15-0.2 in 10 ml of YPAD medium.

Tip: Dilute the overnight culture 1:10 before measuring the OD₆₀₀ to give an accurate reading.

5. Culture at 30 °C with shaking (~225 rpm) until the OD₆₀₀ reaches 0.4-0.5.

Tip: Transformation efficiency is reduced when the OD₆₀₀ is near or above 0.6.

6. Pellet the yeast culture by centrifugation for 5 minutes, 1,000g at 4°C.

Tip: Once the desired OD₆₀₀ is obtained, keep the yeast culture on ice to slow down further growth.

7. Remove the medium and resuspend the pellet in 10 ml of sterile Milli-Q H₂O.

8. Centrifuge again for 5 minutes, 1,000g at 4°C.

9. Remove the supernatant and resuspend the final yeast pellet in 100 µl of freshly made, sterile TE-LiAc. This is the competent yeast cells that can be kept on ice for 2-3 hours.

10. Denature the salmon sperm DNA by incubating at 95°C for 5 minutes and chill on ice immediately for at least 2 minutes.

11. Mix 200 ng of bait plasmid and denatured 100 µg of salmon sperm carrier DNA in a sterile 1.5 ml microtube.

12. Add the DNA mixture from step 11 to 100 µl of competent yeast and mix by flicking the tube.

13. Add 600 µl of freshly prepared sterile PEG-LiAc-TE solution and mix inverting the tube several times.

Critical: It is strongly recommended that TE-LiAc and PEG-TE-LiAc mixtures are prepared fresh for each transformation as the quality of Li-Ac and PEG will affect transformation efficiency.

14. Incubate for 30 minutes at 30 °C with shaking at 225 rpm.
 15. Add 70 µl of DMSO and mix gently by inversion.
 16. Heat shock for 15 minutes in 42 °C water bath.
 17. Place on ice for 2 minutes, fill the microtube with sterile Milli-Q H₂O and pellet the cells by centrifugation for 5 minutes at 3,000g, RT.
 18. Remove the supernatant and resuspend the pellet in 100 µl of sterile 1xTE. Plate 50 µl of the transformed yeast onto a 10 cm YC-W (i.e. lacking tryptophan) plate.
 19. Incubate the plate for 2-3 days at 30 °C.
- Pause Point: These yeast plates are stable for at least one week when wrapped with parafilm and stored at 4 °C.

Validation of bait expression from LexA-antigen fusion plasmid in yeast

20. Pick a single yeast colony from the YC-W plate from step 19 into 5 ml of YC-W media in a 50 ml falcon tube and resuspend by vortexing.
- Critical: Test several formed clones because of the possible heterogeneity of the LexA-bait fusion protein expression level.
- Critical: Good aeration is required for yeast to grow efficiently and a smaller size falcon tube will compromise this.
21. For short-term storage of bait-containing yeast clones streak a small amount from step 19 onto YC-W plate and incubate for 2-3 days at 30 °C.
- Pause Point: The yeast plate is stable for at least one week when wrapped with parafilm and stored at 4 °C. This can be used as a source for screening libraries after checking for the bait expression.
22. Culture the single colonies from step 21 overnight at 30 °C with shaking at 225 rpm.
 23. Take 500 µl of the overnight culture for protein analysis and freeze the remaining culture for long-term storage by adding 1x volume of 30% (v/v) sterile glycerol solution.
 24. Centrifuge 500 µl of yeast culture from step 23 for 1 minute at 3,000g.
 25. Remove the supernatant and wash the pellet in 500 µl Milli-Q H₂O and centrifuge again for 1 minute at 3,000g .
 26. Resuspend the pellet in 300 µl of Milli-Q H₂O and then equal volume of 0.6M NaOH, mix well.
- Critical Step: This two-step procedure is important as direct suspension of cells in 0.3M NaOH causes inefficient extraction.
27. Incubates cells at RT for 10 minutes.
 28. Centrifuge the cells for 1 minute at 3,000g.
 29. Carefully remove the supernatant and resuspend in 20 µl of 5% SDS.
 30. Add 20 µl 2x SDS-PAGE buffer and boil the sample for 5 minutes.
 31. Load 20 µl of the supernatant for western blot (a general western blot method is described elsewhere). Detect the fusion bait protein using anti-LexA antibody.
- Assessing auto-activation- by the LexA-bait fusion protein
32. Streak a single colony from the established bait line from step 21 onto a new YC-W plate and incubate for 2-3 days at 30 °C.
 33. Pick a single colony into 100 µl of sterile 1xTE, resuspend with a pipette.
 34. Use sterile inoculating loops to re-streak the yeast from step 33 onto a series of

YC-WH plates containing various concentrations of 3-AT (ranging from 0-50 mM)

35. Incubate for 2-3 days at 30°C.

36. Assess yeast growth to determine the minimal concentration of 3-AT that prevents selfactivation. First round of screening – Large scale yeast transformation

37. Inoculate 5-10 colonies of the established bait strain from step 32 in 1 ml of autoclaved Milli-Q H₂O in a sterile 15 ml falcon tube and resuspend by vortexing. Critical Step: Ensure that there are no clumps of yeast as this will inhibit cell growth

38. Inoculate the yeast suspension into 200 ml of YC-W media (in a 500 ml Erlenmeyer flask).

39. Culture overnight at 30°C with shaking at 225 rpm.

40. Measure the OD₆₀₀ of the overnight culture and calculate the volume required to dilute this culture to OD₆₀₀ between 0.15-0.2 in 1 L of YPAD medium.

41. Take the volume calculated in step 40 (between 100-200 ml) and dispense this into 225 ml Falcon conical tube.

42. Centrifuge for 5 minutes at 1000g at RT and remove the supernatant

43. Resuspend the yeast pellet in 5 ml YPAD medium and inoculate into 1 L of pre-warmed YPAD medium in 2.5 Erlenmeyer flask.

44. Incubate at 30 °C with shaking at 225 rpm until the OD₆₀₀ reaches between 0.4-0.5 (usually 3-4 hours)

45. Critical Step: The OD₆₀₀ at the end of YPAD culture should not exceed 0.6 as transformation efficiency would drop beyond this OD₆₀₀.

46. Transfer the yeast culture into 50~225 ml conical tubes and pellet the cells by centrifugation for 5 minutes at 1000g at 4°C.

47. Resuspend each pellet in 40 ml of sterile Milli-Q H₂O and combine into one 225 ml conical tube

48. Centrifuge the yeast cells for 5 minutes at 1000g at 4°C.

49. Resuspend the yeast pellet again in 200 ml of sterile Milli-Q H₂O and centrifuge the yeast cells for 5 minutes at 1000g at 4°C.

50. Resuspend the yeast pellet in in 8 ml freshly prepared TE-LiAc. Critical Step: Keep at 4°C until use (no more than 1 hour on ice).

51. Denature the salmon sperm DNA by incubating at 95°C for 5 minutes and chill on ice immediately for at least 2 minutes.

52. Combine 300 µg of a yeast single domain library DNA and 10 mg of denatured salmon sperm DNA and mix well by pipetting (the mixture may be viscous).

53. Add this DNA mixture to 8 ml of competent yeast cells (from step 52) and mix well by inversion.

54. Transfer the DNA-competent yeast mix into 250 ml Erlenmeyer flask and add 60 ml of freshly prepared, sterile PEG-TE-LiAc solution, mix by swirling. Critical Step: The transformation efficiency could be affected by the quality of the PEG, LiAc and carrier DNA. PEG-TE-LiAc mixture and TE-LiAc mixture (in step 50) should be prepared on the same day as the transformation.

55. Incubate for 30 minutes at 30 °C with shaking at 225 rpm.

56. Add 7 ml of DMSO and mix gently by swirling. Critical Step: Do not vortex.

57. Heat shock for 15 minutes at 42°C in a water bath and swirl to mix every 2 minutes.

58. Place on ice for 2 minutes and transfer into 225 ml conical tube.
 59. Fill the tube with sterile Milli-Q H₂O and centrifuge for 5 minutes at 2,500g, RT.
 60. Carefully decant the supernatant without disturbing the pellet, gently resuspend the pellet in 10 ml of sterile Milli-Q H₂O and then fill the tube with 200 ml of sterile Milli-Q H₂O.
 61. Centrifuge for 5 minutes at 2,500g, RT, remove the supernatant by pipetting.
 62. Resuspend the pellet in 10 ml of sterile 1xTE buffer.
 63. To determine the total number of transformants, make 100 μ l serial dilutions of transformed yeast from step 62 (the equivalent of 10 μ l (1:10), 1 μ l (1:100), 0.1 μ l (1:1,000), 0.01 μ l (1:10,000), in 1xTE and plate onto 9 cm YC-WL plates.
 64. Incubate for 2-3 days at 30 °C.
 65. Count the number of yeast colonies and multiply this number by the dilution factor to give the number of transformed yeast (e.g. 150 colonies counted on YC-WL plate spreading equivalent to 0.1 μ l (dilution 1:1000) of yeast is $150 \times 1000 = 1.5 \times 10^5$ per 100 μ l of transformed yeast plated out for colony counting. 100 μ l of total 10 ml transformants (1:100) will have $1.5 \times 10^5 \times 100 = 1.5 \times 10^7$ transformants).
 66. Plate all of the transformed yeast from step 62 onto 5x Nunc bioassay dishes (25cm x 25cm square) with YC-WLH agar (using minimal 3-AT concentration required to prevent selfactivation determined in step 34 if necessary).
 67. Incubate for 4-5 days at 30 °C.
 68. Pick those yeast colonies that have grown to about 2-3 mm in diameter separately into 50 μ l 1xTE.
 69. Re-streak individual colonies separately onto a new 6 cm YC-WLH plate (this will be the master plate)
 70. Grow for 2-3 days at 30 °C.
- Pause Point: The yeast master plates are stable for at least a week when wrapped in parafilm and stored at 4°C. Preparation of yeast plasmid DNA for a second sub-library construction
71. Inoculate all positive yeast colonies from the master plates into 5 ml YC-L medium in a 15ml Falcon tube.
 72. Incubate overnight at 30 °C with shaking at 225 rpm.
 73. Centrifuge culture at 1000g for 5 minutes.
 74. Pour off supernatant and resuspend in 200 μ l YC medium.
 75. Add 20 μ l of lyticase solution (10U/ μ l). Mix by vortexing.
 76. Incubate at 37 °C for 30-60 minutes with shaking at 225 rpm.
 77. Add 10 μ l of 20% SDS and vortexing vigorously.
 78. Put the sample through one freeze/thaw cycle (-20°C) and vortex again to ensure complete lysis of the cells. Pause Point: lysed cells can be stored frozen at -20°C.
 79. Bring the volume of the lysate to 200 μ l in 1xTE buffer.
 80. Add 200 μ l of phenol:chloroform:isoamyl alcohol (25:24:1).
 81. Vortex vigorously for 5 minutes.
 82. Centrifuge at 15,000g for 10 minutes at RT.
 83. Transfer the upper aqueous phase to a fresh tube.
 84. Add 20 μ l of 3 M Sodium acetate and 500 μ l of 100% ethanol.
 85. Place at -70°C or in dry ice for 1 hour.

86. Centrifuge at 15,000g for 10 minutes at RT.
87. Discard the supernatant and dry the pellet for 10-15 minutes at RT.
88. Resuspend the pellet with 20 µl Milli-Q H₂O. Pause Point: The isolated yeast plasmid DNA can be stored at -20°C indefinitely. Construction of the single-domain sub-library with CDR2 randomization for a second round of screening.
89. To randomize the CDR2 of VH (template sources from VH library screenings, ~20-50 ng for each PCR) set up a 50 µl PCR reaction with primerpair A (sFvVP16F and rdmVHCDR2Rev (expected band size 328 bp)) and a 50 µl PCR reactions with primer B (VHCDR2Fw and sFvVP16R (expected band size 360–399 bp)) according to the manufacturer's protocol for Phusion High-Fidelity DNA polymerase. For VL (from VL library screening), set up a 50 µl PCR reaction with primer pair C (sFvVP16F and rdmVLCDR2Rev (expected band size 320 bp)) and a 50 µl PCR reaction using primer pair D (VLCDR2Fw and sFvVP16R (expected band size 345 bp)).
90. Put the PCR reaction mixtures into thermocycler and run the following PCR program: initial 95°C for 2 min, 95°C for 20 s, 55°C for 10 s and 72°C for 20 s for 30 cycles, and final extension step at 72°C for 5 minutes.
91. Take 2 µl of each PCR reaction and run on 1.5% agarose (w/v) gel to confirm the size of the PCR products.
92. Run the remaining PCR reaction on 1.5% agarose (w/v) gel and excise the correct sized band (see step 89)
93. Extract DNA from the gel pieces using QIAquick gel extraction kit according to manufacturer's instructions. The final DNA elution volume should be 30 µl in 1.5 ml microfuge tube.
94. For the assembly of the two PCR products from Step 93, set up 100 µl PCR reactions using 2 µl of 10 µM primers sFvVP16F and sFvVP16R plus 1 µl of each DNA (for VH library 1µl of products from primer pair A and primer pair B or for VL library from 1 µl of products from primer pair C and primer pair D).
95. Carry out the PCR assembly using the same thermocycling conditions as Step 90.
96. Take 2 µl of each PCR reaction and run on 1.5% agarose (w/v) gel to confirm the size of the PCR products.
97. If the analytical gel shows the correct size PCR product (669–708 bp for VH and 645 bp for VL), purify the remaining PCR reaction with a QIAspin PCR purification kit using the manufacturer's instructions. The final DNA elution should be 50 µl in a 1.5 ml micro tube.
98. Add 10 µl of 10× NEB Cutsmart buffer and 2 µl (40 U) of SfiI restriction enzyme to the eluted PCR fragment and mix well. Incubate for at least 3 hrs at 50 °C.
99. At the same time, set up a digestion of 5 µg of pVP16* yeast plasmid, 5 µl of 10× NEB Cutsmart buffer, 2.5 µl (50 U) of SfiI restriction enzyme in a 100 µl total reaction and mix well.
100. Incubate at 50 °C until digested completely (for 3 hrs at least). Critical Step: Complete digestion of the vector should be confirmed by electrophoresis of 2 µl (0.2 µg) of the digestion reaction on a 1–1.5% agarose gel (w/v) compared with the uncut plasmid.
101. For each SfiI digest add 1 µl of 10× cutsmart buffer, 2.5 µl (50U) of NotI-HF enzyme, mix well and incubate for a further 3 hrs at 37°C. Confirm the complete digestion of the pVP16* plasmid by electrophoresis of 2 µl aliquot on a 1.5% agarose (w/v) gel.

102. Add 1 μ l of calf-intestine alkaline phosphatase (CIAP) to the SfiI-NotI digested pVP16* reaction and incubate for a further 30 min at 37 °C. Critical Step: Do not add CIAP to the SfiI-NotI digested PCR fragments.

103. Run the digested DNAs on a preparative 2% agarose gel (w/v) (for PCR products) or 0.8% agarose gel (w/v) (for CIAP-treated linearized pVP16* plasmid).

104. Cut the appropriate band from the gel containing the single-domain DNA fragment (around 333–396 bp) and linearized pVP16* (8.2 kbp).

105. Extract the DNA from the pieces of agarose gel using QIAquick gel extraction kit according to the manufacturer's instructions. Elute the DNA with 30 μ l of elution buffer.

106. Set up a set of 30 μ l ligations in sterile microfuge tubes with 2 μ g of the purified SfiI-NotI linear pVP16* plasmid and 150 ng of the purified SfiI-NotI PCR fragment (vector:insert ratio of ~1:3), 1 μ l of T4 DNA ligase and 3 μ l of 10 \times ligase buffer. Incubate overnight at 15 °C (or on ice). Pause Point: After overnight reaction, the ligation samples can be stored at – 20 °C for up to 2 weeks.

107. Add 59 μ l of H₂O, 1 μ l of yeast tRNA (10 mg ml^{–1}) and 10 μ l of 3 M sodium acetate and mix well.

108. Add 250 μ l of ice-cold absolute ethanol, vortex and incubate on dry ice (or at – 70 °C) for 10 min (or – 20 °C for 2–3 h).

109. Centrifuge for 10 min at 15,000g, RT.

110. Remove the solution and wash the DNA pellet once with 500 μ l of 70% ethanol (v/v) and re-spin at 15,000g, RT.

111. Remove the ethanol and dry the pellet for 10–15 min at RT.

112. Resuspend the DNA pellet in 10 μ l of 1 \times TE, which is now ready for bacterial transformation.

113. Use 1 μ l of ligation reactions for bacteria transformation per 50 μ l of NEB®5-alpha competent E.coli or by electroporation according to the manufacturer's instructions.

114. After transformation, add SOC medium (at RT) to 1 ml and transfer to a 15 ml sterile Falcon tube.

115. Culture for 60 min at 37 °C with shaking (225 r.p.m.).

116. Make 100 μ l of tenfold serial dilutions (from 1:100 to 1:10,000) from the 100 μ l of transformation and plate onto 9 cm TYE + ampicillin (100 μ g ml^{–1}) plates to determine the transformation efficiency. Incubate the plates overnight at 37 °C. Critical Step: The bacterial colonies from these plates can not only be used for calculating the library size but also for checking the quality of library

117. Inoculate a total of 10–20 individual colonies randomly selected from the titration plates (from step 116) in 2 ml of 2 \times TY containing 100 μ g ml^{–1} ampicillin. Culture overnight at 37 °C with shaking (225 r.p.m.), harvest the bacteria from each culture and prepare the plasmid DNA using QIAspin plasmid mini kits according to the manufacturer's instructions.

118. Digest 0.5 μ g of each plasmid with SfiI (at 50 °C for at least 3 h) and NotI (at 37 °C for at least 1 h) as and run the products on a 1.5% agarose gel (w/v).

119. Sequence the plasmids that contain inserts of the correct size (confirmed in step 118) using either sFvVP16F or sFvVP16R primers. Multiply the transformation efficiency from step 116 by the fraction of plasmids with insert determined from sequence data. This is equal to the size of library from 1 μ l of ligation reaction in step

- 112.
120. Calculate the volume of ligation required to make a sub-library size of $>1 \times 10^6$. Repeat the bacteria transformation with the remaining ligation reaction from step 112.
121. Plate the transformation mix onto ten large square TYE plates (25 cm \times 25 cm) containing 100 μ g ml⁻¹ ampicillin. Incubate the plates overnight at 37 °C.
122. Add 5 ml of 2% TY + 100 μ g ml⁻¹ ampicillin onto each plate and scrape all of the bacterial colonies using a sterile spreader.
123. Transfer all of the scraped bacteria suspension into 50 ml Falcon tubes and make bacterial pellets by spinning for 30 min at 2,500g, 4 °C.
124. Extract the plasmid DNA from the recovered cells using a QIAGEN Plasmid Midi or Maxi Kit according to the manufacturer's instructions. The extracted plasmid DNA is the CDR2 randomized sub-library.
125. Measure the quality and concentration of the plasmid DNA by determining the UV spectrum from 220 and 300 nm.
Pause Point: The sub-library DNA can be stored at - 20 °C indefinitely. Second round screening
126. Using the same bait line expressing LexA-antigen from step 36, screen the CDR2 sublibrary using the procedure described in steps 37-65.
127. Plate all of the transformed yeast onto 5x large (25 cm \times 25 cm) YC - WLH plates containing 25, 50, 75 or 100 mM 3-AT (this will allow iDABs with increased in vivo binding affinity to be selected on the higher concentrations of 3-AT). Pause Point: Yeast should be stable for at least 1 week at 4 °C.
128. Incubate for 4-7 d at 30 °C.
129. Pick those yeast colonies that are 1-2 mm in size and separately streak onto a new 14- or 9-cm small round YC - WLH plate (as master plate).
130. Incubate the plates at 30 °C until yeast growth is visible (1-2 d). Pause Point: The plates can be kept at 4 °C for 3-7 d. Preparation of yeast prey plasmids DNA for the third sub-library construction
131. Inoculate the yeast colonies from the YC-WLH plate into 5 ml of YC-L medium.
132. Extract the plasmid DNA from yeast cultures as described in steps 71-88. Measure the quality and concentration of the plasmid DNA by determining the UV spectrum from 220 and 300 nm.
Construction of a CDR1-randomized sub-library for a third round of screening
133. Using the plasmid DNA from step 132 (20-50 ng) set up two 50 μ l PCR reactions as described in step 89 except use the following primer pairs: Primers pairs A: sFvVP16F and rdmVHCDR1Rev and B: VHCDR1Fw and sFvVP16R for VH libraries and primer pairs C: sFvVP16F and rdmVLCDR1Re and D: VLCDR1Fw and sFvVP16R for VL libraries.
134. Carry out the PCR reaction as in step 90.
135. Extract and purify the amplified PCR fragments as in Steps 91-93.
136. Carry out the PCR assembly and digest the assembled PCR fragment with SfiI and NotI as in steps 94-98.
137. Construct the third sub-library by cloning the PCR fragments from step 136 into SfiI and NotI sites of pVP16* DNA as described before in steps 99-125. Third round selection screening
138. Repeat the same protocol as in step- 126-130 except using the library

- constructed in step 137, however, carry out the screening with YC – WLH plates containing a higher concentration of 3-AT than with the second round screening. (Choose the concentration of 3-AT that allowed the growth of 20–100 yeast in Step 127. Extraction of yeast prey plasmid DNA from individual selected colonies
139. Inoculate the yeast colonies from the YC – WLH plate of step 138 into 5 ml of YC – L medium, culture overnight at 30 °C with shaking (225 r.p.m.) and then harvest the yeast and extract the plasmid DNA from yeast culture as described in steps 71–88.
140. Transform 1–5 µl of the DNA stock from Step 139 into bacteria (e.g., DH5α) using electroporation or chemical transformation and plate onto TYE + ampicillin (100 µg ml⁻¹) plates.
141. Grow the bacteria overnight at 37 °C.
142. Inoculate bacterial colonies into 2 ml of 2× TY containing 100 µg ml⁻¹ ampicillin.
143. Extract the plasmid DNA using QIAprep Spin MiniPrep kit according to the manufacturer's protocol. Pause Point: Plasmid DNA can be stored at – 20 °C indefinitely.
144. To check for the identity of yeast single domain-VP16 plasmid, digest 5 µl of DNA with SfiI and NotI enzymes (digest first with SfiI for 3 hrs at 50 °C, cool to 37 °C, add NotI and continue the digest for a further 3 h at 37 °C). Separate the products on a 1.5% agarose gel w/v). The iDAb–VP16 fusion should give a band of 333–396 bp and the vector a band of 8.2 kbp. Re-testing the single domain VH or VL-VP16 plasmid for interaction with LexA protein
145. Prepare competent L40 yeast as described in steps 2–9 and denature salmon sperm DNA as in step 10.
146. Using 200 ng of pBTM116 vector (empty bait vector) mix with 200 ng of the isolated yeast single-domain VP16 plasmid DNAs prepared in step 143 with 100 µg of salmon sperm carrier DNA. Add the DNA mixture into 100 µl of each competent yeast bait and mix well.
147. Follow the same procedure as described in Steps 13–17.
148. Remove the supernatant and resuspend the pellet in 100 µl of sterile 1× TE. Plate 50 µl of the transformed yeast onto a 10 cm YC-WL plate.
149. Incubate the plate for 2–3 days at 30 °C.
Pause Point: These yeast plates are stable for at least one week when wrapped with parafilm and stored at 4 °C.
150. Plate the transformants of all the clones being tested by spotting 10 µl of each resuspended yeast onto one 10 cm YC – WL plate, one 10 cm YC–WLH plate. Incubate at 30 °C for 1–3 d until yeast growth is visible.
151. True positive prey clones will not grow on YC–WLH plate as they should not interact with LexA without antigen fused to it.

Troubleshooting

Yeast transformation efficiency. Low or no colonies on selection plates after yeast transformation are usually associated with:

1. The quality of transformation reagents. As emphasized in the protocol, the TE-LiAc and PEG-TE-LiAc solutions must be made fresh on the day of transformation. The concentration

of PEG3350 is critical for yeast transformation and when transformation is being carried out all reagents must be mixed well to create homogenous solution. An old stock solution of PEG3350 of more than 1 month should not be used as this could affect the final concentration of PEG (~32-33%) due to evaporation during storage.

2. OD600 at the start and end of culture. Starting OD600 of yeast culture should not exceed 0.2 when doing transformation as yeast requires to undergo at least one doubling. The transformation efficiency also significantly reduces when the harvesting point is near or above OD600 of 0.6. This is particularly critical when doing a large-scale transformation for the libraries.
3. Selective plate is incorrect. Always check the formulation of the plate and use media and plate that is less than one month old.

Sub-library construction. Very few bacteria transformants could be due to:

1. Ligation efficiency is low. The vector to insert ratio should be around 1:3 for optimal ligation efficiency as specified in step 106. The T4 ligation buffer must not be repeatedly freeze/thawed as this degrades the ATP component in the buffer. Ensure complete digestion of pVP16* vector to prevent self-ligation and verify that the PCR insert have the correct molecular weight after SfiI and NotI digestion as stated in step 104.
2. Low competency of E.coli. Test the efficiency of competent cells with supercoiled plasmid (e.g. pUC19) and optimize the amount of ligated reaction added to an aliquot of competent cells. It may be necessary to do several transformations to obtain the library size of $>10^6$. Yeast plasmid extraction. No or poor plasmid yield could be caused by improper growth of yeast. Ensure that the yeast is grown in YC-L media (to enforce selective pressure on the "prey" (leucine-synthesizing pVP16*).

Anticipated Results

The number of yeast transformants will depend on the bait protein used and to an extent will correlate with the size of the bait protein. A rule of thumb is that 20-50 transformants are obtained with a bait length of 100 amino acids that does not cause some auto-activation and growth on histidine-minus plates.

References

- 1 Rabbitts, T. H. & Stocks, M. R. Chromosomal translocation products engender new intracellular therapeutic technologies. *Nat Med* 9, 383-386, (2003) doi:10.1038/nm0403-383.
- 2 Tanaka, T. & Rabbitts, T. H. Interfering with protein-protein interactions: potential for cancer therapy. *Cell Cycle* 7, 1569-1574, (2008) doi:10.4161/cc.7.11.6061.
- 3 Rondon, I. J. & Marasco, W. A. Intracellular antibodies (intrabodies) for gene therapy of infectious diseases. *Annu Rev Microbiol* 51, 257-283, (1997).
- 4 Tanaka, T., Lobato, M. N. & Rabbitts, T. H. Single domain intracellular antibodies: a minimal fragment for direct in vivo selection of antigen-specific intrabodies. *J Mol Biol* 331, 1109-1120 (2003).
- 5 Tanaka, T. & Rabbitts, T. H. Intrabodies based on intracellular capture frameworks that bind the RAS protein with high affinity and impair oncogenic transformation. *The EMBO journal* 22,

1025-1035, (2003) doi:10.1093/emboj/cdg106.

6 Visintin, M., Tse, E., Axelson, H., Rabbitts, T. H. & Cattaneo, A. Selection of antibodies for intracellular function using a two-hybrid in vivo system. *Proc Natl Acad Sci U S A* 96, 11723-11728 (1999).

7 Tanaka, T. & Rabbitts, T. H. Protocol for the selection of single-domain antibody fragments

by third generation intracellular antibody capture. *Nat Protoc* 5, 67-92, (2010)

doi:nprot.2009.199 [pii] 10.1038/nprot.2009.199 [doi].

8 Zeng, J., Li, H. C., Tanaka, T. & Rabbitts, T. H. Selection of human single domain antibodies

recognizing the CMYC protein using enhanced intracellular antibody capture. *J Immunol Methods* 426, 140-143, (2015) doi:10.1016/j.jim.2015.08.009.

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Associated Publications

This protocol is related to the following articles:

Protocol for the selection of single-domain antibody fragments by third generation intracellular antibody capture

Tomoyuki Tanaka and Terence H Rabbitts

Interfering with RAS–effector protein interactions prevent RAS-dependent tumour initiation and causes stop–start control of cancer growth

T Tanaka and T H Rabbitts

Single Domain Intracellular Antibodies from Diverse Libraries

Tomoyuki Tanaka, Helen Sewell, Simon Waters, Simon E. V. Phillips, and Terence H. Rabbitts

Conformational flexibility of the oncogenic protein LMO2 primes the formation of the multi-protein transcription complex

H. Sewell, T. Tanaka, K. El Omari, E. J. Mancini, A. Cruz, N. Fernandez-Fuentes, J. Chambers, and T. H. Rabbitts

Selection of human single domain antibodies recognizing the CMYC protein using enhanced intracellular antibody capture

J. Zeng, H.C. Li, T. Tanaka, and T.H. Rabbitts