Human Single Fold scFv Libraries I + J (Tomlinson I + J)

General Introduction to the Libraries

Over the past 10 years Greg Winter's lab at the MRC Laboratory of Molecular Biology and the MRC Centre for Protein Engineering (Cambridge, UK) has created a number of artificial libraries of antibodies that can be used to derive binders to almost any target molecule using phage display and selection. These binders can be used for all the same applications as conventional monoclonal antibodies (ELISA, Western blotting, FACS, immunohistochemistry etc) but can be isolated in a fraction of the time and without the need for animal immunisation. To date these so called "naïve" or "single pot" phage-antibody libraries have been used successfully in hundreds of molecular biology labs world-wide to derive highly specific antibody reagents to a wide range of different proteins, peptides or small molecule compounds.

The latest libraries (Tomlinson I and J) that are being distributed by the MRC HGMP Resource Centre each comprise over 100 million different scFv fragments cloned in an ampicillin resistant phagemid vector and transformed into TG1 *E. Coli* cells (scFv fragments comprise a single polypeptide with the VH and VL domains attached to one another by a flexible Glycine-Serine linker). By carefully following the protocol provided, large numbers of phagemids can be produced and used to select specific binders to target molecules that are attached to the surface of a tube or biotinylated and captured by streptavidin coated beads (so called "panning"). After each round of panning, the non-binders are washed away and the phagemids bound to the target molecule/s are eluted and amplified by infection into fresh TG1 cells. After producing new phagemids from the previous round of panning, the process can be repeated. Typically two or three rounds of panning are required to ensure that more than half the different scFvs in the selected population bind to the target molecule. The monoclonal scFvs can then be screened for binding (using a simple ELISA based protocol) and then used for further analysis of the target molecule. Since all the functional scFvs in the Tomlinson I and J libraries bind Proteins A and L, either of these secondary reagents can be used for detection, purification or immobilisation. Alternatively, secondary reagents that bind the attached myc or HIS6 tags can be used, although in our experience it is better to use the Protein A or L reagents.

Finally, we would like to emphasise that these libraries represent a valuable resource. Whether you are familiar with phage display or not we recommend that you perform test selections and subsequent ELISA screening using the anti-bovine serum albumin and anti-bovine ubiquitin controls provided. Only when these experiments have been successfully carried out should you defrost the libraries and start preparing library phage.

Derivation of libraries

Both libraries are based on a single human framework for V_H (V3-23/DP-47 and J_H4b) and V_κ (O12/O2/DPK9 and J_κ1) with side chain diversity incorporated at positions in the antigen binding site that make contacts to antigen in known structures and are highly diverse in the mature repertoire. The canonical structure (V_H: 1-3, V_κ: 2-1-1) encoded by this framework is by far the most common in the human antibody repertoire. The CDR3 of the heavy chain was designed to be as short as possible yet still able to form an antigen binding surface. Both libraries can be selected and affinity matured without knowing the sequences of selected clones. Libraries are in phagemid/scFv format and have been pre-screened for binding to Protein A and Protein L so that the majority of clones in the unselected libraries are functional.

Tomlinson I

Constructed in pIT2 (HIS myc tag). Diversified (DVT) side chains based mainly on those positions which are diverse in the primary repertoire (total of 18 residues - H50, H52, H52a, H53, H55, H56, H58, H95, H96, H97, H98, L50, L53, L91, L92, L93, L94 and L96). After selection, can be matured by incorporating additional diversity based on somatic mutation.

Library size (with insert): **1.47×10^8**

Percentage insert: **96%**

Tomlinson J

As above but with NNK side chains.

Library size (with insert): **1.37×10^8**

Percentage insert: **88%**

Read carefully before using the libraries

1. Check that you have received:
   a tube of Library I (~500 μl)
   a tube of Library J (~500 μl)
   a glycerol stock of a positive control anti-ubiquitin ScFv in bacterial strain TG1 (labeled TG1-anti ubi)
   a glycerol stock of a positive control anti-BSA ScFv in bacterial strain TG1 (labeled 13CG2)
   a glycerol stock of T-phage resistant *E. Coli.* TG1 for propagation of phage (labeled TG1Tr)
   (K12 *Δ(lac-proAB) supE thi hsdD5/*F' *traD36 proA+B lacIq lacZΔM15*)
   a glycerol stock of *E. Coli.* HB2151 for expression of antibody fragments
   (K12 *ara Δ(lac-proAB) thi/*F' *proA+B lacIq lacZΔM15*)
   Phage KM13[3] (~100 μl with 10^7 pfu/ml)

2. Check the library is still frozen and make sure you keep it frozen at -70°C until needed.

3. Make stock of KM13 according to Protocol G.

4. Run through the protocols using the control clones before you use the library: Streak the controls on TYE plates containing 100 μg/ml ampicillin and 1 % glucose. After overnight growth at 37°C in an incubator pick a single colony from each and grow these overnight (shaking at 37°C) in 5 ml 2xTY[1] containing 100 μg/ml ampicillin and 1 % glucose. Make phage for the positive and negative controls separately (use 500 μl of overnight in D1-D10). Use a 1:100 mixture of phage produced from the positive and the negative controls and perform one round of selection (C1-C11) using 100 μg/ml of ubiquitin[2] in PBS for coating. Check for enrichment of ubiquitin binders (should be over 50% after one round of selection) by monoclonal phage ELISA (E9-E14).

5. Wherever possible use devoted pipettes and disposable plastic ware. The use of polypropylene tubes is recommended as phage may adsorb non-specifically to other plastics.

6. For efficient infection of phage, *E. coli* must be grown at 37°C and be in log phase (OD at 600 nm of 0.4). To prepare this:
   i. Transfer a bacterial colony from a minimal media plate into 5 ml of 2xTY medium (no antibiotics or glucose). Grow shaking overnight at 37°C.
   ii. Next day dilute overnight 1:100 into fresh 2xTY medium. Grow shaking at 37°C until OD 600 is 0.4 (1.5-2 hrs)

7. All centrifugations, except those performed in a micro centrifuge, are performed at 4°C.

8. Libraries I and J must be used separately and preferably in parallel. This will ensure selecting the most antigen binding clones.

Day by day overview of library selection

In advance

Gather all equipment and reagents (product details are given in the notes at the end of all the protocols). Make sure you have all the necessary media and plates for bacterial growth (the large Bio-Assay plates need to be air-dried in a sterile environment for 2 hrs before use).

Plan your time - most of the daily procedures can be performed simultaneously, so read through each protocol carefully before starting.

	Steps	*Procedure*
Day 1 (5 hrs)	A1-A6	Grow libraries I and J and make phage
	B1-B3	Make secondary stock of libraries
Day 2 (6 hrs)	A7-A12	Grow libraries I and J and make phage (cont.)
	C1	Coat immunotubes for 1st round of selection
Day 3 (6.5 hrs)	C2-C11	1st round of selection
Day 4 (3 hrs)	D1-D6	Make phage from 1st round of selection
	C1	Coat immunotubes for 2nd round of selection
Day 5 (6.5 hrs)	D7-D11	Make phage from 1st round of selection (cont.)
	C2-C11	2nd round of selection
Day 6 (3 hrs)	D1-D6	Make phage from 2nd round of selection
	C1	Coat immunotubes for 3nd round of selection
Day 7 (6.5 hrs)	D7-D11	Make phage from 2nd round of selection (cont.)
	C2-C11	3rd round of selection
Day 8 (3 hrs)	D1-D6	Make phage from 3rd round of selection
	E1	Coat 96 well plate for polyclonal phage ELISA
Day 9 (6.5 hrs)	D7-D11	Make phage from 3rd round of selection (cont.)
	E2-E8	Polyclonal phage ELISA

Further characterisation of individual clones can be performed by monoclonal phage ELISA (protocol E), monoclonal ELISA using soluble scFv fragments (protocol F), PCR screening (to check for insert, protocol H) and sequencing (protocol I).

A. Growing the libraries

1. Add the library stock to 200 ml pre-warmed 2xTY containing 100 μg/ml ampicillin and 1 % glucose.
2. Grow shaking at 37°C until the OD 600 is 0.4 (1-2 hrs).
3. Take 50 ml of this and add 2×10^{11} KM13 helper phage[3]. (Use the remaining 150 ml to make a secondary bacterial stock of the library by following protocol B).
4. Incubate without shaking in a 37°C water bath for 30 min.
5. Spin at 3,000 g for 10 min (3,600 rpm in Centra 8 or equivalent). Resuspend in 100 ml of 2xTY containing 100 μg/ml ampicillin, 50 μg/ml kanamycin and 0.1% glucose.
6. Incubate shaking at 30°C overnight.
7. Spin the overnight culture at 3,300 g for 30 min (4,000 rpm in Centra 8 or equivalent).
8. Add 20 ml PEG/NaCl (20 % Polyethylene glycol 6000, 2.5 M NaCl) to 80 ml supernatant. Mix well and leave for 1 hr on ice.
9. Spin 3,300 g for 30 min (4,000 rpm in Centra 8 or equivalent). Pour away PEG/NaCl. Respin briefly and aspirate any remaining dregs of PEG/NaCl.
10. Resuspend the pellet in 4 ml PBS and spin at 11,600 g for 10 min in a micro centrifuge to remove any remaining bacterial debris.
11. Store the phage at 4°C for short term storage or in PBS with 15 % glycerol for longer term storage at -70°C.
12. To titre the phage stock dilute 1μl phage in 100μl PBS, 1μl of this in 100μl PBS and so on until there are 6 dilutions in total. Add 900μl of TG1 at an OD 600 of 0.4 to each tube and incubate at 37°C in a waterbath for 30 mins. Spot 10 μl of each dilution on a TYE[5] plate containing 100 μg/ml ampicillin and 1 % glucose and grow overnight at 37°C. Phage stock should be 10^{12}-10^{13}/ml, enough for at least 10 selections.

B. Growing secondary stocks of the libraries

1. Grow the remaining 150 ml from A3 for a further 2 hr shaking at 37°C.
2. Spin down the cells at 10,800 g for 10 min. Resuspend in 10 ml of 2xTY containing 15 % glycerol.
3. Store this secondary stock in 20x 500 µl aliquots at -70°C. Use one aliquot for each phage preparation according to protocol A - this will only be necessary if you wish to do more than 10 selections.

C. Selection on immunotubes

(Alternatively, phage can be selected using biotinylated antigen in solution or affinity chromatography. For details see Winter *et al.* (1994) *Annu. Rev. Immunol.* **12**, 433)

1. Coat immunotube[6] overnight with 4 ml of the required antigen. The efficiency of coating can depend on the antigen concentration, the buffer and the temperature. Usually 10-100 μg/ml antigen in PBS is used.
2. Next day wash tube 3 times with PBS (pour into the tube and then pour it immediately out again).
3. Fill tube to brim with 2 % MPBS (2 % Marvel milk powder[7] in PBS). Incubate at rt. Standing on the bench for 2 hr to block.
4. Wash tube 3 times with PBS.
5. Add 10^{12} to 10^{13} phage from A11 in 4 ml of 2 % MPBS. Incubate for 60 min at rt. rotating using an under-and-over turntable and then stand for a further 60 min at rt. Throw away supernatant.
6. Wash tubes 10 (round 1)-20 (rounds 2 and 3) times with PBS containing 0.1 % Tween 20.
7. Shake out the excess PBS and elute phage by adding 500 μl of trypsin-PBS (50 μl of 10mg/ml trypsin stock solution[8] added to 450 μl PBS) and rotating for 10 min at rt using an under-and-over turntable.
8. Take 1.75 ml of TG1 at an OD 600 of 0.4 and add 250 μl of the eluted phage (the remaining 250 μl should be stored at 4°C). Incubate for 30 min at 37°C in a water bath without shaking.
9. Spot 10 μl, 10 μl of a 1:10^2 dilution and 10 μl of a 1:10^4 dilution on TYE plates containing 100 μg/ml ampicillin and 1 % glucose and grow overnight at 37°C to titre the phage.
10. *In round 1 if using a complex antigen (eg cells, cell lysates etc):* take the remaining TG1 culture and spin at 11,600 g in a micro centrifuge for 5 min. Resuspend the pelleted bacteria in 1 ml of 2xTY and plate on a large square Bio-Assay dish[9] containing TYE, 100 μg/ml ampicillin and 1 % glucose.

    In round 1 if using a single hapten, carbohydrate or protein antigen and in subsequent rounds for all antigens: take the remaining TG1 culture and spin at 11,600 g in a micro centrifuge for 5 min. Resuspend the pelleted bacteria in 50 μl of 2xTY and plate on a regular TYE plate containing 100 μg/ml ampicillin and 1 % glucose.
11. Grow plates at 37°C overnight.

The first round of selection is the most important. Any errors made at this point will be amplified in subsequent rounds of selection. After each round you should get back at least 100 infective phage. If you get less than this it is probable that a mistake has occurred. If so, try repeating the infection with a freshly grown TG1 culture (from a new overnight) at an OD 600 of 0.4 using the remaining 250 μl of eluted phage from C7. If this still yields less than 100 phage, repeat the selection starting at C1.

D. Further rounds of selection

1. After overnight growth add 7 ml of 2xTY 15 % glycerol to the large square Bio-Assay dish or 2mls to the regular plates and loosen the cells with a glass spreader, mixing them thoroughly. After inoculating 50 μl of the scraped bacteria to 50 ml of 2xTY containing 100 μg/ml ampicillin and 1 % glucose, store 1 ml of the remaining bacteria at -70°C in 15% glycerol.
2. Grow shaking at 37°C until the OD 600 is 0.4 (1-2 hrs).
3. Take 10 ml of this culture and add 5×10^{10} helper phage.
4 Incubate without shaking in a 37°C water bath for 30 min.
5. Spin at 3,000 g for 10 min. Resuspend in 50 ml of 2xTY containing 100 μg/ml ampicillin, 50 μg/ml kanamycin and 0.1% glucose.
6. Incubate shaking at 30°C overnight.
7. Spin the overnight culture at 3,300 g for 15 min.
8. Add 10 ml PEG/NaCl (20 % Polyethylene glycol 6000, 2.5 M NaCl) to 40 ml supernatant. Mix well and leave for 1 hr on ice.
9. Spin 3,300 g for 30 min. Pour away PEG/NaCl. Respin briefly and aspirate any remaining dregs of PEG/NaCl.
10. Resuspend the pellet in 2 ml PBS and spin at 11,600 g for 10 min in a micro centrifuge to remove the remaining bacterial debris.
11. Use 1 ml of this phage for the next round of selection (protocols C and D) and store 1 ml at 4°C.
12. Repeat selection for another 2 rounds.

E. Screening phage particles by ELISA

Populations of phage produced at each round of selection can be screened for binding by ELISA to identify "polyclonal" phage antibodies. Phage from single colonies can then be screened by ELISA to identify "monoclonal" phage antibodies. Alternatively, after a polyclonal phage ELISA you could proceed directly to making monoclonal soluble antibody fragments, see protocol F. In general, we have found that 2% Marvel in PBS is best for blocking during phage ELISA whereas 3% BSA in PBS is best for blocking during scFv ELISA.

(a) Polyclonal phage ELISA

1. Coat a 96 well flexible assay plate[10] overnight with 100 µl per well of antigen in the same buffer and at the same concentration as used for selection.
2. Wash wells 3 times with PBS. Plates can be immersed in a shallow bath containing PBS but you should check that all wells fill with wash solution (if they do not you may create false positives during later washes). Discard liquid by flipping plate over and then shaking it. Add 200 µl per well of 2 % MPBS (2 % Marvel in PBS) or 3% BSA-PBS (3% bovine serum albumin in PBS) to block and incubate for 2 hr at rt.
3. Wash wells 3 times with PBS. Add 10 µl PEG precipitated phage from the end of each round of selection in 100 µl 2 % MPBS (or 3 % BSA-PBS).
4. Incubate for 1 hr at rt. Discard phage solution and wash 3 times with PBS-0.1 % Tween 20.
5. Add 1 in 5000 dilution of HRP-anti-M13[11] in 2 % MPBS (or 3 % BSA-PBS). Incubate for 1 hr at rt., wash 3 times with PBS-0.1 % Tween 20.
6. Add 100 µl of substrate solution (100 µg/ml TMB[12] in 100 mM sodium acetate, pH 6.0. with10 µl of 30 % hydrogen peroxide added to 50 ml of this solution directly before use) to each well and leave at rt. for 2-15 min. A blue colour should develop.
7. Stop the reaction by adding 50 µl 1 M sulphuric acid. The blue colour should turn yellow.
8. Read the OD at 650 nm and at 450 nm. Subtract OD 650 from OD 450.

(b) Monoclonal phage ELISA

9. Inoculate individual colonies from the titration plates from each round of selection (see C11) into 100 µl 2xTY containing 100 µg/ml ampicillin and 1 % glucose in 96 cell-well plates[13]. Grow shaking (250 rpm) overnight at 37°C.
10. Use a 96 well transfer device[14] to transfer a small inoculum (about 2 µl) from this plate to a second 96 cell-well plate containing 200 µl of 2xTY with 100 µg/ml ampicillin and 1 % glucose per well. Grow shaking (250 rpm) at 37°C for 2 hr. (Make glycerol stocks of the original 96-well plate, by adding glycerol to a final concentration of 15 %, and then storing the plates at -70°C).
11. After 2 hr incubation (of the second plate) add 25 µl 2xTY containing 100 µg/ml ampicillin, 1 % glucose and 10^9 helper phage.
12. Shake (250 rpm) at 37°C for 1 hr. Spin 1,800 g for 10 min, then aspirate off the supernatant.
13. Resuspend pellet in 200 µl 2xTY containing 100 µg/ml ampicillin and 50 µg/ml kanamycin. Grow shaking (250 rpm) overnight at 30°C.
14. Spin at 1,800 g for 10 min and use 50 µl of the supernatant in phage ELISA as detailed above.

F. Production of soluble antibody fragments

Individual colonies picked from the various rounds of selection (as plated on the dilution series) can be induced in TG-1 to produce soluble scFv (F2-F6). This will ensure the expression of all selected clones including those in which the scFvs contain TAG stop codons (TG-1 as able to suppress termination and introduce a glutamate residue at these positions). Unfortunately, since the TAG stop codon between the scFv and the gIII gene is also suppressed this leads to co-expression of the scFv-pIII fusion which tends to lower the overall levels of scFv expression, even in clones where there are no TAG stop codons in the scFv itself. To circumvent this problem, the selected phage can be used to infect HB2151 (a non-suppresor strain) which is then induced to give soluble expression of antibody fragments (scFv genes that do not contain TAG stop codons will now yield higher levels of soluble scFv than in TG-1, but those that contain TAG stop codons will not produce any soluble scFv) (F1-F6). The expressed scFvs can then be used in ELISA. Detection of bound scFv can be performed using either Protein A-HRP[15] or Protein L-HRP[16] conjugates.

1. From each selection take 10 µl of eluted phage and infect 200 µl exponentially growing HB2151 bacteria (OD 600 of 0.4) for 30 min at 37°C in a water bath. Plate 50 µl, 50 µl of a 1:10^2 dilution, 50 µl of a 1:10^4 dilution and 50 µl of a 1:10^6 dilution on TYE plates containing 100 µg/ml ampicillin and 1 % glucose and grow overnight at 37°C.
2. Pick individual colonies into 100 µl 2xTY 100 µg/ml ampicillin and 1 % glucose in 96 cell-well plates and grow shaking (250 rpm) overnight at 37°C.
3. Use a 96 well transfer device[14] to transfer a small inocula (about 2 µl) from this plate to a second 96 cell-well plate containing 200 µl 2xTY containing 100 µg/ml ampicillin and 0.1 % glucose per well. Grow shaking (250 rpm) at 37°C until the OD 600 is approximately 0.9 (about 3 hr). (A stock can be made of the first plate, by adding glycerol to a final concentration of 15 % and storing at -70°C).
4. Once OD 0.9 is reached (wells look quite cloudy) add 25 µl 2xTY containing 100 µg/ml ampicillin and 9 mM IPTG (isopropyl β-D-thiogalactoside, final concentration 1 mM IPTG). Continue shaking (250 rpm) at 30°C overnight.
5. Coat a 96 well flexible assay plate overnight with 100 µl per well of antigen in the same buffer and at the same concentration as used for selection.
6. Spin the plate from step F4 at 1,800 g for 10 min and use 50 µl of the supernatant (take care not to transfer any bacteria) for ELISA in 3% BSA-PBS (final concentration) (protocol E) except using a 1:5000 dilution of Protein A-HRP[15] or Protein L-HRP[16] to detect binding in step E5.

G. Production of large quantities of helper phage

1. Infect 200 µl TG1 at an OD 600 of 0.4 with 10 µl of 100-fold serial dilutions of KM13 helper phage[3] (in order to get well separated plaques) in a 37°C water bath (without shaking) for 30 min. Add to 3 ml molten H-top agar (42°C) and pour onto warm TYE plates (no antibiotics). Allow to set and incubate overnight at 37°C.
2. Pick a small plaque into 5 ml of fresh TG1 at an OD 600 of 0.4. Grow for about 2 hr shaking at 37°C.
3. Add to 500 ml 2xTY in a 2 litre flask and grow shaking at 37°C for 1 hr. Add kanamycin to a final concentration of 50 µg/ml (no glucose). Grow overnight shaking at 30°C.
4. Spin overnight culture at 10,800 g for 15 min. Add 100 ml PEG/NaCl (20 % polyethylene glycol 6000, 2.5 M NaCl) to 400 ml supernatent and leave for 1 hr on ice.
5. Spin 10,800 g for 30 min. Pour away PEG/NaCl.
6. Resuspend the pellet in 8 ml PBS and add 2 ml PEG/NaCl. Mix well and leave for 20 minutes on ice.
7. Spin 3,300 g for 30 min. Respin briefly and aspirate any remaining dregs of PEG/NaCl.
8. Resuspend pellet in 5 ml PBS and spin at 11,600 g for 10 min in a micro centrifuge to remove any remaining bacterial debris.
9. Store the helper phage at 4°C for short term storage or in PBS with 15 % glycerol for longer term storage at -70°C.
10. To titre the helper phage take 45µl phage and add 5µl trypsin stock solution. Incubate for 30 mins at 37 °C. Dilute 1µl of trypsin treated phage in 1ml PBS and make five 100 fold serial dilutions of this in 1ml aliquots of PBS. Add 50µl of the six dilutions to six separate tube containing 1ml of TG1 at an OD 600 of 0.4. Mix, add 3 ml molten H-Top and pour evenly onto TYE plates. Perform the same dilution series using 1µl of non-trypsin treated phage. The titre of the trypsin treated phage should be 10^5-10^8 lower than for the non-trypsin treated phage. If not, pick another plaque and repeat helper phage preparation.

H. PCR screening selected clones

Once the libraries have been selected you may wish to check individual clones for the presence of full length VH and V_{κ} insert. All PCRs are at annealing temperature of 55°C. 1 min extension for V_H or V_{κ} on their own, 2 min extension for V_H and V_{κ} together.

For V_H only use	LMB3:	CAG GAA ACA GCT ATG AC
	link seq new:	CGA CCC GCC ACC GCC GCT G
	with insert = 527 bp	
	without insert = 227 bp	
For V_{κ} only use	DPK9 FR1 seq:	CAT CTG TAG GAG ACA GAG TC
	pHEN seq:	CTA TGC GGC CCC ATT CA
	with insert = 368 bp	
	without insert = no band	
For V_H and V_{κ} use	LMB3:	CAG GAA ACA GCT ATG AC
	pHEN seq:	CTA TGC GGC CCC ATT CA
	with insert = 935 bp	
	without insert = 329 bp	

I. Sequencing selected clones

For sequencing of selected clones the following primers are recommended.

For V_H use link seq new	CGA CCC GCC ACC GCC GCT G
For V_{κ} use pHEN seq	CTA TGC GGC CCC ATT CA

J. Vector map and sequence of pIT2 vector

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                                                                     RBS
CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATA ATG AAA TAC CTA
---------------->                                                     M   K   Y   L
      LMB3

                                                               SfiI      __NcoI__
TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCG GCC CAG CCG GCC ATG GCC GAG GTG TTT
 L   P   T   A   A   A   G   L   L   L   A   A   Q   P   A   M   A   E   V   F

                                                      XhoI                  linker
GAC TAC TGG GGC CAG GGA ACC CTG GTC ACC GTC TCG AGC GGT GGA GGC GGT TCA GGC GGA GGT
 D   Y   W   G   Q   G   T   L   V   T   V   S   S   G   G   G   G   S   G   G   G

                                   SalI                                NotI                 HIS-tag
GGC AGC GGC GGT GGC GGG TCG ACG GAC ATC CAG ATG ACC CAG GCG GCC GCA CAT CAT CAT CAC
 G   S   G   G   G   G   S   T   D   I   Q   M   T   Q   A   A   A   H   H   H   H
  <-------------------------
         link seq new

                                                myc-tag
CAT CAC GGG GCC GCA GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT GGG GCC GCA TAG ACT
 H   H   G   A   A   E   Q   K   L   I   S   E   E   D   L   N   G   A   A   *   T
                                                                  <---------------------
                                                                          pHEN seq
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Notes

1. 2xTY is 16g Tryptone, 10g Yeast Extract and 5g NaCl in 1 litre.
2. Bovine ubiquitin (5 mg) is available from Fluka, Chemika-BioChemika, Industriestrasse 25, CH-9470 Buchs, Switzerland, Tel +41 81 755 2511, Fax +41 81 756 5449.
3. KM13 is the protease cleavable helper phage described in Kristensen and Winter, *Folding and Design* **3**, 321-328 (1998).
4. PBS is 5.84 g NaCl, 4.72 g Na_2HPO_4 and 2.64 g $NaH_2PO_4.2H_20$, pH 7.2, in 1 litre.
5. TYE is 15g Bacto-Agar, 8g NaCl, 10g Tryptone, 5g Yeast Extract in 1 litre.
6. Nunc Maxisorp immuno test tubes (Cat. No. 4-44202) are available from Gibco BRL, Life Technologies Ltd., P. O. Box 35, Trident House, Washington Road, Paisley, PA3 4EF, Scotland, U.K, Tel +44 141 814 6100, Fax +44 141 887 1167.
7. 'Marvel' is dried skimmed milk powder.
8. Trypsin (T-1426 Type XIII from Bovine Pancreas - Sigma Chemical Company Ltd., Fancy Rd., Dorset, BH17 7NH, U.K, Tel +44 1202 733114; Fax +44 1202 715460) made up in 50mM Tris-HCl pH7.4, 1mM $CaCl_2$ and stored at -20°C
9. Nunc Bio-Assay dish is available from Gibco BRL (see note 6).
10. Falcon MicroTest III flexible 96 well flat bottomed assay plates are available from Becton Dickinson Labware, Becton Dickinson and Co., 2 Bridgewater Lane, Lincoln Park, NJ 07035, USA.
11. HRP-anti-M13 is available from Amersham International plc, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK. Tel: +44 01494 544000; Fax: +44 01494 542929.
12. TMB is 3,3',5,5'-tetramethylbenzidine and is available from Sigma (see Note 8). A 10 mg/ml stock solution can be made by dissolving the TMB in DMSO.
13. Corning 'Cell Wells' flat-bottomed multiple well tissue culture treated plates are available from Corning Glass Works, Corning N.Y. 14831. USA.
14. The 96 well transfer device is a piece of wood the size of a microtitre plate with a handle on one side and 96 metal pins (each 7 cm long with a concave end) on the other. This can be sterilised between bacterial transfer by immersion in a bath of ethanol and then by flaming (hold well away from your body and any flamable objects). If you haven't got one of these (or something similar) you will have to make one yourself or alternatively use a multichannel pipette for bacterial transfer.
15. Horse Radish Peroxidase conjugated Protein A is available from Amersham International plc (see note 11)

14. Horse Radish Peroxidase conjugated Protein L is available from Actigen Ltd, 5 Signet Court, Swanns Road, Cambridge, CB5 8LA, UK. Tel: +44 01223 319101; Fax: +44 01233 316443.