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Affinity maturation of antibodies using phage display

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1. Introduction

1.1 General considerations

Protocols described in this section will deal with alteration of the properties of existing antibodies, formatted as scFv and displayed on bacteriophage. Although the title implies a focus on improving affinities, provisions are made for alteration in off-rate and specificity. Where the aim of the 'maturation' process is a shift in specificity, this can be achieved by introducing bias into the selection process.

Moreover, it is assumed that there is little or no structural information to guide the process, which in consequence relies heavily on the selection procedures. The apparent simplicity belies the impact the selection procedure may have on the final outcome. Many intermediate protocols are described in detail elsewhere in this book, and in these cases, the reader is referred to the relevant section.

Regardless of the aim, the process involves three distinct steps: production of mutant forms of the antibody (mutagenesis), followed by selection of those with improved properties (selection), and identification of new variants (screening).

1.2 Mutagenesis

In the absence of a known 3-D structure, the inevitable question is which parts of the antibody to mutate. The complementarity-determining regions (CDRs) are not wholly responsible for the properties of the antibody, since residues distant from the combining site ('The Vernier' region, refs 1–3, see also Bendig *et al.*, Chapter 7) can influence antigen binding indirectly (see *Figure 1*). This may be by modifying the conformation of the CDR loop or a contact residue, or influencing the way heavy and light chains pack against one another. Consequently, the emphasis is on non-localized mutagenesis strategies such as chain-shuffling and error-prone PCR. Chain-shuffling is the

Framework residues that interact with CDRs

VL D1.3 amino acid sequence

CDR 1: RASGNIHNYLA; CDR2: YTTTLAD; CDR3: QHFWSTPRT

DIQMTQSPASLSASVGETVTITC RASGNIHNYLA WYQQKQGKSPQLLVY YTTTLAD GVPSRFSGSGSGTQYSLKINSLQPEDFGSYYC QHFWSTPRT FGGGTKLEIK

VH D1.3 amino acid sequence

CDR 1: GYGVN; CDR2: MIWGDGNTDYNSALKS; CDR3: ERDYRLDY

QVQLKESGPGLVAPSQSLSITCTVSGFSLT GYGVN WVRQPPGKGLEWLG MIWGDGNTDYNSALKS RLSISKDNSKSQVFLKMNSLHTDDTARYYCAR ERDYRLDY WGQGTTLTVSS

Figure 1. Amino acid sequence of anti-lysozyme antibody D1.3; framework residues in contact with CDRs are underlined (1).

half-way house between site-directed and general mutagenesis strategies and is described in detail in Chapter 8. PCR mutagenesis methods (2) introduce substitutions at 'random' throughout the sequence.

1.3 Selection

Selection can be any method that enables separation of clones that bind from those that do not; as such there is an endless list of possible selection methodologies, and only the two most commonly used methods are included here. These are panning on antigen adsorbed on to plastic (4, 5, 6), and selection with soluble biotinylated antigen (7). Most often, between two and five rounds of selection are needed to adequately enrich a population. The actual number of rounds is almost impossible to predict in advance being dependent on the degree of enrichment afforded by the selection method and the relative affinities of the antibodies in the population. The degree of enrichment (i.e. ratio of binders to non-binders before and following selection) typically varies from 5–1000-fold at each round, and more rounds of selection are needed to resolve antibodies with similar affinities than those having widely differing affinity constants. All selection methods automatically favour variants with stronger binding, since these are best suited to survive the selection process. These antibodies will have either highest affinity or greatest avidity. The highest affinity antibodies within a population are those which, for a given antibody concentration, bind the greatest proportion of antigen at the lowest antigen concentrations.

Selection may also favour variants that form multimers, particularly when the antigen is polymeric or present at high density. In this case, a low-affinity antibody with a tendency to multimerize may be preferentially selected by virtue of a slow rate of dissociation, caused by the rate of dissociation from antigen being a composite of the off-rates of each component of the multimer. The importance of this effect cannot be emphasized too strongly. For example, whereas a maturation cycle may have resulted in selection of a clone with a 5-fold improved off-rate, these truly improved variants can be obliterated by variants with a tendency to multimerize, where the off-rate can be slowed by 1–2 orders of magnitude with no change whatsoever in the true affinity.

Whatever the purpose of the selection, it is important to check the antigen specificity of the clones and to sample clones from all rounds of selection. Non-specific or polyreactive antibodies may predominate in late rounds of selection in the absence of a high-affinity specific antibody to compete. Moreover, sampling the early rounds of selection ensures adequate diversity, since a single species will eventually dominate any selection.

1.4 Screening

A likely outcome of the mutagenesis and selection steps is a mixture of variants with differing properties, and it may be necessary to screen large numbers of

clones to identify those best suited to your purpose. We have concentrated on ELISA-based assays for specificity and affinity since these have the highest throughput. Ideally, the screening method should be as close as possible to the eventual application. If you cannot process large numbers of crude antibodies this way, then use ELISA, Western blotting, *Bst*NI fingerprinting and sequencing to identify those most likely to have appropriate characteristics.

An important part of the screening process is DNA sequencing, described in Chapter 6. For example, you may have reselected the parental sequence, and it is important to know this before investing a large effort in analysis. Another possible outcome is the selection of 'null' sequence variants, i.e. amino acid substitutions that are neither detrimental nor beneficial. Consider any change in a CDR or Vernier residue significant until proven otherwise, even if the change is relatively minor. We have several examples of conservative substitutions (e.g. Ser → Thr, Leu → Ile) that indisputably alter the properties of the antibody (unpublished data).

2. Mutagenesis

2.1 General considerations

Methods are described here for creating mutant V-gene segments ready for cloning using the methods described in Chapter 1. Whichever procedure is used, aim to create repertoires of $> 10^6$ recombinants to ensure adequate sampling of the mutant population. It is also worthwhile quality-controlling these repertoires at an early stage to ensure that they do indeed contain mutants. *Bst*NI fingerprinting is suitable for chain-shuffled repertoires, whereas sequencing 6–10 randomly picked clones is necessary to verify that the other mutagenesis procedures have worked.

Chain-shuffling is a proven method of introducing diversity into a phage repertoire (3) where a given V_H or V_L chain is recombined with a repertoire of complementary chains. The methodologies for this are not described here but are effectively described in Hoogenboom *et al.*, Chapter 8.

2.2 Error-prone PCR

The following method has been shown to introduce an average of 1–2 base substitutions per V domain as a result of manganese reducing fidelity of Taq polymerase. It is a difficult reaction to optimize for the desired number of mutations per gene, and it is suggested that five separate reactions are set up, containing 0, 0.5 mM, 1.0 mM, 1.5 mM, and 2 mM $MnCl_2$. Concentrations of $MnCl_2$ higher than 2 mM may cause components of the PCR reaction to precipitate; moreover, it may be counterproductive to introduce large numbers of mutations. Highly defective antibodies (frameshifts, etc.) may bind non-specifically and out-compete improved variants.

Protocol 1. Error-prone PCR amplification

Equipment and reagents

- 10 × Taq polymerase buffer as provided by the supplier of the Taq polymerase, *or*: 100 mM Tris, pH 9.0 at 25°C, 500 mM KCl, 60 mM $MgCl_2$, 1% (w/v) gelatin, and 10 % Triton X-100
- 10 mM each dNTPs (10 × stock)
- 10 μM pUC19REV (see Appendix 2)
- 10 μM FDTSEQ1 (see Appendix 2)
- 50 mM $MnCl_2$, freshly-prepared
- DNA thermal cycler for PCR
- Taq DNA polymerase (Boehringer Mannhein, under licence from Perkin Elmer/Cetus)
- Mineral oil (paraffin oil) (Sigma, cat. no. M-3516)
- 'Wizard PCR prep' DNA purification kit (Promega)
- TAE buffer: (see Chapter 1, *Protocol 1*)

Method

1. Set up a PCR reaction with your scFv DNA as template:
   - 10 × PCR buffer — 10 μl
   - dNTPs — to 1 mM each
   - template DNA — 20 ng
   - 10 μM pUC19REV — 5 μl
   - 10 μM FDTSEQ1 — 5 μl
   - H_2O — to 100 μl final volume
2. Add 1–4 μl of freshly-prepared 50 mM $MnCl_2$ to the reaction, mix and overlay with mineral oil.
3. Place the sample in the PCR block and heat to 94°C for 2 minutes before adding 1 μl of Taq polymerase (5 units/μl; Boehringer Mannheim or Cetus) underneath the oil.
4. Perform 30 cycles at 94°C (1 min), 60°C (1 min), 72°C (4 min), followed by 10 min at 72°C after the final cycle.[a]
5. Run 3–6 μl of the PCR reaction on a 1% TAE agarose gel to check that the PCR has worked. Expect to see less product (often none at all) at the highest Mn^{2+} concentrations.
6. Recover the remainder of the reaction and process the fragment as described in Chapter 1, *Protocol 3*, step 7 onwards (purification), *Protocol 7* (restriction digestion/purification). Also Chapter 8, *Protocol 3* (ligation) and Chapter 8, *Protocol 4* (transformation). Verify that the library is mutant by sequencing 6–10 randomly picked colonies (Chapter 6).

[a] The long extension time is necessary to compensate for a reduced polymerization rate in the presence of Mn^{2+} ions.

2.3 Site-directed mutagenesis

Introducing a single, specific alteration or multiple changes simultaneously can be achieved using PCR with mutagenic primers or by oligonucleotide

directed mutagenesis. There are several excellent kits for directed mutagenesis that are commercially available. We recommend the use of Amersham's oligonucleotide-directed *in vitro* mutagenesis kit. Full protocols are provided, but only production of single-stranded DNA template is covered here.

With pUC119 based vectors (e.g. pCANTAB5) the DNA strand that is packaged into phage contains the 'sense' strand of the antibody gene (the coding sequence written 5′ to 3′, N terminus to C terminus). All oligos should therefore be 'antisense', i.e. the reverse complement of the antibody sequence.

Protocol 2. Preparation of single-stranded template DNA

Equipment and reagents

- Phenol (BRL ultrapure redistilled, cat. no. 5509UA)
- Chloroform
- Microcentrifuge
- 95–100% ethanol
- 75% ethanol
- TE buffer: 10 mM Tris–HCl pH 7.4, 1 mM EDTA
- 3 M sodium acetate (pH 6.0)

Method

1. Perform a phagemid rescue[a] from a 10 ml culture, and PEG precipitate the resulting phage as described in *Protocol 8.*
2. Redissolve the pellet in 500 µl of TE buffer and transfer to a 1.5 ml microcentrifuge tube. Add 500 µl of freshly pH adjusted phenol.[b] Vortex for 10 seconds, leave to stand for 10 minutes, then vortex for 10 seconds again before centrifuging at 13 000 *g* for 10 minutes at room temperature.
3. Carefully remove up to 450 µl of the upper aqueous phase to a fresh tube and add 900 µl chloroform. Vortex for 5 sec and centrifuge at 13 000 r.p.m. for 5 min at room temperature.
4. Carefully remove the aqueous layer and measure the volume. Add 1/10th volume of 3 M sodium acetate (pH 6.0), mix, then add 2.5 vol. of ethanol (95 or 100%) and mix again.
5. Place at –20°C overnight or –70°C for 30 min or longer. Recover single-stranded DNA by centrifuging at 13 000 *g* for 20–30 min at 4°C. DNA will be smeared up the side of the tube furthest away from the centre of the rotor.
6. Aspirate the supernatant and add 1 ml 70% ethanol. Centrifuge at 13 000 *g* in a microcentrifuge for 2 min, aspirate the supernatant and, if available, dry the pellet under vacuum for 2–3 min.
7. Redissolve the pellet in 30 µl TE buffer and run 2 µl of this alongside molecular weight markers on a 1% agarose gel. If using λ DNA

digested with *Hin*dIII. A predominant band migrating between the 2.3 kb and 4.3 kb markers should be visible. M13KO7 single-stranded DNA migrates between the 6.5 kb and 9.4 kb markers.

[a] The presence of glucose in the medium sometimes hinders phagemid production and is best omitted.
[b] Melt at 60°C then add 200 ml distilled water to 100 g bottle and mix. Store at 4°C. Before use, remove an aliquot of the lower phenol phase and add 25 µl of 2 M Tris (unbuffered) per ml of phenol, to give a final pH of 7.0–8.0.

2.4 Mutagenesis using 'spiked' PCR primers

2.4.1 V_H CDR3 spiking

Not all CDRs will be of equal importance from one antigen–antibody pair to the next. However, a theme common to many is the importance of the third CDR of the heavy chain (V_H CDR3). This is the most variable CDR and forms the centre of the antigen combining site. It is often the best place to start. In all cases where we have 'spiked' V_H CDR3, antibodies superior to the parent have been isolated.

'Spiking' is achieved by synthesizing oligonucleotides where each phosphoramidite is mixed with a small amount of the other three phosphoramidites before loading on the synthesizer. The resulting oligonucleotide (CDR3SPIKE) will have a small number of changes along its entire length. We usually synthesize spiked oligonucleotides bounded by the invariant cysteine preceding the CDR3 and ending in the fourth framework region (FR4). Those oligonucleotides with changes at the 3′ end (i.e. the part encoding FR3) will amplify poorly during PCR, whereas changes in FR4 are corrected in subsequent amplification steps where an unmutated PCR primer based in FR4 is used.

The overall procedure involves PCR amplification from the parental scFv gene to generate a V_H gene whose end (encoding CDR3) has been spiked and linking this with a fragment encoding a flexible linker peptide and the V_L region. This latter fragment is generated by PCR of the scFv gene using one primer with homology to the end of the heavy chain PCR fragment (to drive the assembly reaction) and the other based in the vector sequences. Usually the parental V_L gene is used in the assembly, but this could equally be any cloned V_L gene or repertoire thereof.

Protocol 3. Synthesis of spiked oligonucleotide

Equipment and reagents

- Oligonucleotide synthesizer and reagents

Method

1. Make up 5 ml stock containing each phosphoramidite at 100 mM.

Protocol 3. *Continued*

2. Decide what error rate you want:

   error rate = number of mistakes per oligo/length of oligonucleotide.

3. Now calculate the volume of equimolar spike mix to add to each bottle:

   volume of spike mix, µl = error rate × 1.33 × 1000 × 5[a];

   e.g. to introduce an average of three errors into a 100-base oligonucleotide:

   0.03 × 6650 = 200 µl spike mix per 5 ml of phosphoramidite.

4. Synthesize the antisense of V_H CDR3 sequence and purify.

[a] 1.33 is a correction factor due to the mix containing all four phosphoramidites; 1000 converts millilitres to microlitres; 5 ml is the final volume of phosphoramidite in the bottle.

Protocol 4. PCR amplification of V_H genes and assembly into scFv

Equipment and reagents

- See *Protocol 1*
- Spiked oligonucleotide

Method

1. Set up the following reaction to amplify the V_H segment with the spiked oligonucleotide.
   - 10 × PCR buffer — 10 µl
   - 4 mM dNTP — 5 µl
   - 10 µM pUC19 reverse[a] (see Appendix 2) — 5 µl
   - 10 µM spiked oligo[a] — 5 µl
   - template DNA — 20 ng
   - H_2O — to 100 µl
   - Taq polymerase — 5 U
2. Amplify using 25 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min followed by 10 minutes at 72°C.
3. Analyse 3–5 µl of the PCR reaction on a 1.5% gel. If a discrete band of 350 bp is visible, gel-purify the remainder of the PCR reaction as described in Chapter 1, *Protocol 3*, step 7 onwards.
4. Set up the following PCR reaction to amplify the parental (or other) light chain together with the scFv linker.
   - 10 × PCR buffer — 10 µl
   - 4 mM dNTP — 5 µl
   - 10 µM reverse JH[b] (see Chapter 1, *Table 2*) — 5 µl
   - 10 µM FDTSEQ1[b] (see Appendix 2) — 5 µl

- template DNA — 20 ng
- H_2O — to 100 µl
- Taq polymerase — 5 U

5. Amplify using 25 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, followed by 10 min at 72°C.
6. Analyse 3–5 µl of the PCR reaction on a 1.5% agarose gel. If a discrete band of 400 bp is visible, gel-purify the remainder of the PCR reaction as described in Chapter 1, *Protocol 3*, step 7 onwards.
7. Assemble and pull-through V_H and linker–V_L segments using *Protocol 5* in Chapter 1, but omitting the linker DNA since this is already present on the light chain fragment. The primers used should be pUC19reverse and FDTSEQ1. This will generate a scFv gene with *Sfi*I and *Not*I restriction sites already in place.
8. Digest with *Sfi*I and *Not*I (Chapter 1, *Protocol 7*) prior to gel purification and cloning (Chapter 8, *Protocols 3* and *4*).

[a] The primer pUC19reverse is located in the plasmid sequences upstream of the antibody insert. The heavy chain spiked oligo is located in CDR3 and in framework 4. This pair generate a CDR3 spiked heavy chain.
[b] The human primer reverseJH is located in framework 4 and is complementary to the spiked oligo used to generate the heavy chain fragment above. (The mouse equivalent is LINKBACK, Chapter 1, *Table 1.*) The primer FDTSEQ1 is located in *gene 3*. This pair generates a fragment encoding the end of framework 4 of the heavy chain together with the linker sequence and the light chain.

2.4.2 V_L CDR3 spiking

Spiking light chain CDR3 can be performed in a similar manner to that described above, though no assembly reaction is necessary since the mutated region is close to the end of the scFv gene. In this case amplify the scFv with a primer based in the vector upstream of the antibody gene and the *Sfi* cloning site (e.g. pUC19reverse) and a 'spiked' light chain primer; this oligonucleotide should extend from CDR3 into the framework region FR4 such that the spiked fragment can be 'pulled-through' with an extended FR4 based primer, appending a *Not*I site for cloning (Chapter 1, Section 3.5).

3. Selection of antibodies with altered properties

There is no substitute for selection schemes based on the unique properties of the system under investigation, and the reader is encouraged to develop inventive selection schemes to derive antibodies with the desired characteristics. Any selection method will automatically favour antibodies of higher affinity, since by definition, these will be more long-lived interactions and bind antigen at low concentrations. The main complication is avidity, caused by two or more copies of the antibody on the phage head interacting with

adjacent epitopes. Most scFvs will dimerize to a certain extent and it is important to bear in mind that the selection method may favour poor antibodies with a tendency to dimerize. The form of the antigen is the most obvious way to control this. If the antigen is a hapten coupled to a carrier, derivatize at low density. If the antigen is a repeating polymer, then selection on fragments or synthetic versions containing fewer repeat units can be used. For soluble proteins, selections incorporating solution capture are recommended.

At each round of selection, infect *E. coli* TG1 cells with selected phagemids and plate out on media containing ampicillin and glucose, then grow overnight at 30°C. The next day, scrape the plate and rescue an aliquot as described in Chapter 1, *Protocol 8*.

3.1 Selection of phage by panning

This technique is effectively a preparative ELISA. It is particularly good at discriminating between binder and non-binder, but is poor at discriminating between antibodies of similar affinity and can result in the selection of high-avidity antibodies due to high antigen density.

Protocol 5. Solid-phase selection of phage antibodies (see also Chapter 1, *Protocol 12*)

Equipment and reagents

- 75 × 12 mm Nunc-immunotubes (Maxisorp, cat. no. 4-44202)
- Phosphate buffered saline (PBS)
- PBS containing 2% skimmed milk powder (MPBS)
- PBS containing 0.1% Tween-20
- 100 mM triethylamine, freshly made
- 1.0 M Tris–HCl, pH 7.4
- Log-phase TG 1 cells
- 1.5 ml Eppendorf tubes
- 2TYAG medium: see Chapter 1, *Protocol 8*
- Agar plates made with 2TYAG medium

Method

1. To an immunotube add 1–4 ml of an appropriate concentration of antigen in the appropriate buffer.[a, b, c, d]
2. Leave to coat overnight at 4°C.
3. Wash the tube three times with PBS (pour PBS in and pour out again immediately).
4. Block by filling tube to the brim with PBS containing 2% skimmed milk powder. Cover with Parafilm and incubate at 37°C for 2 h.
5. Wash tube three times with PBS.
6. Add 10^{10} to 10^{13} TU phage in 4 ml of MPBS and incubate for 1 hour at room temperature.[e]
7. Wash tubes with 20 washes of PBS, 0.1% Tween-20, then repeat with 20 PBS washes. Each washing step is performed by pouring buffer in and out immediately, best achieved using a wash bottle.

8. Elute phage from tube by adding 1 ml freshly-made 100 mM triethylamine and leaving for 10 minutes at room temperature.
9. Recover supernatant and immediately transfer to a 1.5 ml Eppendorf tube containing 0.5 ml 1.0 M Tris–HCl, pH 7.4, and mix.
10. Mix 750 µl of eluted phage with 10 ml of log-phase TG1 grown at 37 °C (OD_{600} around 1.0). Incubate at 37 °C for 30 minutes and remove an aliquot for titration on 2TYAG plates. Centrifuge the remainder at 2000 *g* for 10 minutes and plate on a large 2TYAG plate. Incubate all plates at 30 °C overnight.
11. Store the remaining selected phagemids at 4 °C.

[a] Antigens are usually coated at 1–10 µg/ml, but concentrations as high as 3 mg/ml are used in the case of certain proteins, e.g. lysozyme and coating is sometimes better in 50 mM sodium hydrogen carbonate, pH 9.6 (pH adjusted with NaOH), than in PBS. ELISAs performed with the antigen are usually a good guide to the best coating conditions.
[b] Peptides often bind poorly to plastic and it is best to couple them to a carrier such as BSA prior to immobilization. Pierce sell a range of preactivated carriers and these are highly recommended.
[c] An alternative to non-specific adsorption is to covalently couple the antigen to the support. This may avoid large conformational changes that can result with direct immobilization. Several companies sell such cross-linking reagents.
[d] Biotinylated antigen (see below) may also be immobilized to a solid surface via streptavidin to avoid conformational changes on adsorption. Streptavidin itself adsorbs poorly to plastic regardless of pH. Covalent coupling, binding to plates coated with biotinylated BSA, or commercially available streptavidin matrices are recommended.
[e] To select against an undesirable cross-reactivity, include soluble antigen at ≥ 10-fold higher concentration than that used for coating.

3.2 Solution capture on soluble antigen

Solution capture on 'tagged' antigen is a powerful method for selecting for the highest affinity antibodies in a population, since the concentration of antigen can be controlled. The highest affinity antibodies are, by definition, those that bind most antigen at the lowest antigen concentration. The principle of the method has been described in detail (7). In brief, a low concentration of antigen which is in molar excess over phage is added; for example, to select antibodies of 10 nM or higher affinity, use antigen at 10 nM or lower concentration to select 10^{11} or fewer phage (6×10^{11} phage/ml is 1 nM, 6×10^{10} phage/ml is 0.1 nM, etc.) in a 1 ml volume.

The antigen is tagged to enable recovery of bound phage, the nature of the tag depending on the antigen in question. The usual method is biotinylation; Pierce supply many reagents for biotinylation, all of which are supplied with detailed protocols and these are recommended. The example given below is for biotinylation of a peptide by coupling of an NHS ester to lysine residues. We find it is best to add just one or two biotins to each molecule of antigen to avoid destroying epitopes.

Protocol 6. Biotinylation of proteins using NHS S–S biotin

Equipment and reagents

- NHS S–S biotin (Pierce)
- 1 M $NaHCO_3$, pH 8.5
- avidin–HABA (4-hydroxyazobenzene-2-carboxylic acid)

Method

1. Calculate the reagent concentrations, e.g.

   5 mg/ml stock of peptide mol. wt 3337 is 1.5 mM = 1.5 nanomoles/µl.
   1 mg of NHS S–S biotin dissolved in 1 ml of H_2O = 1.65 nanomoles/µl.[a]

2. To singly biotinylate 150 nanomoles of peptide, set up the following reaction:

• peptide stock	100 µl
• 1 mg/ml NHS S–S biotin	90 µl
• 1 M $NaHCO_3$, pH 8.5	50 µl
• H_2O	760 µl

3. Incubate on ice for 2 h.
4. Remove unincorporated biotin from the sample using, for example, column chromatography.[b]
5. Determine the efficiency of the coupling reaction using avidin–HABA reagent as given in the Pierce booklet or by comparing in Dot blots with a known standard. Expect the reaction to be 15–30% efficient.

[a] The NHS reagent should dissolve readily in water. If not, then it may have absorbed water due to inappropriate storage and will couple at lower efficiency. Best results have been obtained if it is < 6 months-old.
[b] The sample can be used without removal of unincorporated biotin if the coupling is performed at low density, since a vast excess of streptavidin is used in capturing phage. Note that the HABA reagent cannot then be used to determine coupling efficiency.

Protocol 7. Capture of phage on soluble biotinylated antigen

Equipment and reagents

- Streptavidin magnetic beads (Dynal, cat. no. M280) and magnetic rack
- PBS/2% skimmed milk powder (MPBS)
- 50 mM DTT
- PBS/2% skimmed milk powder/0.5% Tween–20
- Log-phase TG1 cells
- 1.5 ml microcentrifuge tubes

Method

1. Mix phage and biotinylated antigen,[a, b] in 500 µl PBS/2% skimmed milk powder/0.5% Tween-20 in a 1.5 ml microcentrifuge tube and incubate for 1 h at room temperature.[c]

2. Dispense 250 µl of streptavidin magnetic beads and collect on a magnet. Aspirate the supernatant and resuspend the beads in 250 µl of PBS/2% skimmed milk powder (MPBS) and rotate end over end to block for 1 hour at room temperature.
3. Add the streptavidin magnetic beads to the phage and incubate at room temperature for 5–15 minutes.
4. Place tubes in the magnetic rack for 1–2 min to allow the beads to migrate. Aspirate the supernatant and resuspend the beads in 1 ml of MPBS.
5. Recover the beads and repeat wash steps. Use four × 1 ml MPBS washes followed by two PBS washes. Transfer the beads to a fresh tube after the third wash to avoid recovering trapped phage.
6. After the last PBS wash, resuspend an aliquot of the beads in 100 µl of 50 mM DTT, mix and leave for 5 min at room temperature to elute the phage (the biotin reagent contains a disulfide bond separating the biotin moiety from the antigen). Store the remainder of the beads in 100 µl of PBS at 4 °C, in case you need to return to them.
7. Add DTT containing eluted phage to 10 ml log-phase TG1 cells (OD_{600} around 1.0), and treat as in *Protocol 5*, steps 10 and 11.

[a] As a starting point, use 10^{10}–10^{11} phage and 100 nM antigen. Reduce phage input and antigen concentration in later rounds of selection.
[b] To select against an undesirable cross-reactivity, include unlabelled antigen at ≥ 10-fold molar excess.
[c] Temperature and incubation times and conditions can be varied as appropriate to the end-use.

3.3 Off-rate selection

One parameter that often distinguishes primary from affinity-matured antibodies is the rate of dissociation from the antigen, the 'off-rate', which is often slower for high-affinity antibodies. Off-rate selections can be performed in a modification of the above method, and is described in detail in ref. 7. Phage are first equilibrated with biotinylated antigen then a molar excess of unlabelled antigen added, such that phage dissociating from the biotinylated antigen will most likely rebind to a non-biotinylated moiety and are therefore lost. Slower off-rate antibodies will remain bound to biotinylated antigen for longer and are isolated when phage are captured at later time-points. The point of maximum discrimination has to be determined experimentally, since background binding eventually reduces discrimination. Unlike the previous protocol, the actual concentrations of antigen used are not the major issue; as a guide, use biotinylated antigen at a concentration 2–3 times the estimated K_d (20–30 nM for an antibody with an estimated dissociation constant of 10 nM). Antigen should be in molar excess over phage.

Protocol 8. Off-rate selection

Equipment and reagents

As for *Protocol 7*

Method

1. Mix phage and biotinylated antigen and allow to equilibrate.
2. Add at least a 10-fold molar excess of unlabelled antigen.
3. Capture aliquots on streptavidin dynabeads at various time-points, from 5 minutes to 1 hour.
4. Reinfect *E. coli* as described in the previous protocol, and sample clones from different time-points in an affinity screen.

4. Screening selected populations of antibodies

Specificity and affinity should now be checked as a matter of routine. It is vitally important to check that the antibody binds specifically. If possible, grow clones in a 96-well format and test them by ELISA, either as phage or as soluble scFv (Chapter 1, *Protocol 13*). Note that selections can go too far, such that those antibodies with desirable characteristics can disappear from later rounds of selection. Some means of sampling up to hundreds of clones from each round is desirable. Consequently, the best screening procedures use a 96-well format such as ELISA, principally because many clones can be screened in parallel. Even if the end-use is different, a crude 96-well screen can be used to focus on the best candidates which can then be analysed in detail. Though the antigen used in selections needs to be as pure as possible, that used in screening need not be as pure if availability is limited.

Clones can be grown up in 96-well arrays and tested for reactivity and specificity in ELISA as described in Chapter 1, *Protocol 13*. The method below is a rapid screen for affinity based on methods described in Chapter 4, and is for ranking clones on the basis of their equilibrium dissociation constant (K_d).

4.1 Affinity screen for phage antibody clones (K_d assay)

The underlying principle of the procedure is that the higher the affinity of the antibody, the greater the proportion that will be complexed with antigen at equilibrium. In practice, aliquots of each crude antibody are mixed either with no antigen or with soluble antigen at two concentrations. The proportion of antibody remaining free at equilibrium is determined by ELISA. The highest affinity clones will manifest the greatest signal reduction with the lowest concentrations of antigen, regardless of the absolute signal. The assay

should be resistant to the two most common artefacts that affect affinity measurement, namely expression level and dimerization, since these are selected against. Variations in specific activity do not affect this assay either, since it is the active fraction that is assayed. Factors affecting this screen are discussed in more detail in Chapter 4.

A number of criteria must be met before the assay can be used:

- Temperature must be constant throughout as this affects the K_d.
- Less than 10% of the available antibody must bind to the coated antigen to prevent readjustment of the equilibrium.
- The absorbance read in the ELISA must be proportional to the antibody concentration.
- The concentration of antibody employed in the assay should be less than or equal to the K_d of the antibody tested. Note that 1.0 μg/ml of an scFv is 40 nM. Yields of scFv in crude culture supernatant range from 2 nM (50 μg/litre, e.g. for a non-induced culture) upwards.

We find that it is best if you perform a standard ELISA on the clones to be tested first, not only in order to work out which are the positives, but also to categorize them into high, medium, and low signal (usually = high, medium, and low expression level). Chapter 1, *Protocols 9, 11*, and *13* describe growing clones in 96-well arrays and performing ELISAs; note that peroxidase described in *Protocol 13* is an unsuitable detection method since colour development is non-linear. Modify the primary ELISAs for development with alkaline phosphatase as described below.

Protocol 9. Determination of per cent binding and linearity of ELISA signal

Equipment and reagents

- Microtitre plate reader
- 2% skimmed milk powder in PBS (MPBS)
- Tween-20
- ELISA plates
- Microtitre tray (for growth of culture)
- 9E10, antibody by Cambridge Research Biochemicals, cell line by ATCC (CRL1729)
- 0.9% NaCl
- Alkaline phosphatase conjugated goat anti-mouse (Pierce, cat. no. 31322X)
- PBS
- *p*-Nitrophenyl phosphate tablet (Sigma, cat. no. N-2765)
- Substrate buffer: 0.1 M glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10.4

Method

1. Pick one or more 'representative' clone(s) and grow and induce in a microtitre tray as for a standard ELISA.
2. Coat two ELISA plates with your test antigen using your normal coating volume and conditions, and block both for at least an hour with 2% skimmed milk powder in PBS (MPBS).

Protocol 9. *Continued*

3. Harvest the culture supernatants and add Tween-20 to 0.1% final concentration. Make dilutions from the neat supernatant ranging from 1 µl to 90 µl of supernatant per 100 µl sample in the appropriate concentration of MPBS (for plates coated with 100 µl of antigen).
4. Add your test samples, in triplicate, to one of the two coated plates and allow the antibody to bind (e.g. 37 °C for 1 h).
5. After the binding step transfer the samples to the second coated plate and allow the samples to bind as before. Meanwhile wash the first plate: two quick rinses in PBS/0.1% Tween-20 followed by three × 2 minute washes in PBS/Tween-20 followed by three quick rinses in PBS with no detergent. After the last wash, tap out all the fluid and store the plate inverted on damp tissue paper.
6. Wash the second plate as above, then process both plates simultaneously thereafter.
7. Incubate the samples with 10 µg/ml 9E10 in MPBS for 1 h at room temperature and wash as in step 5.
8. Dilute alkaline phosphatase conjugated goat anti-mouse 1/5000 in MPBS and incubate for 1 h room temperature.
9. Wash (as in step 5), but use saline instead (0.9% NaCl) for the final rinse to remove the phosphate buffer—the detection step is pH dependent.
10. Dissolve one × 20 mg tablet of *p*-nitrophenyl phosphate in 20 ml of substrate buffer and add 100 µl per well.
11. Read absorbance at 405 nm. Don't worry too much about which time-point to use; the reaction remains linear whichever time you use so long as the absorbance of your most concentrated sample does not exceed 2.0.
12. Plot the antibody concentration (i.e. supernatant volume) versus *OD*, at your preferred time-point. The graph should be linear over at least part of the range. We find that the [*Ab*] versus *OD* is linear in the range 2–20 µl of culture supernatant. Less than 2 µl generally results in > 10% of the antibody binding.
13. Calculate how much of the antibody in each sample has bound by comparing the *OD*s of the same samples on the first and second plates; you can plot readings with the two plates on the same graph and determine the 10% cut-off by the difference in slope. However, the cut-off is usually obvious from simple comparison of the *OD*s.

Protocol 10. K_d assay

Equipment and reagents

- ELISA plates (Falcon)
- Polypropylene microtitre trays (Greiner)
- 1% Tween-20 in PBS
- MPBS (*Protocol 5*)
- Plate reader

Method

1. Coat ELISA plates with antigen; you will need three times the number of ELISA plates as you have microtitre tray arrays.
2. Harvest the supernatants and place in polypropylene microtitre trays containing 1/10th volume of 1% Tween-20 in PBS. Mix the supernatant with the Tween buffer and keep the plates on ice while you are setting up the assay.
3. Make dilutions of the antigen in MPBS. The concentrations of antigen to use depends on the expected/target K_ds. We typically use 1 nM and 10 nM concentrations.
4. Aliquot the antigen solutions into polypropylene microtitre trays: we usually put MPBS in the first four columns (1–4), the lowest antigen concentration in the next four columns (5–8), and the highest concentration in the last four (9–12). Each full tray of clones therefore translates into three polypropylene assay plates and three ELISA plates. We find that the assay works best if the polypropylene microtitre trays are rinsed in PBS and blocked in MPBS for an hour or so at room temperature prior to use.
5. Transfer aliquots of the test samples into the antigen solutions and mix by pipetting up and down a few times.
6. Allow the binding to come to equilibrium, e.g. 37 °C for 1 hour.
7. Transfer the equilibrated samples to coated ELISA plates and allow uncomplexed antibody to bind, e.g. 37 °C for 1 hour.
8. Develop as for a standard ELISA and read *OD* at 405 nM.
9. Calculate the per cent reduction in signal for each of the clones. The best clones will show the greatest signal reduction at the lowest concentration of soluble antigen.

5. Sequence and fingerprint analysis

An analysis of the genes in the selected clones can often prove revealing. *BstN*I fingerprinting before and after selection gives a 'quick and dirty'

snapshot of whether there is in fact selection for particular clone types and how many different types of clones are being selected (Chapter 8, *Protocol 6*). Sequence analysis is definitive however, and is particularly revealing when sequence alterations are correlated with altered properties. All changes mapping to CDR or Vernier residues (*Figure 1*) should be considered significant until proven otherwise, and in our experience, there is no such beast as a 'conservative substitution'. Nevertheless, some sequence alterations may have been selected by virtue of their being 'null', i.e. neither beneficial nor detrimental. In the ideal case, a particular clone type will score highest in the K_d assay and will have been observed to comprise an increasingly greater proportion of the population as the selections have progressed. Refer to Chapter 6 to put the changes observed in your antibodies into context of those observed *in vivo*.

The acid test is whether the antibody has been improved. Improvements in affinity can be measured as described in Chapter 5, with changes in kinetic constants most readily observed by surface plasmon resonance. Changes in specificity are more readily tested, though beware that apparent loss of an undesirable cross-reactivity may be the result of a drop in affinity, such that cross-reaction is now below the level of detection.

Finally, if the antibody has not been improved check that there were sufficient mutants there to begin with; characterize the selected clones immunologically and by sequence analysis; and try changing the selection conditions. In our experience some antibodies are very difficult to improve further, though these are a minority. Improved antibodies have to be there before they can be selected, but if the selection conditions are unfavourable, they may never get selected.

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4

Measuring antibody affinity in solution

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1. General considerations

Determination of the affinity of a monoclonal antibody (mAb) for its antigen (Ag) is of considerable importance. It gives a quantitative indication of how strong the interaction is between the Ab and the Ag. It is the basic experimental parameter in a variety of studies, such as the analysis of mAb/Ag binding mechanism (1, 2) or the use of mAbs as conformational probes (3, 4). Hence the need for convenient and rigorous methods to determine the affinity. The affinity, K_a ($K_a = 1/K_d$), of a mAb for its antigen is defined by the Law of Mass Action as:

$$K_d = [Ag] \times [Ab]/[complex];$$

where K_d is the equilibrium dissociation constant, [*complex*] is the concentration of saturated antigen or mAb sites, [*Ag*] is the concentration of free antigenic sites on the antigen, and [*Ab*] is the concentration of free binding sites on the antibody. These concentrations are *binding site concentrations in the solution at equilibrium*. This has three important consequences, as described in Sections 1.1–1.3.

1.1 K_d does not directly reflect association or dissociation kinetics

K_d depicts an equilibrium property, and therefore does not reflect the speed at which equilibrium is reached. Yet, K_d does depend on the association and dissociation rate constants.

In many cases, the relationship between the equilibrium and rate constants is simple. When binding is a simple one-step reaction, $K_d = k_{off}/k_{on}$, where k_{on} and k_{off} are the association and dissociation rate constants. However, when a significant conformational change of either the mAb or the Ag occurs upon association, important deviations from that simple equation can be observed

(1). For such cases, measuring only k_{on} and k_{off} and using $K_d = k_{off}/k_{on}$ would provide an erroneous estimate of the affinity.

1.2 True K_d cannot be determined when the mAb or the Ag is immobilized in a solid-phase assay

Several convenient methods such as ELISA or surface plasmon resonance, with the Ag or the mAb immobilized on the titration plate or on the 'sensor chip' (e.g. gold covered with a carboxymethylated dextran hydrogel), are often used to provide affinity values. Such measurements yield real values of K_d only rarely. One reason is that immobilization often results in a partial denaturation of the protein, thus modifying its binding properties (see ref. 5 for review). Secondly, K_d is defined in solution, with both the mAb and the Ag diffusing and rotating freely in solution, but in the solid-phase assay one of the partners is immobile. This can result in estimates of K_d by solid-phase assay being orders of magnitude different from the real affinity in solution.

One should emphasize, however, that measurements of an 'apparent binding constant' by solid-phase assays may, for some purposes (such as comparative studies), be sufficient. In some instances, the binding constant obtained by solid-phase assays might even be of more significance than the K_d determined with the Ag in solution. Thus, with respect to diffusion and rotation, an antigen on the surface of a large eukaryotic cell may behave more like the Ag immobilized in a solid-phase assay than the Ag in solution, provided that precautions have been taken to avoid denaturation of the Ag upon immobilization.

1.3 The determination of K_d must take into account the valency of the mAb and of the Ag molecule

The Law of Mass Action ($K_d = [Ag] \times [Ab]/[complex]$) expresses the concentrations of reagents in terms of reactive sites, that is, antibody-binding sites (free or saturated) and antigenic sites on the antigen molecule (free or saturated). To determine the affinity from binding experiments, one must therefore determine these values. Thus, one must measure not only the antibody and antigen concentrations, but also their valencies (number of sites per molecule) and understand how this valency influences the experimental determination of the binding. This is discussed in a recent review (6).

2. Overview of methods to measure affinities in solution

Measuring K_d consists of mixing the mAb and Ag at various initial concentrations, bringing to equilibrium, then measuring the concentrations of free and saturated sites at equilibrium and analysing the binding curve. The

experimental difficulty resides in distinguishing the free and bound states of either the mAb or the Ag. Several methods can be used such as:

- equilibrium dialysis for haptens and dialysable antigen
- radioimmunoassay using precipitation with salts or other agents
- filtration
- fluorescence measurements
- ELISA- or RIA-based methods.

In this chapter, we discuss the practical aspects of measurement using fluorescence, ELISA, or RIA and describe, in detail, the ELISA- and the RIA-based competition methods for measuring affinity in solution.

2.1 Fluorescence

The use of fluorescence to determine K_d requires that either the mAb or the Ag be fluorescent, and that a change in fluorescence should occur upon formation of the mAb/Ag complex. The fluorescence signal used can be either intrinsic (e.g. tryptophan residues from the mAb and, possibly, from protein antigens; some prosthetic groups such as pyridoxal phosphate, NADH, flavins, etc.) or result from prior fluorescent labelling of the Ag or the mAb with a fluorochrome. The fluorescent change observed upon association may be one of the following:

- wavelength shift
- fluorescence quenching
- fluorescence transfer
- change in fluorescence polarization.

We shall not discuss the practical aspects of these experiments because each mAb/Ag complex poses unique problems, and hence the exact procedure depends on the fluorochromes used. Thus, the wavelengths of fluorescence excitation and emission vary from mAb to mAb and from Ag to Ag, even when the same fluorochrome is studied. The procedure used also depends on the nature of the change observed (quenching, transfer, polarization, shift) and on the type of fluorimeter used. Many unlabelled mAb and Ag complexes do not give rise to a fluorescence change and individual labelling procedures must be devised for each. There is no reliable way to predict whether or not a given label will give rise to a measurable fluorescent change upon complex formation. It is therefore impossible to provide a rule of thumb as to which fluorochrome one should use and how exactly one should proceed to measure the affinity.

We shall, however, briefly discuss some of the pitfalls that should be avoided when using fluorescence for affinity measurements:

(a) Reagents used for fluorescence labelling should be highly purified because of the intense fluorescence signals sometimes produced by impurities.

(b) The sensitivity of current fluorimeters sets a lower limit of 10^{-8} to 10^{-9} M on the K_d that can be determined. Some mAbs have much higher affinities for their Ag.

(c) Labelling of antibody or antigen often gives rise to heterogeneously labelled products which require purification, since both the number of fluorochromes and their position on the molecule affect the fluorescence signal.

(d) The label may affect the binding characteristics, either by steric hindrance or through a change in conformation, thus modifying the K_d. This should *always* be checked by performing competition experiments with the unlabelled molecule.

(e) When reading the fluorescence in the fluorimeter, one should take into account the absorbance of the solution at the wavelength of excitation or emission when this absorbance is *not* negligible, i.e. above 0.05 (effect referred as to inner filter effect). Keeping these precautions in mind, fluorescence measurements should provide precise affinity values (7).

2.2 ELISA- and RIA-based methods

A method that is frequently used for estimating the affinity of a mAb for a macromolecular antigen is to measure the amount of mAb bound to an Ag-coated ELISA plate after incubation with different concentrations of mAb. In these experiments, the mAb concentration that yields half-maximal binding on the coated antigen is taken as the reciprocal of the affinity. This approach does not, however, allow determination of real affinity because equilibrium is attained at the liquid/solid interface rather than in solution (see Section 1.2). Moreover, as already discussed (see Section 1.2), coating the antigen on to the plastic by use of the conventional adsorption methods may alter its conformation (5, 8), which affects the mAb/Ag interaction and hence the affinity. Thus, although providing an estimate of the relative efficiency of the binding of a mAb to the *immobilized* antigen, such direct ELISA- or RIA-based methods fail to provide the real affinity for the *native* antigen.

Two main ELISA-based methods have been described (see refs 7 and 9) for studying the association/dissociation equilibrium *in solution* and have been recently reviewed (6). Both rely on the following principle. Mixtures of the mAb at a fixed concentration and the antigen at varying concentrations are incubated until equilibrium is reached. A solid-phase assay using Ag-coated plates is used to determine the concentration of the free (i.e. not associated with antigen) mAb at equilibrium. The methods differ in two main respects; the conditions under which equilibrium is established (in the presence or absence of the coated antigen) and the conditions under which the ELISA is performed. Thus, Friguet *et al.* (7) first incubate the mAb and the

Ag in solution for a time sufficient to reach equilibrium, and only then transfer the equilibrated solution into an ELISA plate to determine the concentration of free mAb. The amount of Ag coated in each well and the incubation time for the ELISA are such that only a small fraction (at most 5–10%) of the unsaturated mAb at equilibrium is trapped by the coated antigen. This ensures that, during the ELISA, equilibrium in solution is not significantly modified. By this means, the observed equilibrium constant corresponds to the real affinity. In the other method (see for example ref. 9) where the antibody is in contact with the soluble and immobilized antigen simultaneously, uncontrolled kinetic factors are likely to affect the relative binding of the antibody to the antigen in the liquid and solid phases, and no care is taken to avoid a shift of the equilibrium in the liquid phase when some of the free mAb becomes bound to the solid phase. This method therefore often yields underestimates of the real affinity. Thus, the method of Friguet *et al.* (7) is recommended when determination of the real affinity is required and this method is described in detail in Section 3.

3. Affinity measurements in solution by competition ELISA

3.1 Theoretical aspects

The antibody site to antigenic site association reaction can be written as follows:

$$\text{antibody} + \text{antigen} \leftrightarrow \text{complex};$$

with the concentration of antibody sites, antigen sites, and complex at equilibrium given as $[Ab]$, $[Ag]$, and $[x]$, respectively.

The concentration of antibody sites $[Ab]$ and the antigen sites $[Ag]$ at equilibrium are related to the *total* antibody sites $[Ab_t]$ and the *total* antigen sites $[Ag_t]$ by:

$$[Ab] = [Ab_t] - [x] \quad [1]$$

$$[Ag] = [Ag_t] - [x]. \quad [2]$$

K_d, the dissociation constant, is defined by:

$$K_d = [Ag]\,[Ab]/[x].$$

If $[Ag_t]$ is varied while $[Ab_t]$ is kept constant:

$$K_d = [Ag]([Ab_t] - [x])/[x];$$

consequently:

$$[x]/[Ab_t] = [Ag]/([Ag] + K_d) \quad [3]$$

Several linear plots of Equation 3 have been proposed, the most commonly used being the following:

$$\text{Scatchard equation: } [x]/[Ag] = ([Ab_t] - [x])/K_d \quad [4]$$

$$\text{Klotz equation: } [Ab_t]/[x] = K_d/[Ag] + 1. \quad [5]$$

If Ab_t and Ag_t are known, the experimental determination of K_d (or $K_a = 1/K_d$) requires precise measurement of only one of the three concentrations $[Ab]$, $[Ag]$, or $[x]$.

3.2 Rationale

The method we have developed requires two steps.

(a) In the first step, the antibody, at a constant concentration, and the antigen, at various concentrations, are incubated in *solution* until equilibrium is reached.

(b) In the second step, the concentration of the antibody that remains free at equilibrium is measured by a classical indirect ELISA in which the antigen is coated on the microtitration plate.

The state (native or partially denatured upon coating) of the coated antigen and whether or not it is recognized by the mAb differently from the soluble antigen is not important as long as the coated antigen can specifically and quantitatively trap the free antibody.

3.3 Requirements for the determination of K_d

For correct determination of the free antibody concentration at equilibrium, several requirements must be fulfilled.

(a) The absorbance obtained in the last step of the indirect ELISA, which reflects the free antibody concentration, must be proportional to the antibody concentration tested.

(b) Only a small percentage of the free antibody molecules (i.e. less than 10%) must bind to the coated antigen, to prevent any significant disruption of the equilibrium in the liquid phase.

(c) Since the dissociation constant K_d is generally dependent on the temperature, the temperature must be kept constant throughout.

To satisfy requirements (a) and (b), it is necessary to determine (using the indirect ELISA procedure described in *Protocol 1*) the total (i.e. initial) antibody concentration range that must be used, the concentration of the coated antigen, and the optimal incubation time of the antibody solutions in the coated wells.

Protocol 1. Indirect ELISA procedure

Equipment and reagents

- ELISA plate spectrophotometer or fluorimeter (Labsystems, Dynatech, SLT Lab-instruments)
- Plate washer, Handiwash type (Titertek, Dynatech)
- 96-well flat-bottom microtitration plates and plate sealers
- 5 ml glass tubes
- Repeater pipettes and tips (e.g. Eppendorf Multipette)
- Immunoconjugate: antibody directed against mouse immunoglobulins linked to alkaline phosphatase or β-galactosidase[a] (e.g. from Biosys, Southern Biotechnology Associates Inc., Promega).
- Substrate solution 1: for alkaline phosphatase.[b] Prepare this solution fresh just before use. Dissolve 20 mg of disodium *p*-nitrophenylphosphate (PNPP) in 10 ml DEA solution (1 M diethanolamine supplemented with 1 mM magnesium sulfate and adjusted with HCl to pH 9.8). The reaction can be stopped by adding 1 M sodium phosphate.
- Substrate solution 2: for β-galactosidase.[b] Prepare 0.4% (w/v) *o*-nitropheny1-β-D-galactopyranoside (ONPG) stock solution in PM_2 buffer (70 mM disodium phosphate, 30 mM monosodium phosphate, 1 mM magnesium sulfate, 0.2 mM manganese sulfate, 2 mM EDTA magnesium salt, to pH 7.0 with HCl). To prepare a working solution just before use, add 5 ml of 0.4% (w/v) ONPG stock solution to 20 ml PM_2 buffer and then add 18 µl 2-mercaptoethanol. The ONPG stock solution can be kept in the dark at 4°C until it becomes yellowish.
- Substrate solution 3: fluorogenic substrate solution for β-galactosidase.[b] Prepare a 20 mM MUG (methyl-umbelliferyl-β-galactopyranoside) stock solution by dissolving 34 mg of MUG in 5 ml dimethylformamide in a glass test tube. If necessary, warm gently by passing the tube quickly through the pilot light of a Bunsen burner until the MUG is dissolved. Keep the stock solution in the dark at – 20°C. To prepare a working solution, mix 0.25 ml of the 20 mM MUG stock solution to 25 ml PM_2 buffer and add 18 µl 2-mercaptoethanol.
- Specific antibodies (at the desired concentrations in the buffer which will be used to determine the K_d in *Protocol 3*
- Antigen 1 µg/ml in coating buffer
- Washing buffer: PBS (pH 7.4) supplemented with 0.05% w/v Tween-20. Dissolve 8 g NaCl, 0.2 g KH_2PO_4, 2.8 g $Na_2HPO_4.12H_2O$ and 0.2 g KCl in distilled water to 1 litre final volume.
- Coating buffer: 50 mM carbonate buffer, pH 9.6. Dissolve 1.59 g Na_2CO_3 and 2.93 g $NaHCO_3$ in distilled water to 1 litre final volume.

Method

The assays must be done in triplicate, plus a control with a non-coated well (see legend to *Figure 1*).

1. Add 0.1 ml of a 1 µg/ml antigen solution (in coating buffer) to the well of an ELISA microtitration plate following the scheme in *Figure 1*. Do not coat the first vertical line of wells, which will be blank wells containing only the substrate solution.
2. Incubate the plate, covered with a plate sealer, for at least 3 h at room temperature or overnight at 4°C. Usually a blocking step by overcoating with BSA (0.5%) is not needed.
3. Empty the plate by turning it upside down and shaking it over a sink, then hitting it hard several times on a pile of paper towels.
4. Wash out all wells with washing buffer three times, allowing a 3 min incubation between washes at the chosen temperature.
5. After the third wash, add 0.1 ml of each antibody solution to three

Protocol 1. *Continued*

coated wells and one non-coated well. Incubate the covered plate for 30 min at the chosen temperature.

6. Wash the wells three times, as in steps 3 and 4, at room temperature.
7. Add, to each well, 0.1 ml of the immunoconjugate previously diluted in washing buffer. Depending on the immunoconjugate supplier, use a 500- to 1000-fold dilution. Incubate for 30 min at room temperature.
8. Wash the wells three times, as in steps 3 and 4, at room temperature.
9. Add 0.1 ml of the appropriate substrate solution to each well.
10. Cover the plate with an adhesive plate sealer to avoid substrate evaporation, which can lead to erroneous results. Incubate at room temperature. If the reaction is too slow at room temperature, incubate the plate at 37°C.
11. Follow the appearance of the product by measuring (without the plate sealer) the absorbance or fluorescence at the relevant wavelength for each substrate. Thus, for substrate solutions 1 or 2, measure the change in absorbance at 405 nm. When using substrate solution 3, measure the fluorescence at 480 nm (excitation wavelenght 355 nm).
12. If needed, stop the reaction by adding 0.05 ml of the appropriate stopping solution: 1 M sodium phosphate, pH 7, for substrate solution 1, 1.43 M Na_2CO_3 (no pH adjustment) both for substrate solution 2, and for substrate solution 3.

[a] These two enzymes are preferred to peroxidase because of their linearity in the assay response.
[b] Three substrate solutions are listed, one for use with an immunoconjugate linked to alkaline phosphatase and two for β-galactosidase linked immunoconjugates. Prepare only the appropriate substrate solution.

Protocol 2. Quantification of the amount of antibody trapped on to the coated antigen

Equipment and reagents

As for *Protocol 1*

Method

1. Prepare two coated plates as described in *Protocol 1*, step 1, using an antigen concentration of 1 μg/ml.
2. Prepare antibody solutions at different concentrations (e.g. from 10^{-8} M to 10^{-11} M) in the buffer used for the antigen, and *supplemented* with 0.02% bovine serum albumin.

3. Wash the first coated plate as described in *Protocol 1*.
4. Add 0.1 ml (per well) of each antibody solution, prepared in step 2, to three coated wells and one non-coated well of the first plate (the assays are carried out in triplicate to have enough solution to make duplicates in step 7). Incubate for 30 minutes at the temperature chosen for the affinity measurement.
5. Carefully pipette the antibody solution out of each well and pool the triplicate samples corresponding to the same antibody concentration. Follow the ELISA procedure described in *Protocol 1* from step 6 for this first plate.
6. Wash the second coated plate as in step 3.
7. Add 0.1 ml of each of the pooled antibody solutions that were recovered from the first plate to the wells. Carry out the assays in duplicate. Incubate for the *same time* and at the *same temperature* as in step 4.
8. Follow the ELISA described in *Protocol 1* from step 6 for the second plate. The enzymatic reaction must be stopped after the *same incubation time* in the two plates. Plot the absorbance read in each plate versus the initial antibody concentrations used for the first plate.

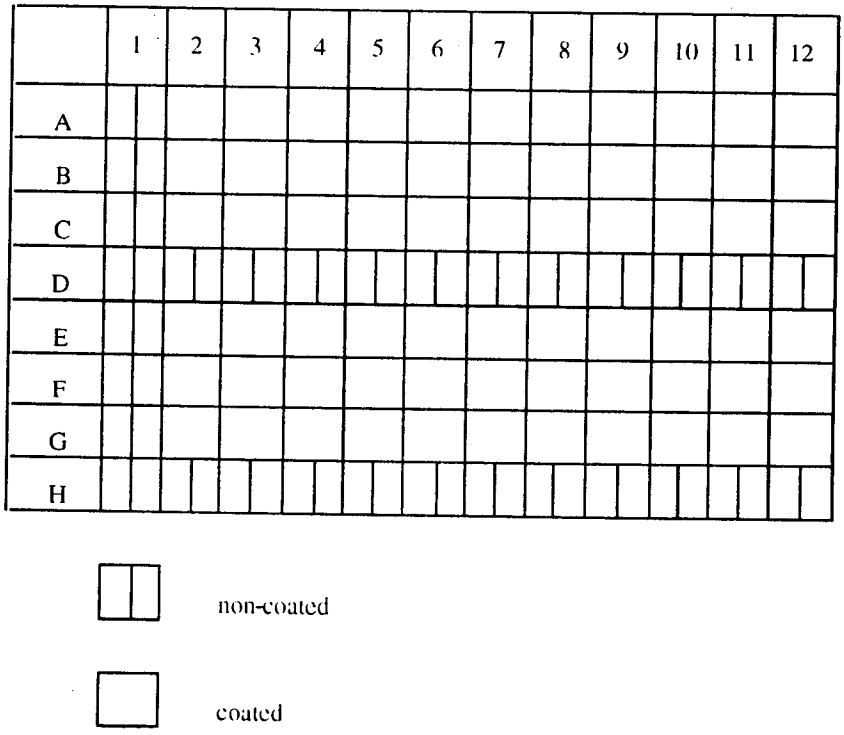

Figure 1. Scheme for coating ELISA plates. The first non-coated vertical line A1 to H1 will become the blank wells containing only the substrate solution, when measuring absorbances in the spectrophotometer. The wells used for assays (triplicate plus control in a non-coated well) are A2 to D2 for the first sample, A3 to D3 for the second one, etc.

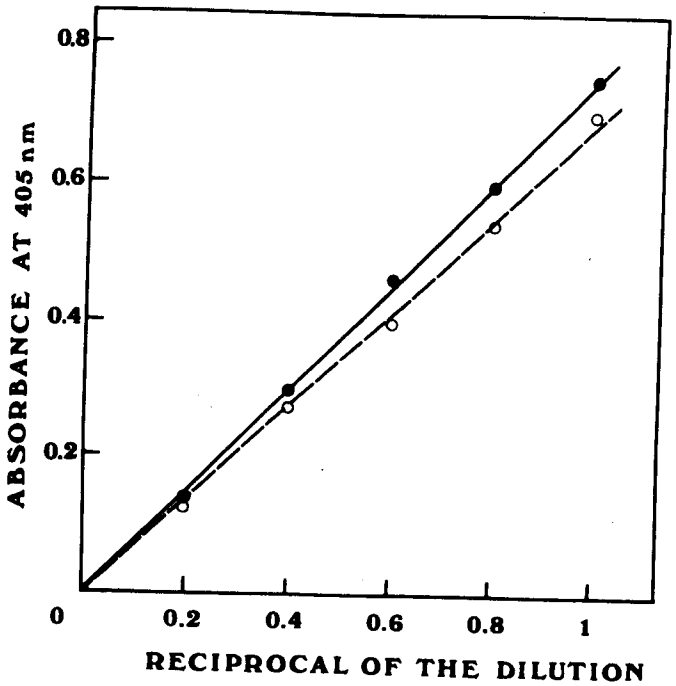

Figure 2. An example of the determination of the amount of antibody retained on a coated antigen. The mAb 46–9 directed against the β_2 subunit of *E. coli* tryptophan synthase was incubated for 1 h at 20°C in plates previously coated with 1 µg/ml β_2. The range of antibody concentrations was 10^{-10} to 7×10^{-10} M. After incubation, the liquid in the wells was transferred to a second coated plate and incubated under identical conditions (see *Protocol 2*). The absorbance at 405 nm was monitored after 2.5 h. Closed and open symbols correspond, respectively, to the absorbance values obtained with the first and the second plate.

The plot obtained from protocol 2, step 8 is linear over a limited concentration range of antibody and reaches a plateau. The constant concentration of antibody used for the determination of K_d must be chosen from the linear part of this plot. In this linear range (see *Figure 2*), the fraction of antibody retained on the coated antigen in the first plate is deduced from the ratio $(S_1 - S_2/S_1)$, where S_1 is the slope obtained with the first plate and S_2 with the second plate. In the case described in *Figure 2*, the amount of antibody retained on the coated antigen represents 6% of the total amount of antibody incubated in the plate.

If the amount of antibody retained is higher than 10% of the total amount of antibody, it is *absolutely necessary* to repeat the experiment with a lower concentration of antigen in the coating step 1 and/or to reduce the incubation time of the antibody solutions on the plate in step 4, until conditions are found under which the two slopes differ by no more than 10%.

3.4 Determination of K_d

Binding equilibrium studies require that the total concentration of antibody $[Ab_t]$ should be close to, or lower than the value of the dissociation constant

(K_d). Since the dissociation constant is a priori not known, the total antibody concentration should be chosen to be as small as possible. Choose the lowest antibody concentration that gives an absorbance of 1 for the enzymatic reaction after a reasonable time. Do not forget that this antibody concentration should be in the linear part of the plot established in *Protocol 2*.

The results are unsatisfactory if the total antibody concentration [Ab_t] is higher than the K_d. The sensitivity of the immunoenzymatic assay with a chromogenic substrate limits the minimum total antibody concentration to about 10^{-10} M. Thus for an antibody with very high affinity, the experiment should be carried out with the β-galactosidase immunoconjugate and its fluorogenic substrate which increase the sensitivity of the assay and permits the use of lower antibody concentration. Taking into account these considerations determine the K_d as described in *Protocol 3*.

Protocol 3. Determination of dissociation constant (K_d)

Equipment and reagents

As for *Protocol 1*

Method

1. Prepare a coated plate as described in *Protocol 1*, step 1, with the antigen at a concentration of 1 µg/ml, or the one determined in *Protocol 2*.
2. Prepare 3 ml of the antibody solution at twice the concentration required (since it will be diluted 2-fold with the antigen) as determined in *Protocol 2*. The antibody and antigen solutions are prepared in the buffer usually required for the antigen and *supplemented* with 0.02% bovine serum albumin.
3. Prepare the antigen solutions (0.3 ml for each concentration) at 10 different concentrations (e.g. by serial dilutions from 10^{-7} to 10^{-10} M). The value of [Ag_t] corresponds to the total concentration of antigenic sites (for oligomeric proteins, it is the concentration of protomers if each protomer binds one antibody molecule).
4. Prepare 11 tubes, 10 containing 0.25 ml of the antigen at different concentrations (prepared in step 3) and one containing 0.25 ml of incubation buffer (no antigen).
5. Add 0.25 ml of the antibody solution prepared in step 2, to each tube and incubate at the desired constant temperature until equilibrium is reached (e.g. overnight).
6. When equilibrium is reached wash the coated plate prepared in step 1, as described in *Protocol 1*, steps 3 and 4.
7. Add 0.1 ml (per well) from each tube to three coated wells and one

Protocol 3. *Continued*

non-coated well. Incubate at the same temperature as in step 5 and for the time determined in *Protocol 2*.

8. Follow the ELISA procedure described in *Protocol 1* from step 6. Since the antibody concentration was chosen in a range where the absorbance is proportional to the antibody concentration, the absorbance read for each well will correspond to the free antibody concentrations.

3.5 Calculations

K_d is calculated from either a Scatchard plot (using Equation 4 (Section 3.1) or Klotz plot (using Equation 5 (Section 3.1)). First, the relative absorbance readings at various antigen concentrations are used to calculate the amount of complex and free antigen at each point as follows: A0 is the absorbance measured for the well containing the total antibody concentration $[Ab_t]$ in the absence of antigen. A1, A2, ... A10 are the absorbances measured for the wells containing the total antigen concentrations $[Ag_{t1}]$, $[Ag_{t2}]$ ... $[Ag_{t10}]$.

$[Ab]$, the free antibody concentration in each well, is related to the absorbance A measured in the ELISA by the following equation:

$$[Ab] = [Ab_t]\,(A/A_0),$$

therefore:

$$[Ab_1] = [Ab_t]\,(A_1/A_0);$$

$$[Ab_2] = [Ab_t]\,(A_2/A_0); \ldots$$

$$[Ab_{10}] = [Ab_t]\,(A_{10}/A_0).$$

$[x]$, the antigen–antibody complex concentration, is related to the total antibody site concentration $[Ab_t]$ and the free antibody site concentration $[Ab]$ (see Equation 1 in Section 3.1):

$$[x] = [Ab_t] - [Ab] = [Ab_t] - [Ab_t]\,[A/A_0] = [Ab_t]\,(A_0 - A)/A_0,$$

therefore:

$$[x_1] = [Ab_t]\,(A_0 - A_1)/A_0;$$

$$[x_2] = [Ab_t]\,(A_0 - A_2)/A_0; \ldots$$

$$[x_{10}] = [Ab_t]\,(A_0 - A_{10})/A_0.$$

$[Ag]$, the free antigen concentration, is related to the total antigen site $[Ag_t]$ and the antibody–antigen complex concentration $[x]$ (see Equation 2 in Section 3.1):

$$[Ag] = [Ag_t] - [x],$$

therefore:

$$[Ag_1] = [Ag_{t1}] - [x_1];$$

$$[Ag_2] = [Ag_{t2}] - [x_2]; \ldots$$

$$[Ag_{10}] = [Ag_{t10}] - [x_{10}].$$

Since as indicated above $[x] = [Ab_t]\ (A_0 - A)/A_0$,

$$[Ag_1] = [Ag_{t1}] - [Ab_t]\ (A_0 - A_1)/A_0;$$

$$[Ag_2] = [Ag_{t2}] - [Ab_{t2}]\ (A_0 - A_2)/A_0; \ldots$$

$$[Ag_{10}] = [Ag_{t10}] - [Ab_t]\ (A_0 - A_{10})/A_0.$$

For the Scatchard equation calculate the values of $[x]$ and $[Ag]$ using the absorbance obtained for each triplicate.

The Scatchard equation (see Equation 4 in Section 3.1) can be also written:

$$[x]/[Ab_t]\ [Ag] = (1 - [x]/[Ab_t])/K_d;$$

$[x]/[Ab_t]$ (the fraction of bound antibody) is usually referred as υ.

Plot $\upsilon/[Ag]$ versus υ. The straight line obtained (see *Figure 3*) has a slope equal to K_a. The K_d can be calculated since $K_a = {}^1/K_d$.

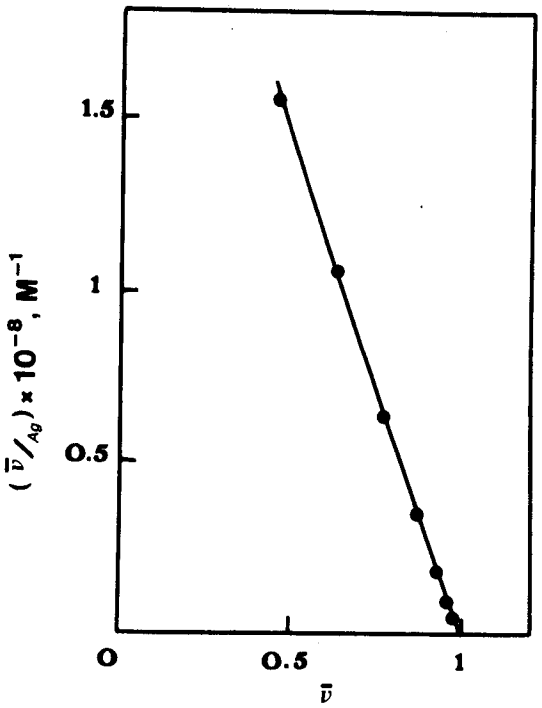

Figure 3. Scatchard plot of the binding of the holo-β_2 subunit of tryptophan synthase to mAb 46–9 measured by ELISA at 20°C. ν is the fraction of bound antibody $[x]/[Ab_t]$ and $[Ag]$ the concentration of free antigen at equilibrium. The total concentration $[Ab_t]$ of antibody sites was 3×10^{-10} M. The slope of the straight line is equal to the $K_a = 1/K_d$). The value of K_d is 3.6×10^{-9} M.

For Klotz representation (see Equation 5 in Section 3.1) calculate $1/[Ag]$ and $[Ab_t]/[x]$ using the absorbance obtained for each triplicate.

Plot $[Ab_t]/[x]$ versus $1/[Ag]$. The straight line obtained has a slope equal to $K_d = (1/K_a)$.

Moreover, by using programs now readily available for any standard microcomputer, a non-linear regression method allows the extraction of K_d directly from the saturation curve in solution (e.g. plotting $(A_0 - A)/A_0$ versus $[Ag]$).

3.6 Determination of K_d with impure antibody

The high sensitivity of the ELISA permits the total antibody concentration $[Ab_t]$ to be very low compared to the total antigen concentration $[Ag_{t1}]$, $[Ag_{t2}]$ ... $[Ag_{t10}]$ (e.g. $[Ag_t] = 10[Ab_t]$). In such a case, the free antigen concentration can be approximated by the total antigen concentration, since the antibody–antigen complex concentration becomes negligible compared to the total antigen concentration. For example, for the total antigen concentration $[Ag_{t1}]$ the equation:

$$[Ag_1] = [Ag_{t1}] - [Ab_t]\,(A_0 - A_1)/A_0$$

becomes: $[Ag_1] = [Ag_{t1}]$,

if $[Ab_t]$ is sufficiently smaller than $[Ag_{t1}]$.

Using the approximation $[Ag_t] = [Ag_{t1}]$ and replacing $[x_1]$ $[Ab_t]$ by its expression given by the absorbances:

$$[x_1]/[Ab_t] = (A_0 - A_1)/A_0,$$

the Scatchard equation (see Equation 6 in Section 3.5) can be written:

$$(A_0 - A_1)/A_0.1/[Ag_{t1}] = 1/K_d.(1 - (A_0 - A_1)/A_0); \qquad [7]$$

and the Klotz equation (see Equation 5 in Section 3.1) can be written:

$$A_0/(A_0 - A_1) = K_D/[Ag_{t1}] + 1. \qquad [8]$$

Equations 7 and 8 no longer contain the total antibody concentration $[Ab_t]$. Therefore, when $[Ag_1]$ can be approximated by $[Ag_{t1}]$, Equations 7 and 8 each permit the determination of the dissociation constant K_d even if the antibody site concentration is not known, such as in ascitic fluids, impure monoclonal antibody, or non-titrated Fab (see the Klotz plot of *Figure 4*). Remember that this is true only if the antigen is in large excess over the antibody (e.g. 10-fold).

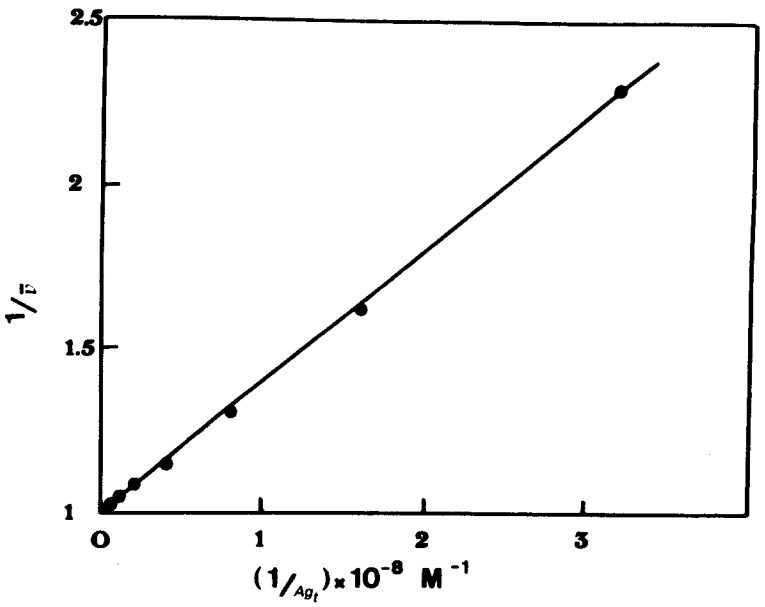

Figure 4. Klotz plot of the binding of holo β_2 to mAb 46–9 at 20°C measured by the ELISA with impure antibodies. ν is the fraction of bound antibody $[x]/[Ab_t]$ and $[Ag_t]$ the total antigen concentration. The concentration of impure antibody was 10^{-4} mg/ml. The slope of the straight line is equal to the K_d. The value of K_d is 3.8×10^{-9} M.

4. Affinity measurement in solution by an RIA-based method

4.1 Rationale

The rationale of the competition ELISA described has been adapted to an RIA-based method where the antigen is available as a radiolabelled molecule in minute amounts and is not necessarily pure. It has been designed to measure the affinity of polypeptide chains synthesized in a cell-free system in the presence of radioactive amino acids (see ref. 10). Chemically radiolabelled antigen can also be used, providing that the conformation of the protein is not altered upon labelling. In principle, the radiolabelled antigen at a constant concentration and the antibody at various known concentrations in large excess over the antigen concentration are incubated in solution until equilibrium is reached. The concentration of the free antigen (that has not reacted with the antibody) is then determined by using immunobeads, i.e. dextran beads, (instead of the ELISA coated plate) to which the same antibody is covalently attached. The free antigen molecules are specifically trapped by the immobilized antibody and the radioactivity of the immunotrapped antigen fraction is then determined.

4.2 Requirements for the determination of K_d

The same requirements that have been underlined for determining the

free antibody concentration at equilibrium in the competition ELISA must be also fulfilled for determining the free antigen concentration in this method:

(a) The concentration of antigen trapped on the immunobeads must be proportional to the concentration of antigen free in solution.

(b) Only a small percentage of the free antigen molecules in the equilibrium solution must bind to the antibody immobilized on the beads to prevent any readjustment of the concentrations at equilibrium.

These two requirements are fulfilled by comparing the total radioactivity of the antigen in solution to the radioactivity bound to the beads during the RIA (see *Protocol 4*). Assuming that the antigen is the only radioactive molecule present in the incubation mixture, a simple radioactivity measurement in a scintillation counter can be done. In a more complex situation where the antigen is present in a heterogeneous mixture of radioactive molecules, a separation step, such as gel electrophoresis, has to be carried out followed by the quantification of the amount of radioactive antigen on the gel.

Protocol 4. Coupling antibody on dextran beads

Equipment and reagents

- Coupling buffer: 0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl
- Wash buffers:
  —0.1 M Tris–HCl, pH 8;
  —0.1 M sodium acetate, pH 4, containing 0.5 M NaCl;
  —0.1 M Tris–HCl, pH 8, containing 0.5 M NaCl
- Cyanogen bromide activated Sepharose 4B beads commercially available from Pharmacia have been routinely used for coupling the antibody by following the instructions given by the supplier

Method

1. Suspend 1 g of freeze-dried powder in 1 mM HCl and filter-wash with 200 ml of 1 mM HCl.
2. Make up 5 mg of immunoglobulin in 2 ml coupling buffer and mix it with 2 ml of the swollen gel prepared in step 1.
3. Rotate the mixture end-over-end for 2 h at room temperature.
4. Check that no free immunoglobulin is present in the coupling buffer after pelleting the beads and measuring the protein concentration in the supernatant.
5. Resuspend the coupled beads in 15 ml of 0.1 M Tris–HCl buffer, pH 8, to block the remaining active groups on the beads. Incubate for 16 h at 4 °C.
6. Wash the antibody-coupled Sepharose beads with three cycles of

alternating pH: each cycle consists of a wash with 0.1 M sodium acetate buffer, pH 4 (containing 0.5 M NaCl), followed by a wash with 0.1 M Tris–HCl buffer, pH 8 (containing 0.5 M NaCl).

7. Store the immunobeads at 4°C as a 50% suspension in 0.1 M Tris–HCl buffer, pH 8 (containing 0.5 M NaCl).

Protocol 5. Quantification of the amount of antigen trapped on to the immunobeads

Equipment and reagents

- Immunobeads (see *Protocol 4*)
- Microcentrifuge for 1.5 ml plastic micro test tubes
- Incubation and wash buffer: PBS or any buffer with pH and ionic strength compatible with antibody/antigen interaction and supplemented with a non-ionic detergent such as 0.1% Nonidet P-40 to minimize non-specific protein–protein interaction
- Radiolabelled antigen (see section 4.1)
- Liquid scintillation radioactivity counter
- Electrophoresis sample buffer (if required): 10% SDS, 20% 2-mercaptoethanol, 16% glycerol, 0.04% Bromophenol Blue, 4 mM EDTA, and 40 mM Tris–HCl, at pH 8.0
- Electrophoresis apparatus and power supply (if required)
- Radioactivity scanner (if required)

Method

NB: Carry out steps 1 and 2 at an appropriate fixed temperature.

1. In a series of micro test tubes, mix a constant volume (50 µl) of the immunobeads (prepared as described in *Protocol 4*) with increasing amounts of the radiolabelled antigen in a final volume of 200 µl.
2. Incubate the mixtures for a constant period of 1 min (or a little more if required to increase the sensitivity of the radioactive signal).
3. Add 850 µl of wash buffer and immediately spin at 10 000 *g* for 15 sec in a microcentrifuge. Remove the supernatant and repeat this washing procedure twice with 1 ml of wash buffer.

   If the antigen is the only radiolabelled molecule present in the antigen solution, proceed as indicated in step 4 and then go directly to step 8; otherwise, skip step 4 and proceed from steps 5 to 7.
4. Suspend the pelleted beads in incubation buffer and count the amount of radiolabelled antigen trapped by the immunobeads in a scintillation counter.
5. If the antigen is not the only radiolabelled molecule, add 10 µl SDS sample buffer to the pelleted beads to dissociate the antigen bound to the beads.
6. Run an SDS gel to separate the antigen of interest from the other radiolabelled molecules.

Protocol 5. *Continued*

7. Determine the radiolabelled antigen concentration by quantitatively scanning the gel (after drying the gel).
8. For each antigen concentration, compare the amount of antigen trapped by the immunobeads with the total amount of antigen initially present in the solution.

A constant fraction of the free antigen initially present in the solution must be recovered in the pelleted immunobeads. Moreover, this fraction must be low enough (not more than 10%) to ensure that the equilibrium is not significantly perturbed in the immunobeads binding step. As already pointed out for the affinity ELISA method, it is critical that this condition should be fulfilled to avoid underestimation of the affinity constant. To minimize the fraction of antigen bound to the immunobeads, several parameters can be adjusted—such as the amount of immunobeads used, the density of antibody molecule on the beads, or the incubation time of the antigen with the immunobeads.

4.3 Determination of K_d

As was the case for the antibody concentration in the ELISA (see Section 3.4), the antigen concentration for the RIA should be choosen to be close to or lower than the K_d value. Using the RIA under the conditions described in Section 4.2, the affinity of a monoclonal antibody for its antigen can be determined as described in Protocol 6.

Protocol 6. Determination of the K_d by an RIA-based method

Equipment and reagents

- Immunobeads (see *Protocol 4*)
- Microcentrifuge for 1.5 ml plastic micro test tubes
- Incubation and wash buffer: PBS or any buffer with pH and ionic strength compatible with antibody/antigen interaction and supplemented with a non-ionic detergent such as 0.1% Nonidet P-40 to minimize non-specific protein–protein interaction
- Radiolabelled antigen (see section 4.1)
- Stock solution of the monoclonal antibody
- Electrophoresis sample buffer (if required): 10% SDS, 20% 2-mercaptoethanol, 16% glycerol, 0.04% Bromophenol Blue, 4 mM EDTA, and 40 mM Tris–HCl, pH 8.0
- Electrophoresis apparatus and power supply (if required)
- Radioactivity scanner (if required)

Method

NB: Carry out steps 1 to 3 at an appropriate temperature. Run the experiment in *duplicate* at least and include control samples with Sepharose beads coupled with a non-specific antibody to evaluate non-specific binding of the antigen to the immunobeads.

1. Make up the radiolabelled antigen (1.4 ml) at twice the required concentration (as determined in *Protocol 5*).

2. Make up the antibody solutions at about seven different concentrations (0.2 ml for each concentration) ranging, for instance, from 10^{-7} to 10^{-10} M.
3. Prepare micro test tubes containing 75 µl of the antibody solution at different concentrations (as prepared in step 2) and two tubes containing 75 µl incubation buffer (zero antibody).
4. Add 75 µl of the antigen solution prepared in step 1 to each micro test tube containing the antibody solutions and also to the tubes containing the incubation buffer. Incubate at a constant temperature until equilibrium is reached. In most cases, 2 hours has been found to be enough.
5. Add 50 µl of the immunobeads suspension prepared as described in *Protocol 4*.
6. Follow the procedure of *Protocol 5* from steps 2 to 7..

If R_0 is the radioactivity obtained for the samples without antibody, R_i the radioactivity for the samples with the monoclonal antibody at a concentration M_i(M_i = concentration of antibody-binding sites), the fraction v of bound antigen is equal to:

$$v = (R_0 - R_i)/R_0.$$

This expression is obtained assuming that the non-specific binding of the radiolabelled antigen to the immunobeads is negligible, otherwise the background values obtained with the control samples have to be subtracted from both R_0 and R_i. If M_i has been kept much larger than the total concentration of antigen, M_i can be considered as practically equal to the concentration of free antibody sites. Thus, plotting $R_0/(R_0 - R_i)$ as a function of $1/M_i$ results in a straight line, the slope of which gives the K_d.

5. Conclusions

The indirect competition ELISA method described above offers many advantages, as long as it is used under conditions where equilibrium in solution is reached and not perturbed by the solid-phase immunoassay. Only minute amounts of mAb and antigen are needed. Only one of the components (antigen or mAb) needs to be purified or titrated. It provides the real saturation curve in solution. The species at equilibrium in solution are not labelled, which ensures that the observed affinity is characteristic of *native* mAb and antigen.

The rationale of the competition ELISA described in Section 2 has been adapted to an RIA-based method aimed at measuring the affinity of polypeptide chains synthesized in cell-free systems in the presence of radioactive amino acids (see Section 4 and ref. 10).

With the indirect competition ELISA or RIA, the two main problems one may encounter are related to the sensitivity of the solid-phase assay (which sets a higher limit to the affinity values that can be measured) and to the complexity of the experimental curves obtained with multivalent antibodies (which complicates the extraction of the affinity constant from the experimental data).

The lower limits to the K_d values that can be determined by ELISA using classical chromogenic substrates are about 10^{-10} M. However, commercially available fluorogenic substrates for ELISA increase the assay sensitivity by a factor of about 100, and render it possible to determine K_d of 10^{-11}–10^{-12} M (3). Higher affinities could certainly be approached by RIA-based indirect competition methods, using very high specific activity radiolabelled antigens.

The multivalency of antibodies and antigens, and sometimes the heterogeneity of the antibodies, complicate the determination of affinity. This has become the object of much attention (11, 12). The simple data analysis proposed by Friguet *et al.* (7) provides satisfactory results even when divalent immunoglobulins are used, as long as one extracts the affinity value only from that part of the saturation curve obtained at high saturation of the mAb by the antigen.

In summary, the competition ELISA method described above is the method of choice to determine the real equilibrium dissociation constants of mAb–Ag complexes *in solution* because of its simplicity, its modest requirements in terms of equipment, as well as quantity and purity of antigen or monoclonal antibody. Its rationale adapted to an RIA-based method permits the measurement of the affinity of monoclonal antibodies for radioactive polypeptide chains present in minute amounts in crude heterogeneous mixtures.

These methods can be transposed to the study of protein–protein interactions (see, for example, ref. 13) that do not involve antibodies.

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Antibody engineering

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