Chapter 42
Anti-Histidine Antibodies as Tools for Reversible Capturing of His-Tagged Fusion Proteins for Subsequent Binding Analysis

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42.1 Introduction

Protein-binding reagents, most commonly polyclonal or monoclonal antibodies, are essential tools for protein characterization in basic research, biotechnology, medical therapy, and diagnostics. Antibody-based therapeutics has become available as a means to efficiently target several human diseases such as cancer treatment where antibody-based immunotherapy complements chemotherapy (Adams and Weiner 2005). Two prerequisites for this application of antibodies are high specificity and high affinity to the target molecule. In a recent study, Tang and co-workers showed that high affinity of an antibody like anti-HER2/neu is associated with antibody-mediated cellular cytotoxicity (Tang et al. 2007). In addition, the neutralization ability of antibodies against infectious agents like HIV-1 (human immunodeficiency virus type 1) or SARS coronavirus have been demonstrated to simultaneously increase with their affinity (Nelson et al. 2007; Rogers et al. 2008), thus emphasizing the need for highly accurate binding data not only for basic research but also for diagnostics and therapy.

Optical biosensors provide an excellent tool for in-depth characterization of the interactions of protein-binding reagents in real time. For more than about 20 years now, chip-based Surface Plasmon Resonance (SPR) biosensors, such as those manufactured by Biacore (GE Healthcare), have been used for high-resolution, time-resolved interaction measurements of biomolecules. These instruments do not only allow the determination of affinities (e.g. equilibrium dissociation constant, K_D) but also provide distinct rate constants for association (k_a) and dissociation (k_d) with high accuracy. Intersystem comparisons of biosensors have demonstrated the reliability of these instruments (Katsamba et al. 2006). In a recent study, reproducibility was tested by Myszka and co-workers thus, demonstrating that differences

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between users were negligible when testing the same set of immunosamples on different biosensors world-wide (Rich et al. 2008).

42.1.1 Application of SPR in Antibody Engineering

SPR-based Biacore technology was originally used to investigate antibody-antigen binding (Fagerstam et al. 1990; Karlsson et al. 1991). Since then, dozens of other applications combining antibodies and Biacore systems have been generated (for an overview, see Rich and Myszka 2008). One approach is to determine the antibody concentration in sera as shown by Pol et al. (2007) or the antibody isotyping as described (Swanson et al. 2004). Recently, these instruments have also been used to screen for high-affinity antibodies from crude cell lysates (Canziani et al. 2004; Steukers et al. 2006). Here, the authors used either covalently coupled anti-human-Fc IgG antibodies or immobilized protein A to capture antibodies from various hybridoma supernatants and subsequently determined the Fab concentration from extracts. With this strategy, Canziani and coworkers, using Biacore 2000 or 3000 instruments, were able to test three antibodies from three supernatants. In a similar study, it was demonstrated that Biacore A100 and Flexchip units (array format) were suitable for increased to high throughput applications (Safsten et al. 2006; Wassaf et al. 2006).

42.1.2 Principle of a Biacore Analysis

The principle work flow of a Biacore-based interaction analysis consists of (1) the immobilization of a ligand (molecule 1) on a sensor chip, (2) the injection of an analyte (molecule 2) in the flow phase over the sensor surface, and (3) the regeneration of the chip surface. Interaction of the analyte with the immobilized ligand causes a change in the refractive index close to the sensor chip surface which, in turn, is detected as a change in SPR-signal (in response units, RU) and plotted against the time in a so-called sensogram (for details, see Gesellchen et al. 2005).

For the immobilization of ligands, several strategies exist. The standard approach is the covalent coupling of a ligand via primary amines (free N-termini or lysine residues) utilizing NHS/EDC (*N*-hydroxysulfosuccinimide/*N*-ethyl-*N*′-dimethylaminopropyl-carbodiimide) chemistry or via thiol groups (Gesellchen et al. 2005). Non-covalent, reversible capturing can be achieved by employing genetically engineered fusion tags (for example, GST (Glutathion S-Transferase), FLAG (DYKDDDDK peptide) or Strep-tag (WSHPQFEK peptide)) once an appropriate binding reagent (antibody), specifically targeted against the fusion tag, has been attached to a sensor chip. Biotinylated molecules can also be captured site-directed on a streptavidin surface with high affinity; however, because of their high affinity, a complete regeneration applying rather mild conditions may

be problematic. For reversible capturing of GST-tagged fusion proteins, a commercially available anti-GST antibody (GST capture kit, BR-1002-23, Biacore) is commonly used (Zimmermann et al. 2008). Thus, the interaction pattern and/or the functionality of the fused protein can be influenced by the high molecular weight of the GST-fusion tag (~26 kDa). Proteolytic cleavage of the fusion tag using a specific protease may be a suitable strategy (Terpe 2003) to remove the fusion part still requiring extra steps and may result in loss of protein.

The commonly used polyhistidine-fusion tag is smaller in size than the GST-tag. Several strategies exist for site-specific capturing/immobilization. The first strategy is comprised of reversible, non-covalent binding to a specific NTA (Nitrilotriacetic acid) chip (BR-1004-07, Biacore; (Gershon and Khilko 1995) in the presence of Ni^{2+} ions; additionally, covalent coupling on those NTA chips can be performed (Willard and Siderovski 2006; Diskar et al. 2007; see also Method section). Slow dissociation from the polyhistidine capturing surface is fundamental for performing a sandwich type binding assay. Reversible binding has to be tested for every His-tagged protein in order to determine if the dissociation rate from the polyhistidine capturing surface is slow enough for subsequent interaction analysis. Insufficient capturing (fast off-rate) of polyhis-tagged proteins is especially problematic when using Ni^{2+}-NTA sensor chips (Herberg, personal communication). Immunotools, that are, antibodies against His_6-tagged fusion proteins, may provide an alternative that is superior to the Ni^{2+}-NTA-approach.

Besides immobilization, efficient regeneration is crucial in order to provide a defined ligand concentration for accurate binding analysis. There are two general modes of regeneration. One is to gently remove just the analyte from the ligand and the other is to dissociate both molecules from the sensor surface in a single step-procedure. In case of a covalently coupled antibody, acidic conditions are often useful to dissociate the ligand and analyte simultaneously (see Methods section for details).

In this study, we tested the binding of 12 RGS-$(His)_6$-tagged (Arg-Gly-Ser-$(His)_6$) proteins (Table 42.1) to two anti-$(His)_6$, one anti-$(His)_5$ and one anti-RGS-$(His)_4$

Table 42.1 Description, molecular weight, and Swiss-Prot accession number of the 12 RGS-$(His)_6$-tagged proteins

	MW (kDa)	SwissProt acc.	Description
His_9482_6E10	70	O43742	InaD-like protein
His_9146_6B2	50	O60239	SH3 domain-binding protein 5
His_9448_6E4	30	Q96H25	hypothetical protein (fragment)
His_9522_6F3	40	Q96F30	similar to src homology 3 domain-containing protein hip-55
His_9142_6B1	30	Q6PJ62	eEF-1B gamma (fragment)
His_9326_6C9	30	AAP35323	Eukaryotic translation elongation factor 1 gamma
His_9502_6F2	50	Q9UI47	Similar to catenin (cadherin-associated protein)
His_9605_6F10	110	P21333	Filamin A, alpha
His_9189_6B9	17	P43080	Guanylyl cyclase activating protein 1 (gcap 1)
His_9413_6D9	20	O08765	Ganglioside expression factor 2 (gef-2) (gate-16)
His_9427_6D11	26	O43639	Cytoplasmic protein nck2 (nck adaptor protein 2)
His_9617_6F11	48	P12277	Creatine kinase, b chain (ec 2.7.3.2)

antibody. The goal was to identify an antibody suitable for stable, but reversible capturing of His-tagged proteins in order to allow further interaction analysis with other proteins (sandwich assay).

42.2 Materials

42.2.1 Biacore Consumables

- CM5 sensor chips (research grade), store at 4°C
- NTA sensor chips, store at 4°C
- 100 mM NHS (*N*-hydroxysuccinimide), store at −20°C
- 400 mM EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride), store at −20°C
- 1 M Ethanolamine hydrochloride, pH 8.5, store at 4°C

42.2.2 Buffers

Coupling buffer
- 10 mM sodium acetate, pH 4–6, store at 4°C
- 100 mM N-hydroxysuccinimide (NHS), store at −20°C
- 400 mM *N*-ethyl-*N*′-(dimethylaminopropyl)-carbodiimide (EDC), store at −20°C
- 1 M ethanolamine hydrochloride, pH 8.5, store at 4°C

NaMOPS Biacore running buffer
- 20 mM MOPS (3-(N-morpholino) propanesulfonic acid), pH 7.0.
- 150 mM NaCl.
- 0.005% Tween 20 (Fluka) (store at 4°C and equilibrate to room temperature before use).

HBS-EP Biacore running buffer
- 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4
- 150 mM NaCl
- 0.005% Tween 20
- 0.05 mM EDTA (store at 4°C and equilibrate to room temperature before use)

Regeneration buffer for antibody surfaces
- 10 mM glycine-HCl, pH 1.5–2.5, store at 4°C (see also trouble shooting)

Regeneration buffer for NTA surfaces
- 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 8.3
- 150 mM NaCl
- 0.005% Tween 20
- 350 mM EDTA (storage at 4°C and equilibrate to room temperature before use)

42.2.3 Anti-His Tag Antibodies

- anti-$(His)_6$ antibody, directed against terminal $(His)_6$-epitope, monoclonal, mouse, IgG1, unconjugated, BSA-free, Dianova GmbH, Hamburg, DIA 910
- anti-$(His)_6$ antibody, Trend Pharma & Tech Inc., Canada, IgG2b
- Penta-His antibody, directed against terminal or internal $(His)_5$-epitope, monoclonal, mouse, IgG1, unconjugated, BSA-free, lyophilized from PBS buffer, Qiagen, Hilden
- RGS-His antibody, directed against RGS-$(His)_4$-epitope, monoclonal, mouse, IgG1, unconjugated, BSA (Bovine serum albumin)-free, lyophilized from PBS (Phosphate buffered saline) buffer, Qiagen, Hilden
- The antibodies from Qiagen were purchased as Anti-His Antibody Selector Kit (3×3 micrograms of Penta-His Antibody, Tetra-His Antibody, and RGS-His Antibody, respectively).

42.3 Methods

42.3.1 Purification of His-Proteins in a 96-Well Format

The codons for the RGS-$(His)_6$ tag/epitope (Arg-Gly-Ser-$(His)_6$) can be fused to a gene of interest using the pRSET-vectors (Invitrogen) or the pQE-vectors (Qiagen). N-terminal RGS-$(His)_6$-tagged proteins were overexpressed in *E. coli* SCS1 cells and purified with Ni^{2+}-NTA agarose in a 96-well plate format, as described by Büssow et al. (Bussow et al. 2004), except for substituting 50 mM Tris pH 8.0 by 50 mM Hepes pH 8.0. Eluates from four independent purifications were pooled for Biacore experiments to compensate for differences in expression level and purification yield. Proteins were concentrated with an Amicon Ultra 10 device to 2 mg/ml and the molecular weight of each protein was determined using SDS-PAGE, ranging from 17–110 kDa.

42.3.2 Amine Coupling of Anti-His Tag Antibodies

Analysis were performed with a Biacore 2000 instrument at 25°C and the data were evaluated with BIAevaluation 4.01 (Biacore) and Graphpad (Graphpad Prism 4.0,

San Diego). NHS, EDC, and CM 5 sensor chips were obtained from Biacore. All buffers were degassed and sterile filtered.

Anti-His tag antibodies were coupled to CM5 (carboxymethylated dextran) sensor chips on two flow cells. The other flow cells served as negative controls. One chip was used to test the two Qiagen antibodies. On the other chip, antibodies from Dianova and Trend Pharma were immobilized. To achieve comparable coupling densities, the antibodies from Qiagen had to be subjected to short-term dialysis (1 h, 4°C) against the coupling buffer (10 mM sodium acetate, pH 5.0) in order to adjust the concentration and pH of the solution. Antibodies (30 $\mu g\ ml^{-1}$) in the coupling buffer were injected for 7 min at a flow rate of 5 $\mu l\ min^{-1}$ over the NHS/EDC-activated surface to generate surface densities of 3060 RU (anti-RGS-$(His)_4$ antibody from Qiagen), 5970 RU (anti-Penta-His antibody from Qiagen) and 14310 RU (anti-$(His)_6$ antibody from Dianova), and 15370 RU (anti-$(His)_6$ antibody from Trend Pharma).

Procedure in detail:

- Insert CM5 sensor chip equilibrate to room temperature into a Biacore instrument and prime the system with running buffer.
- Start the sensogram and wait until the baseline is stable.
- Inject 15 µl 10 mM NaOH (three times) at a flow rate of 30 $\mu l\ min^{-1}$.
- Decrease the flow rate to 5 $\mu l\ min^{-1}$ and address only the flow cell where the antibody should be immobilized.
- In most cases, a pH scouting is necessary before trying to couple a protein. By this procedure, one determines the pH and protein concentration at which the electrostatic interaction to the dextran matrix works out best for subsequent immobilization. Therefore, dilute the ligand to concentrations ranging from 1 to 30 $\mu g\ ml^{-1}$ in the coupling buffer with different pH values. Generally, a pH from 4.5 to 5.5 is useful to couple antibodies.
- Perform short injections (1 min) of the diluted ligand and observe where the electrostatic attraction is suitable to obtain an increase in SPR-signal (RU).
- Mix the thawed NHS and EDC solution in a 1:1 ratio and inject the mixture for 7 min. After the injection, record an increase in the response signal of 180–250 RU (CM5 chip on a Biacore 2000 system) for the activation of the surface.
- Inject the ligand stepwise until the desired surface density is reached.
- Finally, inject ethanolamine hydrochloride for 7 min to quench unreacted esters and to remove non-covalently bound ligand.
- The control surface (in general flow cell 1) should at least be activated with NHS/EDC and treated afterwards with ethanolamine hydrochloride, as described above.

Application notes:

- The needle and microfluidics cartridge (IFC) have to be rinsed after each injection (extraclean) to avoid a cross-contamination.
- A sensor chip with an already coupled ligand can be stored at 4°C under buffer in a 50-ml screw cap tube.

42.3.3 Reversible Binding of His-Tagged Proteins to Ni^{2+}-NTA

For measurements on NTA sensor chips, HBS-EP (Biacore) running buffer can be used.

Detailed procedure:

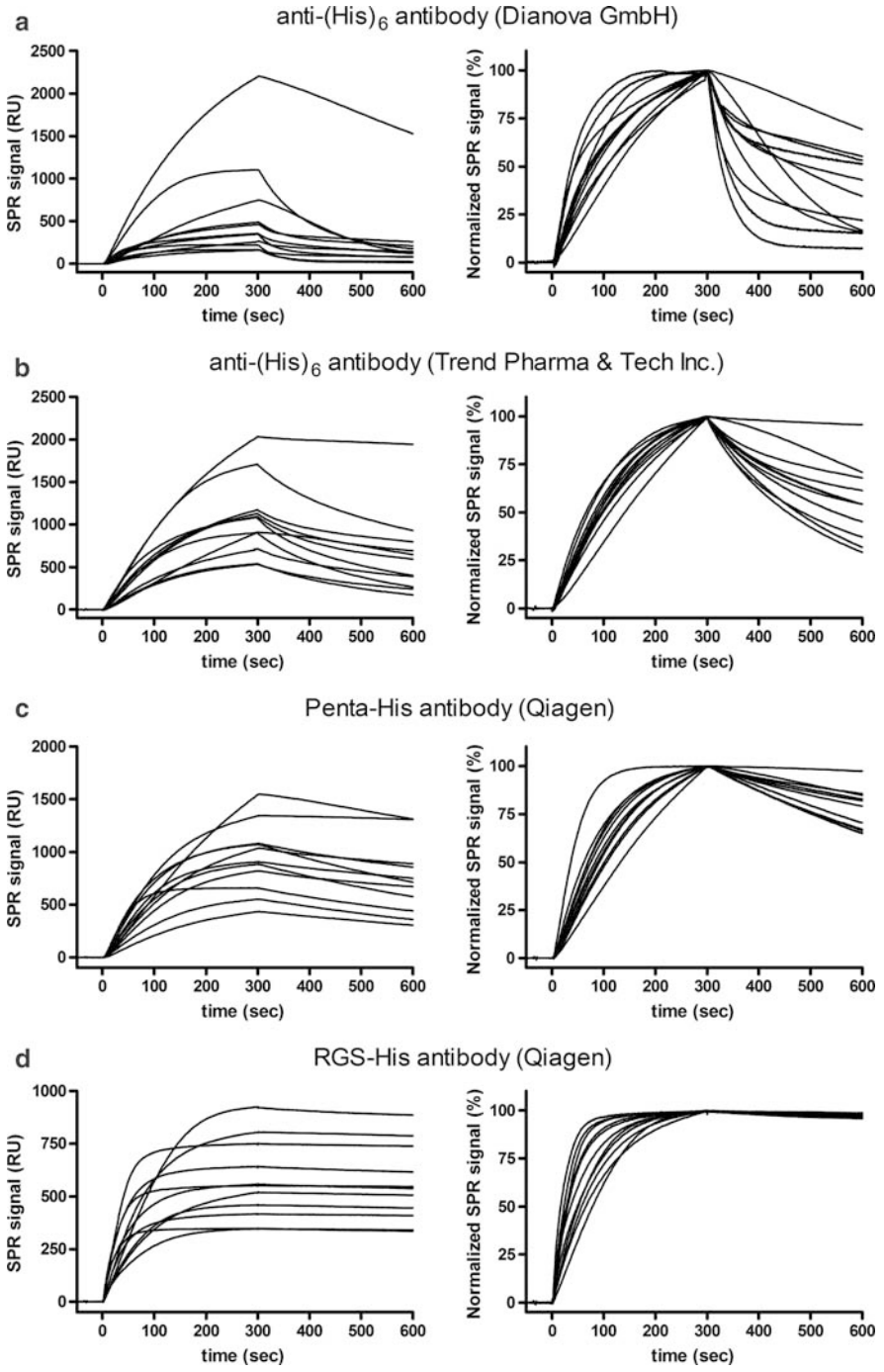
- The first step is to chelate Ni-ions by the NTA surface. Therefore, inject $NiCl_2$ (500 μM) in HBS-EP running buffer for 1 min at a flow rate of 20 μl min^{-1} into at least two flow cells.
- Switch the flow path to the flow cell where the polyhistidine-tagged protein will be captured is going to be bound and decrease the flow rate to 5–10 μl/min.
- Inject the purified histidine-tagged fusion protein diluted in HBS-EP buffer until the desired surface density is reached. Monitor the baseline to assure the stability of the bound ligand (as slow dissociation).
- Via short injections (several seconds) of the $NiCl_2$ solution, it is possible to strip minor amounts of the fusion protein from the surface, and by this, fine-tune the surface coverage.
- For subsequent interaction analysis, direct the non-His-tagged protein (analyte) over both flow cells (reference and the ligand-bound surface) at a flow rate of 30 μl min^{-1}.
- Inject the analyte, as described (Sect. 42.3.5), but omit subsequent cleaning steps.
- Remove the analyte and ligand by chelating Ni-ions with a pulse of 350 mM EDTA pH 8,3 (regeneration buffer for NTA surfaces). Several prolonged injections (2–5 min) might be necessary.

42.3.4 Covalent Coupling of His-Tagged Proteins on NTA

Covalent coupling of His-tagged fusion proteins via primary amine groups on a NTA sensor chip surface was originally described by Willard and Siderovsky (Willard and Siderovski 2006). This method, in contrast to the standard covalent coupling via primary amines, ensures a site directed immobilisation (N-terminal amine group) and can be performed in physiological buffer conditions (e.g. HBS). Initially, the NTA surface has to be saturated with Ni-ions such as described for the reversible binding of polyhistidine-tagged proteins to the NTA surface (see Sect. 42.3.3). In a second step, coupling via the primary amines is performed.

Detailed procedure:

- Inject $NiCl_2$ (500 μM) in HBS-EP running buffer for 1 min at a flow rate of 20 μl per min over only the flow cell where the ligand is to be covalently coupled.
- Decrease the flow rate to 5 μl/min and inject the 1:1 NHS/EDC mixture (see Sect. 42.3.2) for 7 min.


a
anti-(His)6 antibody (Dianova GmbH)
SPR signal (RU)
Normalized SPR signal (%)
time (sec)
b
anti-(His)6 antibody (Trend Pharma & Tech Inc.)
SPR signal (RU)
Normalized SPR signal (%)
time (sec)
c
Penta-His antibody (Qiagen)
SPR signal (RU)
Normalized SPR signal (%)
time (sec)
d
RGS-His antibody (Qiagen)
SPR signal (RU)
Normalized SPR signal (%)
time (sec)

- Subsequently, inject adequate dilutions of polyhistidine-tagged protein in HBS-EP buffer until the surface density of choice is reached.
- Inject ethanolamine hydrochloride for 7 min.
- Next, remove Ni-ions and non-covalently coupled proteins and inject the regeneration buffer for NTA surfaces for 3 min at a flow rate of 20 μl/min.

42.3.5 Surface Plasmon Resonance (SPR) Studies

Procedure for capturing RGS-$(His)_6$-tagged proteins: The 12 recombinant RGS-$(His)_6$-tagged proteins from the protein expression library were diluted 500-fold into running buffer and were injected sequentially over the antibody-decorated surfaces at a flow rate of 30 μl min^{-1}. Binding responses between 200 and 2,500 RU were reached after injection of the analyte (Fig. 42.1 *left panel*). Association and dissociation phases were monitored for 5 min each. Every cycle was completed with two injections of 10 mM glycine pH 1.9 (20 s) to remove the captured his-fusion proteins from the antibody surface.

Detailed procedure:

- Prior to injection, centrifuge all samples at 4°C for 10 min at 10,000×*g*. This procedure removes particles, possibly clogging the integrated fluidics of the instrument.
- Start a sensogram and switch the flow path to the reference flow cell and the antibody-decorated flow cell, respectively.
- Inject a series of different analyte concentrations over the ligand and reference surfaces.
- Regenerate the surface after each cycle by short injections (20–30 s) of glycine-HCl (pH 1.5–2.5). The appropriate pH must be determined previously for each and every antibody (see also trouble-shooting section).
- Additionally, a blank run with the running buffer can be carried out and subtracted from the binding curves (double referencing).

42.3.6 Sandwich Interaction Assay

A sandwich interaction analysis describes stable and reversible binding of one molecule (the ligand) to a chip surface via a specific antibody or Ni^{2+}-NTA in

Fig. 42.1 Binding of RGS-$(His)_6$-tagged proteins to immobilized anti-$(His)_6$ (a and b), anti-penta-His (c), and anti-RGS-$(His)_4$ (d) antibody. The uncorrected sensograms are shown in the left panel and the normalized data are in the right panel. Association and dissociation were carried out in HBS-EP for 300 s each. Normalization was performed with Prism 4.0 (Graphpad Prism 4.0, San Diego). The SPR signal (RU) before injection was set to 0% and the signal at the end of the association (300 s) was set to 100%. (**a**) anti-$(His)_6$ antibody (Dianova), (**b**) anti-$(His)_6$ antibody (Trend Pharma), (**c**) Penta-His antibody (Qiagen), and (**d**) RGS-His antibody (Qiagen)

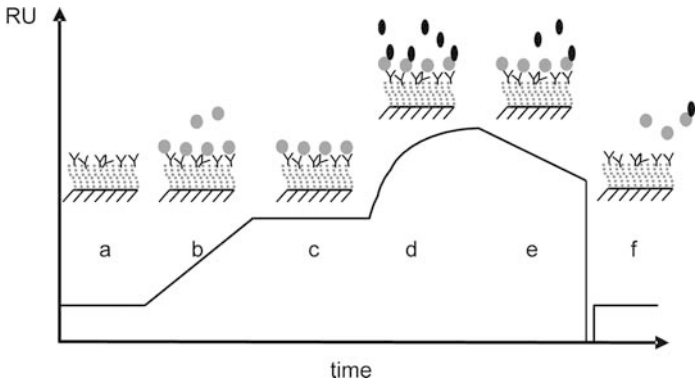


Fig. 42.2 Schematic workflow of a sandwich interaction analysis with RGS-His antibodies used for capturing. (**a**) Equilibration 1: Baseline prior to the injection of a ligand over the RGS-His antibody surface. (**b**) Step 1: Injection of the RGS-$(His)_6$-tagged ligand. (**c**) Equilibration 2: Baseline after ligand capturing. (**d**) Step 2: Association of the analyte. (**e**) Step 3: Dissociation of the analyte. (**f**) Step 4: Regeneration of the captured ligand and bound analyte from the antibody surface

order to be able to determine the binding kinetics for another molecule (the analyte) (see Fig. 42.2). To test for non-specific binding of the analyte to the capturing antibody, this antibody should also be covalently coupled to a reference flow cell. Subsequently, the ligand has to be captured only to one of the antibody surfaces, whereas the other surface serves as a reference.

Detailed procedure:

- To capture a determined amount of protein on an antibody surface, inject the ligand at low flow rates (5–10 µl min^{-1}) until the desired response (RU) is reached (Fig. 42.2b).
- When performing kinetic experiments, set the flow rate at a minimum of 30 µl/min not only to overcome mass-transport-limited interactions, but to reduce rebinding during the dissociation phase. In the same line, reducing the ligand density on the sensor surface may diminish mass transport limitation effects (for details, see Gesellchen et al. 2005).
- Inject the analyte over the reference surface and the analyzing surface (Fig. 42.2d).
- Remove the captured ligand and bound analyte with pulses of glycine-HCl (pH 1.5–2.5) (Fig. 42.2f). See also buffer-scouting procedure for regeneration (Sect. 42.5. trouble shooting).
- Always perform a blank run (buffer injection) for double referencing when carrying out a sandwich type interaction assay.
  Note: For a subsequent analysis cycle with a different analyte concentration, the same surface density has to be accomplished in order to allow a (pseudo first order) kinetic evaluation.

Table 42.2 Evaluation of the dissociation rate constants for the RGS-$(His)_6$-tagged proteins applied to the different antibody-decorated surfaces

RGS-His_6-tagged protein	Dissociation rate constant k_d ($\times 10^{-3}s^{-1}$)				Dissociation after 5 min (%)
	anti-$(His)_6$ antibody (Dianova)	anti-$(His)_6$ antibody (Trend Pharma)	anti-penta-His antibody (Qiagen)	anti-RGS-$(His)_4$ antibody (Qiagen)	
His_9482_6E10	16.4	4.82	1.4	0.13	4.3
His_9146_6B2	3.18	0.4	0.44	0.17	4.25
His_9448_6E4	0.84	0.18	0.064	0.03	1.2
His_9522_6F3	25.1	4.9	1.42	0.1	3.7
His_9142_6B1	18.9	4.11	1.37	0.11	3.5
His_9326_6C9	8.34	3.36	1.38	0.068	2.9
His_9502_6F2	8.06	2.46	0.64	0.052	2.5
His_9605_6F10	7.27	3.28	1.1	0.06	2.9
His_9189_6B9	8.02	3	0.81	0.044	2.2
His_9413_6D9	9.52	2.93	0.7	0.086	3.8
His_9427_6D11	6.96	3.52	0.89	0.016	1.3
His_9617_6F11	3.79	1.34	0.476	–	–

The dissociation rate constants were fitted within an interval of 30 s up to 100 s starting 4 s after the end of the injection assuming a 1:1 Langmuir dissociation model (BIAevaluation software version 4.01). Additionally, the percentage of protein dissociated from the anti-RGS-$(Hi)_{S4}$ antibody after 5 min is presented in the table.

42.4 Results

To select an optimum antibody for a sandwich type assay (Fig. 42.2), four different anti-His tag antibodies were covalently immobilized on CM5 sensor chips (Biacore GE Healthcare) and subsequently tested with 12 RGS-$(His)_6$-tagged proteins from a proteome-wide human expression library where proteins ranged from 17 to 110 kDa in molecular weight (Seitz et al. 2006) (Table 42.1). The overall requirement for this type of sandwich assay is a slow dissociation rate constant (k_d) of the captured his-tagged protein from the antibody to provide a stable ligand surface.

A specific interaction of the RGS-$(His)_6$-tagged proteins with each antibody was obtained suggesting that the fusion tag was accessible for binding to the capture antibody. Dissociation rate constants (k_d) for each antibody were calculated (see Table 42.2) assuming a 1:1 Langmuir model (BIAevaluation 4.01, Biacore). When comparing the dissociation rate constants of all proteins from the tested antibodies, a reduction in the k_d value in the order anti-$(His)_6$-antibody (Dianova) > anti-$(His)_6$-antibody (Trend Pharma) > anti-$(His)_5$-antibody (Qiagen) > anti-RGS-$(His)_4$-antibody (Qiagen) was observed demonstrating increased affinity for the epitope (Fig. 42.1). Only in case of the RGS-$(His)_4$-antibody from Qiagen, association with the RGS-$(His)_6$-tagged proteins was very rapid and dissociation very slow (Table 42.2). This suggested that the interaction with the RGS-$(His)_4$-antibody was extremely stable and suited best for a sandwich assay in order to determine binding kinetics with other, non-RGS-$(His)_6$-tagged proteins as analytes (Fig. 42.3). In contrast to the anti-RGS-$(His)_4$-antibody, the anti-penta-His antibody from

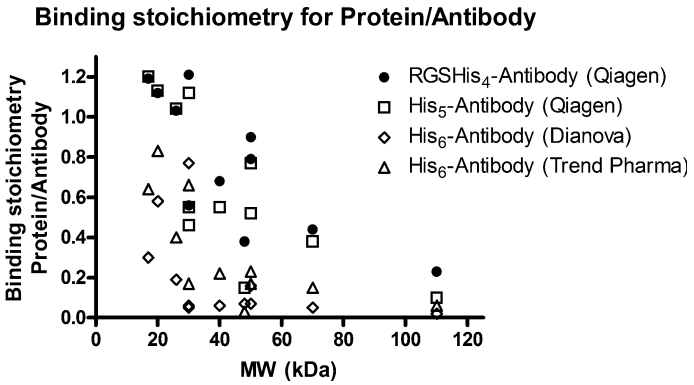


Fig. 42.3 Molecular weight correlation of the RGS-$(His)_6$-tagged protein with the binding stoichiometry for protein/antibody. Binding stoichiometries were calculated on the basis of the molecular weight of ligand and immunoglobulin G. The calculated binding stoichiometry was plotted for each anti-His antibody (depicted in the figure) against the molecular weight of the RGS-$(His)_6$-tagged protein

Qiagen showed a lower capability for capturing, mainly due to the faster dissociation rate (see Table 42.2 and Fig. 42.1). The Tetra-His antibody from Qiagen displayed an even faster off rate for the RGS-$(His)_6$-tagged proteins (Hahnefeld et al. 2004). The antibody distributed by Trend Pharma worked better than the anti-$(His)_6$ antibody from Dianova, that is, the dissociation rate for the RGS-$(His)_6$-tagged proteins was lower, but definitely higher when comparing it even with the anti-penta-His antibody. In general, more than 50% of the bound RGS-$(His)_6$-tagged proteins dissociated in a short period of time from the Dianova antibody (see Fig. 42.1a *right panel*), thus making the antibody less suitable to capture His-tagged proteins when compared with the other antibodies tested.

It could be generally deduced from the binding curves that, for the most part, the lower the molecular weight of a protein the higher the observed binding stoichiometry was (Fig. 42.3). Several RGS-$(His)_6$-tagged proteins were associated with a binding stoichiometry of ~1. However, this 1:1 binding stoichiometry with smaller proteins was only attained in case of the anti-His tag antibodies from Qiagen, probably also due to the steric conditions on the medium-dense antibody-surface. The anti-His antibodies from Dianova and Trend Pharma showed much lower binding stoichiometries for the RGS-$(His)_6$-tagged proteins, most likely due to the more dense antibody packing, thus imposing steric constraints (crowding effect).

Furthermore, we tested the stability of the covalently coupled antibodies after multiple regeneration steps based on residual binding capacity. Therefore, we injected a particular RGS-$(His)_6$-tagged protein on freshly decorated antibody surfaces and the same protein after more then 90 runs (data not shown). The binding capacity of the anti-RGS-His antibody versus the anti-penta-His antibody was significantly more altered. After 24 hours of interaction measurements and

regeneration steps, a ~60% reduction of the SPR signal (RU) was observed for the anti-RGS-His antibody. Still, the dissociation kinetics were unchanged (k_d (first run) = 3×10^{-5} s^{-1}, k_d (run after 24 h) = 3.2×10^{-5} s^{-1}). In contrast, the capacity of the anti-penta-His antibody surface was only reduced by ~25% with also unchanged off rates. The difference in binding capacity after more than 90 cycles of injections and regenerations most likely results from differences in aging of the antibodies under the regeneration conditions applied. Using milder regeneration conditions (i.e., higher pH), the stability of the antibody on the surface might be less impaired, thus allowing to perform even more injection cycles.

42.5 Troubleshooting

42.5.1 Covalent Coupling of Antibodies

The standard buffer for NHS/EDC-mediated covalent coupling via primary amines (free N termini or lysine residues) is 10 mM sodium acetate (pH 4–6). It is obligatory that primary amines (i.e. TRIS-containing buffers) are not present in the coupling buffer. Other options are phosphate or MES-buffers (2-(*N*-Morpholinoethanesulfonic acid)) in the same pH range.

42.5.2 Running Buffers

In some cases, the running buffer has to be optimized in order to reduce non-specific binding or electrostatic attraction to the dextran surface. These effects can be diminished by adding CM-dextran (i.e. 1 mg/ml or more) in the running buffer or by increasing the ionic strength (up to 500 mM NaCl). Furthermore, bovine serum albumin (e.g. 1 mg/ml or more) can be used to prevent adhesion of analyte, especially at low concentrations, to the sample tubes and the flow system of the instrument.

42.5.3 Regeneration of Antibody Surfaces

A general rule for regeneration of the antibody surface is to start with mild conditions and, if necessary, increase to harsher conditions. When using high-pH glycine-HCl (pH 2.5), continue with several short injections. Lowered pH can be tried to optimize the efficiency of the regeneration also by performing short injections (10–15 s) with 100 mM phosphoric acid (pH not adjusted). Short injections with up to 0.05% SDS may help to complete the regeneration. After using SDS

(Sodium dodecyl sulphate), apply several injections with running buffer to remove the residual detergent.

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