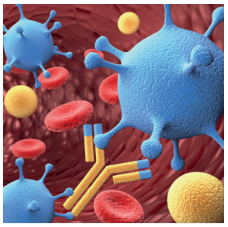
HORIBA Scientific

QVQ Quality in Antibodies

20 YEARS 1819-2019 HORIBA JOBIN YVON

Surface Plasmon Resonance imaging (SPRi)

Production of a single domain antibody Q17c directed against recombinant HER2 protein and its binding study by Surface Plasmon Resonance imaging technology



Application Note

Pharmaceuticals SPRi 42

Karen Mercier[1], Raimond Heukers[2] and Chiraz Frydman[1]

[1]HORIBA Scientific, Palaiseau, France; [2]QVQ Holding B.V., Utrecht, The Netherlands

This application note shows how binding kinetics of single-domain antibodies can be studied with SPRi technology. QVQ produces single-domain antibodies from camelids (sdAbs or VHH). Like conventional Abs, sdAbs are able to bind specific epitopes with high binding affinity. The small size of sdAbs allows for enhanced tumor penetration and fast blood clearance, features that are favorable for *in vivo* imaging applications. Here, the binding of the anti-HER2 sdAb Q17c to recombinant HER2 protein was assessed using Surface Plasmon Resonance imaging (SPRi). An optimization of immobilization conditions was performed for Q17c and its specific antigen HER2 using a single SPRi-Biochip.

Key words: Single domain antibody (sdAb), VHH, HER2/ErbB2, Surface Plasmon Resonance imaging (SPRi), affinity.

Introduction

Since their discovery one quarter century ago by Professor Raymond Hamers at the Vrije Universiteit Brussel (VUB)[1], single domain antibodies from camelids (sdAbs or VHH, also referred to as Nanobodies®) are considered as the "third generation" of antibodies after conventional monoclonal antibodies (mAbs) and antibody fragments (Fab and scFv)[2]. sdAbs are the variable domains of a particular class of heavy chain-only antibodies found in animals from the *camelidae* family (i.e. camels, llamas and alpacas) (Figure 1). These heavy chain-only antibodies (HcAbs) are devoid of light chains and undergo normal selection and maturation by the animals' immune system. For this reason, HcAbs, and therefore the sdAbs, can exhibit high affinities (nM range) and serum stability.

With a molecular weight of only 12-15 kDa (peptide-chain of about 110 amino acids long), sdAbs are much smaller than common antibodies (150-160 kDa) which are composed of two heavy chains and two light chains, and are even smaller than Fab fragments (~ 50 kDa) and single-chain variable fragments (scFv, ~ 25 kDa). sdAbs have molecular dimensions of approximately 4 x 2.5 x 3.5 nm whereas a full-sized antibody has estimated molecular dimensions of 15 x 7 x 3.5 nm.

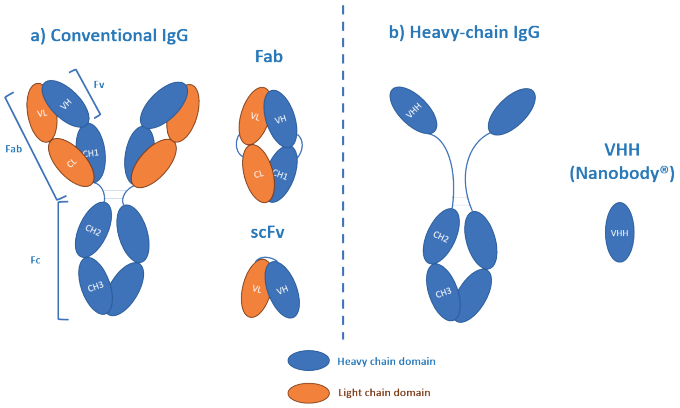


Figure 1: Schematic diagram of conventional (a) and heavy-chain (b) antibodies and fragments thereof.

Because of their small size (10-fold smaller than mAbs), they are differentiated from conventional antibodies with respect to straightforward selection, production and engineering, but also enhanced tissue penetration and a rapid blood clearance.

Thus far, many of such sdAbs (more than 120) have been developed and studied for their use in a wide range of applications, including, but not limited to imaging,

[1] Hamers-Casterman C. et al. (1993) Naturally occurring antibodies devoid of light chains, Nature 363 :446-448
[2] Arbabi-Ghahroudi M. (2017) Camelid Single-Domain Antibodies: Historical Perspective and Future Outlook, Front. Immunol. 8:1589

diagnostics and therapy (oncology and infectious-, inflammatory- and neurodegenerative diseases[3]), consumer products and chromatography.

The first VHH that entered phase 1 of clinical trials in 2007, has now reached the clinic under the name of Caplacizumab (Cablivi), a bivalent anti-blood glycoprotein vWF (von Willebrand Factor) nanobody to control platelet aggregation and rare blood clotting disorders[4].

Beyond medical applications, applications in biotechnology, like biosensor areas, are also emerging. sdAbs contain structural properties that are well-adapted for assays development in biosensing. One is that the quantity of molecules immobilized onto surfaces of µm scale can be significantly higher with small sdAbs compared to whole antibodies, leading to an enhanced detection sensitivity[5].

Contrary to conventional antibodies, sdAbs are single domains that don't require pairing with a light chain, and sdAbs are able to refold to a functional state after denaturation. These features make sdAbs more heat-resistant and stable towards detergents and urea.

One QVQ VHH targeting the tumor-related receptor HER2, commercially designated as Q17c, was studied using Surface Plasmon Resonance imaging (SPRi) technology using the XelPleX™ system. To follow binding kinetics between Q17c and HER2, immobilization of both molecules in different conditions (immobilization system, pH, concentration) was tested on a single SPRi-Biochip™. Affinity of the model could be determined thanks to EzFit™ software.

Production of a single domain antibody Q17c directed against recombinant HER2 protein

QVQ, founded in 2010, is a CRO for custom development of sdAbs with its own pipeline of VHH molecules for research and imaging. QVQ produces VHH in bacteria *(E.Coli)* and yeast *(S.cerevisiae).* For site directional conjugation (without loss of functionality) to dyes, chelators or matrixes like chips, these sdAbs can be equipped with a C-terminal tag (C-Direct tag) containing an extra free cysteine.

Q17 (QVQ product) is a recombinant monoclonal sdAb/VHH that recognizes the extracellular domain of HER2 protein. HER2, also known as epidermal growth factor receptor 2, ErbB2 or Neu, is a single membrane spanning receptor tyrosine kinase. HER2 is one of the 4 ErbB family members and is activated by heterodimerization. It is regarded as a proto-oncogen and overexpression of HER2 is observed in a large number of cancers and therefore serves as a target for tumor-imaging and therapy. Q17 was generated by immunization with MCF-7 tumor cells and selection on recombinant ectodomain[6]. This VHH has been validated for binding to recombinant HER2 ectodomain (ELISA), HER2 on cells (IF, cell-based ELISA, immuno-EM) and HER2 in tumors (*in vivo* optical molecular imaging, image-guided surgery)[6, 7, 8]. The anti-HER2 VHH Q17 was recloned into the pyQVQ11 yeast production vector, which provided Q17 with a C-terminal cysteine-containing C-Direct tag (generating Q17c). This cysteine allows for directional coupling to chips, enzymes, biotin, chelators, fluorescent dyes and other molecules without compromising the binding characteristics of the sdAb. Q17c was produced in *S. cerevisiae* and was purified by affinity chromatography (protein A) and size exclusion chromatography. The purified antibody was stored in PBS and was checked for correct size and purity (SDS-PAGE), concentration (UV-VIS) and integrity (binding to HER2 in ELISA).

In the following SPRi binding study of HER2 and Q17c, the two molecules were immobilized on a same type of SPRi-Biochip™. The multiplexing capability of SPRi technology enables a fast optimization of the immobilization step using a variety of conditions (immobilization system, pH, concentration).

Binding study between HER2 recombinant protein and a specific single domain antibody Q17c by Surface Plasmon Resonance imaging technology

1. Materials and methods

1.1. Immobilization on a SPRi-Biochip™ CH-HD

The SPRi-Biochip™ CH-HD is made of a self-assembled monolayer of reactive polyoxide ethylene glycol at high density. The SPRi-Biochip™ CH-HD was activated using an EDC/sulfo-NHS solution in preparation for amine coupling.

The two proteins, Q17c and HER2, respectively framed in blue and in red on Figure 2, were immobilized using two spotting systems: the SPRi-Arrayer™ and the SPRi-CFM, in order to determine the optimal approach of spotting. The two spotting techniques were tested on the same sensor chip.

The SPRi-Arrayer™ is an automatic and compact system used in the HORIBA Scientific SPRi platform for immobilizing ligands in a multiplex format onto a SPRi-Biochip™ or a SPRi-slide™. This versatile instrument uses direct contact spotting and is suitable for printing on bare or 2D-functionalized SPRi-Biochips™ or SPRi-Slides™. Contact spotting allows for fast and flexible microarray printings. The diameter of the printing pin can be adapted to the number of required spots in the matrix. Here, the diameter of the printing pin was 500 µm.

The SPRi-CFM uses continuous flow deposition to immobilize up to 48 molecules in a single printing run. Three printing runs can be performed on a single biochip (and up

[3] Harmsen M. M. et De Haard H. J. (2007) Properties, production, and applications of camelid single-domain antibody fragments, Appl. Microbiol. Biotechnol. 77:13-22

[4] https://www.ema.europa.eu/en/medicines/human/EPAR/cablivi

[5] Saerens D. *et al.* (2008) Antibody Fragments as Probe in Biosensor Development, Sensors 8:4669-4686

[6] Kijanka *et al.* (2013) Rapid optical imaging of human breast tumor xenografts using anti-HER2 VHHs site-directly conjugated to IRDye800CW for imaging-guided surgery, Eur J Nucl Med Mol Imaging, 40(11):1718-29

[7] Kijanka *et al*. (2016) Optical imaging of pre-invasive breast cancer with a combination of VHHs targeting CAIX and HER2 increases contrast and facilitates tumor characterization, EJNMMI Res., 6:14

[8] Kijanka *et al*. (2017) A novel immuno-gold labeling protocol for nanobody-based detection of HER2 in breast cancer cells using immune-electron microscopy, J Struct Biol, 199:1-11

to 144 spots per chip can be generated). The microfluidic immobilization improves the spot homogeneity and gives a higher immobilization level. For this experiment, the flow rate of the SPRi-CFM was set to 15 µL/min and the contact time to 30 minutes.

Prior to the spotting with the SPRi-Arrayer™, Q17c protein and its respective negative control (irrelevant VHH) were prepared in a 10 mM PBS buffer at pH7.4 at three different concentrations following a two-fold dilution series: 7 µM, 3.5 µM and 1.75 µM.
The two VHHs at each concentration were immobilized in quadruplicate on the SPRi-Biochip™ CH-HD activated surface.
HER2 protein and its respective negative control (hIgG) were prepared in 10 mM PBS buffer at pH7.4 at a concentration of 1 µM.
An HFR positive control antibody was also prepared in 10 mM PBS buffer at pH7.4 at a concentration of 7 µM to check for chip reactivity.
HER2, hIgG and HFR Ab were immobilized in 6 replicates on the SPRi-Biochip™ CH-HD activated surface.

Prior to the printing with the SPRi-CFM, Q17c protein and its respective negative control (irrelevant VHH) were prepared in 10 mM sodium acetate buffer at two different pHs (4.0 and 5.0). For each buffer, the two VHHs were prepared at three different concentrations following a two-fold dilution series: 667 nM, 333 nM and 167 nM.
HER2 protein and its respective negative control (hIgG) were prepared in 10 mM sodium acetate buffer at two different pHs (4.0 and 5.0) at a concentration of 130 nM.

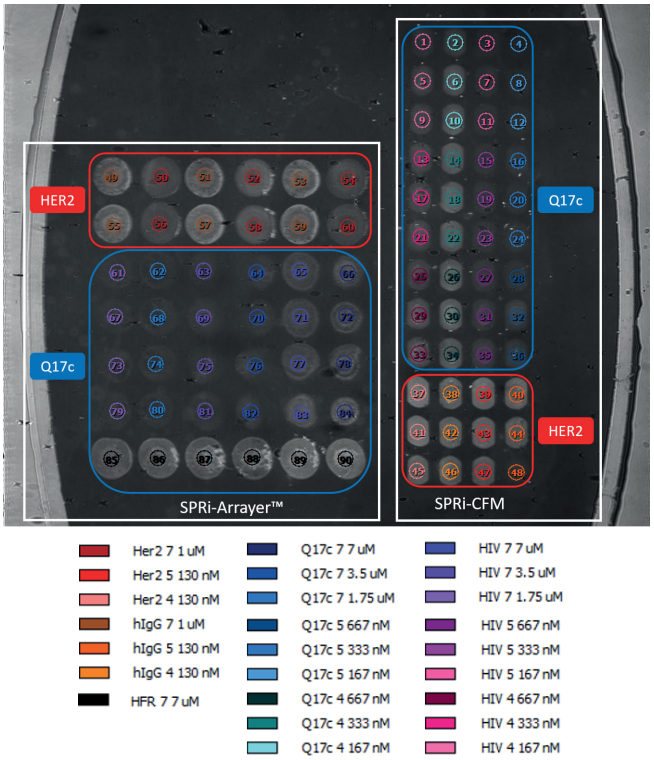


Figure 2: Image of the printed SPRi-Biochip™ CH-HD.

Each preparation of Q17c, irrelevant VHH, HER2 and hIgG was immobilized in triplicates on the SPRi-Biochip™ CH-HD activated surface.
After the immobilization procedure, the SPRi-Biochip™ was blocked using 1 M ethanolamine.
Figure 2 shows an SPR image of the printed SPRi-Biochip™ CH-HD.

1.2. SPRi experimental details

The printed SPRi-Biochip™ was loaded into the XelPleX™ system. The interactions were monitored using EzSuite software. The running buffer was 10 mM PBS pH 7.4 and the working temperature was set to 25°C.
Then, 200 µL of Q17c and then of HER2 were injected into the fluidic system at a flow rate of 50 µL/min.
Q17c was injected at four increasing concentrations, following a two-fold dilution series: 17, 33.5, 67 and 134 nM.
HER2 was injected at four increasing concentrations, following a three-fold dilution series: 2, 6, 17 and 52 nM.
A regeneration cycle was performed between each protein injection by flowing a 0.1 M glycine-HCl pH2.0 solution with a contact time between 30 seconds and 4 minutes.

2. Results and discussion

2.1. Q17c injections

2.1.1. Q17c protein binding monitoring

SPRi technique allows an image display of the binding response. Figure 3 corresponds to a difference image saved during the dissociation phase of Q17c injection at 134 nM. Specific binding was observed on the spots of HER2 protein whatever the immobilization conditions while injecting Q17c at 134 nM.

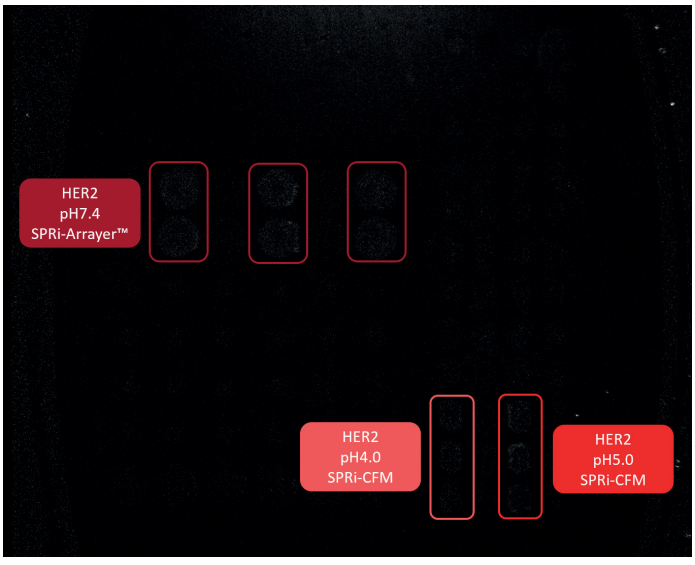


Figure 3: Difference image obtained during the rinsing step of Q17c injection at 134 nM

2.1.2. Optimization of the immobilization conditions for HER2 protein

HER2 protein was immobilized at three different pH levels and using two different immobilization systems on a single biochip in order to evaluate and select the best immobilization conditions.

The large working area of the SPRi-Biochip™ and the multiplexing capabilities of SPRi systems allow for the immobilization of different molecules and/or testing of different immobilization conditions on a single biochip.

Specific Q17c protein binding responses retained on the spots of HER2 protein are represented in Figure 4. Q17c protein was injected at 4 different concentrations following a two-fold dilution series: 17, 33.5, 67 and 134 nM. For each injected concentration, the specific binding responses were measured during the dissociation phase of Q17c injections at the same time point. Values are reference-subtracted and spot-averaged. Highest binding responses were obtained for HER2 immobilized at 1 µM prepared in 10 mM PBS buffer at pH7.4 and using contact spotting.

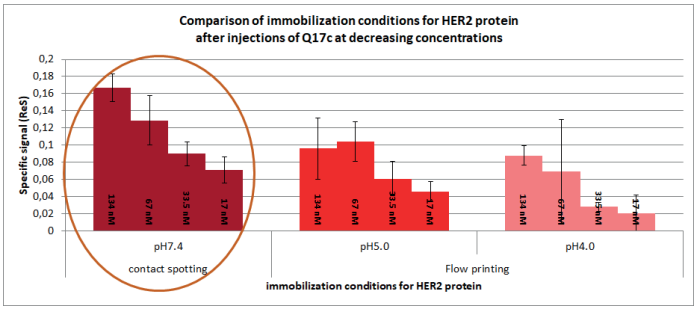


Figure 4: Specific responses retained for HER2 protein immobilized at three different pH levels and using two different immobilization systems after the injections of Q17c at 17, 33.5, 67 and 134 nM.

2.1.3. Kinetic analysis of HER2 (ligand) / Q17c (analyte) interactions

The kinetic curves for HER2 protein immobilized in the optimal conditions were analyzed using the EzFit software. This software is suitable for processing multiplexed data intuitively. The SPRi signal obtained on reference spots (i.e. hIgG) were used for referencing. Then, the data were fitted locally (i.e. Rmax (maximum of reflectivity) different for each curve) using a 1:1 interaction model (see Figure 5; orange curves correspond to the 1:1 model fits). The affinity between HER2 immobilized and Q17c injected is estimated around 0.7 nM. The study of the molecules in the reverse approach immobilizing Q17c and injecting HER2 was also studied in the next part.

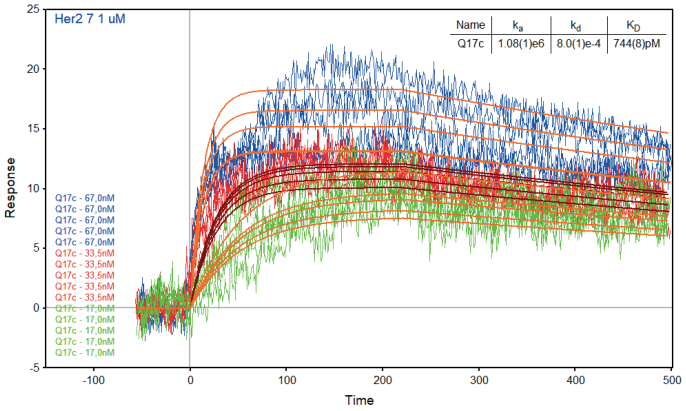


Figure 5: Kinetic analysis of HER2 (ligand) immobilized at 1 µM in 10 mM PBS pH7.4 using contact spotting (optimal conditions) / Q17c (analyte) interactions (local fits using a 1:1 interaction model; Q17c injection at 134 nM is excluded in the analysis)

2.2. HER2 injections

2.2.1. HER2 protein binding monitoring

Q17c protein was immobilized using two different immobilization systems, at three different pH levels (4.0, 5.0 and 7.4) and at six different concentrations (167, 333 and 667 nM with fluidic printing and 1.75, 3.5 and 7 µM with contact spotting) on a single biochip (Figure 2).

Figure 6 shows the averaged and reference-subtracted kinetic curves obtained for Q17c immobilized in the different conditions after the injection of HER2 at 52 nM.

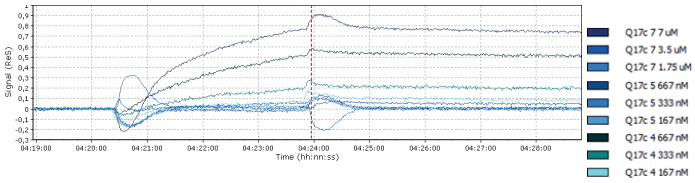


Figure 6: Averaged and reference-subtracted kinetic curves obtained for Q17c immobilized in different conditions on the functionalized SPRi-Bio-chip™ CH-HD after the injection of HER2 at 52 nM.

Specific binding monitoring was observed while injecting HER2 protein at 52 nM for Q17c immobilized at:

- 7 µM, in 10 mM PBS pH7.4 using contact spotting and
- pH4.0 whatever the concentration using flow printing.

No binding was observed for Q17c immobilized in the other conditions.

Some strange “bumps-like” can be observed on the kinetic curves. These “bumps” occur at the beginning and at the end of the HER2 protein injection in the flow cell of the XelPleX™ system. They are due to the big difference of refractive index between the compositions of HER2 stock solution and of the experimental running buffer.

2.2.2. Optimization of the immobilization conditions for Q17c protein

Specific HER2 protein binding responses retained on the spots of Q17c are represented in Figure 7. HER2 protein was injected at 4 different concentrations following a three-fold dilution series: 2, 6, 17 and 52 nM. For each injected concentration, the specific binding responses were measured during the dissociation phase of HER2 protein injections at the same time point. Values are reference-subtracted and spot-averaged. Optimal binding responses of HER2 protein were obtained for Q17c immobilized at 7 µM in 10 mM PBS pH7.4 using contact spotting system.

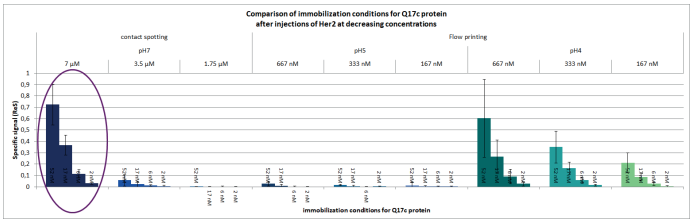


Figure 7: Specific responses retained for Q17c protein immobilized using two different spotting systems, at three different pH levels and at six different concentrations after the injections of HER2 protein at 2, 6, 17 and 52 nM.

2.2.3. Kinetic analysis of Q17c (ligand) / HER2 (analyte) interactions

The kinetic curves obtained for Q17c protein immobilized in the optimal conditions after injections of HER2 protein at 6, 17 and 52 nM were analyzed using the EzFit software. The SPRi signal obtained on reference spots (i.e. irrelevant VHH) were used for referencing. Then, the data were fitted locally (i.e. Rmax (maximum of reflectivity) different for each curve) using a 1:1 interaction model (see Figure 8; orange curves correspond to the 1:1 model fits). The affinity between Q17c immobilized and HER2 injected is estimated around 0.7 nM as in the reverse approach presented here in the part 1 of "Results and Discussion" paragraph (HER2 immobilized and Q17c injected).

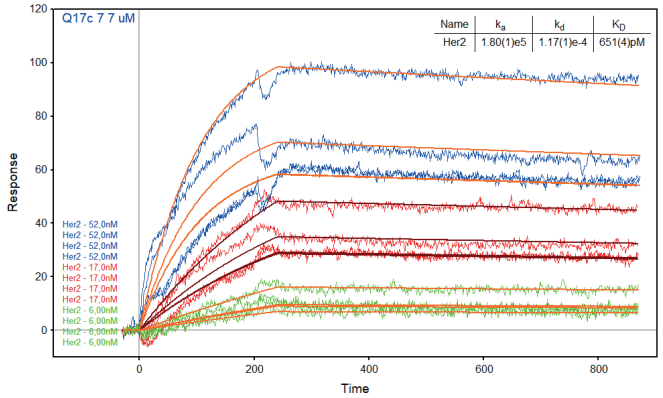


Figure 8: Kinetic analysis of Q17c (ligand) immobilized at 7 µM in 10 mM PBS pH7.4 using contact spotting (optimal conditions) / HER2 (analyte) interactions (local fits using a 1:1 interaction model; HER2 injection at 2 nM is excluded in the analysis)

Conclusion

Q17c, a camelid-derived single-domain antibody targeting the extracellular domain of the tumor-related receptor HER2 was studied using Surface Plasmon Resonance imaging thanks to the XelPleX™ system.

Based on the multiplexing feature of SPRi technology, the binding between Q17c and HER2 was analysed immobilizing either one of the two molecules on a single SPRi-Biochip™. This multiplexing capability was further exploited by employing immobilization optimization using a variety of conditions. More specifically, Q17c was immobilized under 9 different conditions, i.e. two different spotting systems, three different pH levels and six different concentrations of Q17c. This leads to a rapid optimization of immobilization conditions and a biochip design that could be used for the follow up experiments. Indeed, during this 5 hours-experiment, 12 different conditions and 720 sensorgrams were generated using a single biochip. This data shows that one can simply conceive a chip with multiple different VHH molecules on one biochip.

The binding affinity of Q17c for HER2 was also determined by the SPRi setup. This was done using two molecular configurations: immobilization of HER2 and injection of Q17c versus immobilization of Q17c and injection of HER2. In both cases, the binding affinity was estimated to be ~0.7 nM, which is in the range of those found for conventional antibodies and correlates well with the published value for this molecule[6].

This application note shows that single-domain antibodies molecules serve well for SPRi purposes. Also, using the SPRi setup, similar specificity and affinity values were obtained as published previously. Moreover, the small size of sdAbs, their high specificity, affinity and stability make these molecules particularly well adapted for use in biosensors like the SPRi technology.

HORIBA Scientific

info.sci@horiba.com

USA: +1 732 494 8660
UK: +44 (0)1604 542 500
China: +86 (0)21 6289 6060
Taiwan: +886 3 5600606
France: +33 (0)1 69 74 72 00
Italy: +39 06 51 59 22 1
India: +91 (80) 4127 3637
Brazil: +55 (0)11 2923 5400

www.horiba.com/scientific

Germany: +49 (0) 6251 8475 0
Japan: +81(75)313-8121
Singapore: +65 (6) 745-8300
Other: +33 (0)1 69 74 72 00