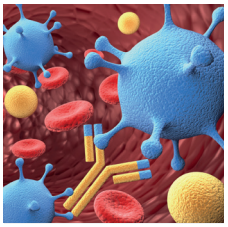
HORIBA Scientific

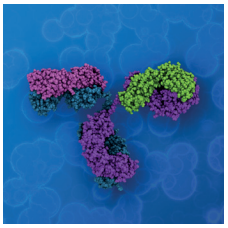
Diaclone

SynAbs Singular Antibodies

Surface Plasmon Resonance imaging (SPRi)

Production of a rescued recombinant monoclonal antibody directed against a steroid hormone and its binding study thanks to Surface Plasmon Resonance imaging technology





Application Note

Biology
SPRi 40

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A monoclonal antibody (mAb) highly specific to a steroid hormone (undisclosed) of 290 Daltons was analyzed thanks to SPRi technology. This application note highlights the sensitivity of the XelPleX™ system for the detection of small molecules.

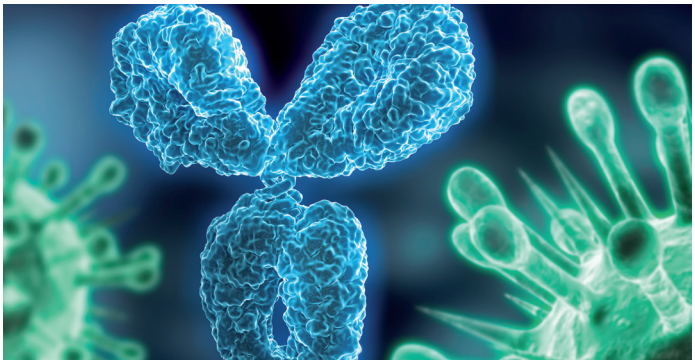
Key words: monoclonal antibody (mAb), recombinant antibody, steroid hormone, antibody rescue, Surface Plasmon Resonance imaging (SPRi), affinity.

Introduction

Monoclonal antibodies represent therapeutics of great importance in clinical medicine and are used to treat various conditions such as cancer, inflammatory disease, organ transplantation, cardiovascular disease, infection, respiratory disease and even ophthalmologic disease.
Monoclonal antibodies have also been widely used in biological research and in-vitro diagnosis (IVD) for over 40 years. Köhler and Milstein[1] were the first to generate a monoclonal antibody in mice in 1975 using the hybridoma technique when they fused single B-cells from the spleen of a mouse to myeloma (cancer) cell line.

The main feature of monoclonal antibodies is their mono-specificity. They bind to a single epitope of their target as they are produced by a single B-lymphocyte clone.
This method of producing monoclonal antibodies is still the standard. However, in recent years, alternative methods of molecular biology linked with recombinant technologies have appeared, and new recombinant versions of the antibody can be validated for specificity to the target. This engineering approach has the big advantage of avoiding the loss of precious clones that do not have reliable stability.

Diaclone has over 30 years of expertise in mAb development by hybridoma technique and has a team of molecular biologists dedicated to antibody rescue and genetic engineering, as well as phage display technology. To secure some high valued antibodies obtained by the hybridoma technique and to avoid troubles like hybridoma instability, molecular biology is very useful in the optimization of the coding sequence and in the reformatting of some promising antibodies



At the end of the engineering process, the recombinant antibody needs to be tested against the target for validation. The XelPleX™ system from HORIBA Scientific is a perfect tool for this validation step. This technology belongs to a new generation of label-free interaction analysis platforms and is capable of accurate affinity determination in a multiplex mode.

A monoclonal antibody developed at Synabs SA (Belgium) by a new hybridoma technology for guinea pig mAb has been rescued and produced by Diaclone. That monoclonal antibody was highly specific to a steroid hormone (undisclosed) of 290Da molecular weight and was analysed using the XelPleX™ system.

The affinity of the hormone / antibody model, as well as the limit of detection of the hormone, was determined. Thanks to the array-based format of the SPRi sensor chips, it is easy to extend these results to multiple interactions and to quickly integrate the XelPleX™ system into the validation of rescued recombinant monoclonal antibodies from Diaclone.

20 YEARS 1819-2019 HORIBA JOBIN YVON

[1] Köhler G. and Milstein C., 1975, Continuous cultures of fused cells secreting antibody of predefined specificity, Nature 256, 495-497.

Rescue process of a monoclonal antibody directed against a 290Da steroid hormone

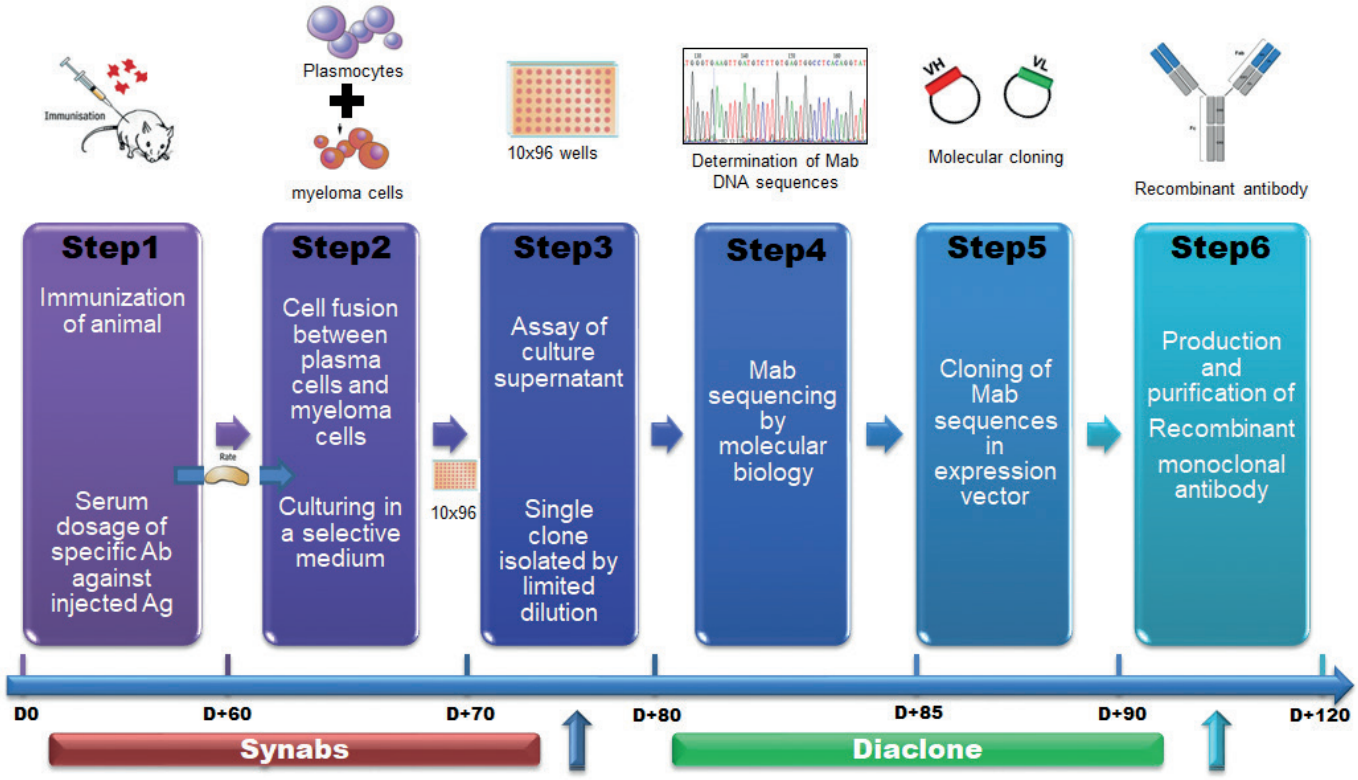


Figure 1: Scheme of monoclonal antibody rescue and recombinant production process

As seen in Figure 1, the 3 first steps of the mAb development process are immunization, followed by cell fusion, and then cell cloning to obtain monoclonal clones by limited dilution. The rescue step begins by performing molecular biology experimentations: cell pellets from a hybridoma mAb candidate is used to obtain Heavy (VH) and Light (VL) nucleotide sequences.

The VL and VH sequences are then sub-cloned in expression vectors which are used for transient transfection in mammalian cells to produce the recombinant antibody. After 14 days of production, the antibody is purified by affinity chromatography.

Among recombinant monoclonal antibodies rescued by Diaclone according to the process described above, one well-characterized antibody binds specifically to a 290 Daltons steroid hormone. Thanks to the XelPleX™ system (based on SPRi technology), this was evaluated.

Binding study between a 290 Daltons hormone and a specific recombinant antibody by Surface Plasmon Resonance imaging (SPRi) technology

Materials and methods

Antibodies immobilization using SPRi-CFM on a SPRi-Biochip™ CMD-200MD

The SPRi-Biochip™ CMD-200MD is a hydrogel made of carboxymethyl dextran. The SPRi-Biochip™ CMD-200MD is characterized by a thickness of 200nm and a medium density. It is activated using an EDC/NHS solution in preparation for amine coupling.

Prior to the immobilization process, the antibody of interest was prepared at a concentration of 0.7µM in 10mM sodium acetate at pH4.0 and at pH5.0. These antibody preparations were used to immobilize the antibody on the SPRi-Biochip™-activated surface using the SPRi-Continuous Flow Microspotter (SPRi-CFM).

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 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.

Two reference antibodies were also immobilized:

- a Diaclone negative control antibody which was prepared at the same concentration of 0.7µM in 10 mM sodium acetate buffer at pH5.0 only, for referencing purposes;
- and an HFR positive control antibody which was prepared at 0.7µM in 10mM sodium acetate at pH4.0, to check for chip reactivity.

Each antibody was immobilized in triplicates (Figure 2).

After the immobilization procedure, the SPRi-Biochip™ was blocked using 1 M ethanolamine.

Figure 2: Image of the printed SPRi-Biochip™ CMD-200MD. Spots of the antibody of interest immobilized at pH4.0 are framed in pink, spots of the antibody of interest immobilized at pH5.0 are framed in purple, negative control spots are framed in green and positive control spots are framed in blue.

Antibodies immobilization using SPRi-Arrayer™ on a SPRi-Biochip™ CH-LD

The SPRi-Biochip™ CH-LD is made of a self-assembled monolayer of polyoxyde ethylene glycol. A low density (LD) of reactive polyoxyde ethylene glycol is mixed together with non-reactive polyoxide ethylene glycol. The SPRi-Biochip™ CH-LD was activated using an EDC/sulfo-NHS solution in preparation for amine coupling.

The antibody of interest and a negative control antibody (prepared in 10mM sodium acetate at pH 5.0) were immobilized in replicates on the SPRi-Biochip™-activated surface at a concentration of 7µM using the SPRi-Arrayer™ (Figure 3).

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 500µm.

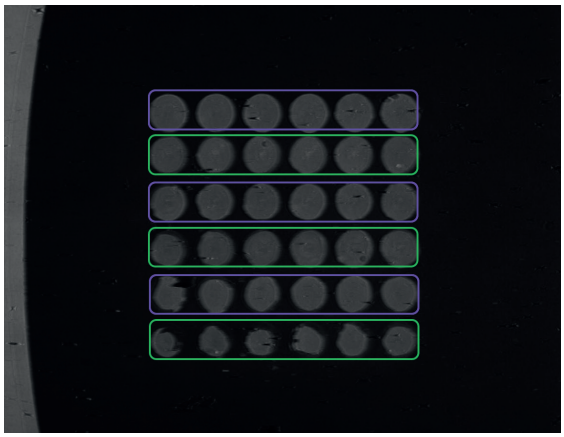


Figure 3: Image of the printed SPRi-Biochip™ CH-LD. Spots of the antibody of interest are framed in purple and negative control spots are framed in green.

After the immobilization procedure, the SPRi-Biochip™ was blocked using 1M ethanolamine.

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, 200µL of the hormone were injected into the fluidic system at a flow rate of 50µL/min. The hormone was injected at six increasing concentrations, following a three-fold dilution series: 0.5, 1.5, 4.6, 14, 41 and 125nM. A regeneration cycle was performed between each hormone injection by flowing a 0.1M glycine-HCl pH2.0 solution with a contact time of 30 seconds.

Results and discussion

Optimization of the immobilization buffer pH for the antibody of interest

The antibody of interest was immobilized using two different immobilization buffers on a single biochip in order to evaluate and select the best immobilization buffer.

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.

Figure 4 compares the averaged and reference-subtracted kinetic curves obtained for the antibody of interest immobilized using 10mM sodium acetate buffer at pH4.0 and at pH5.0.

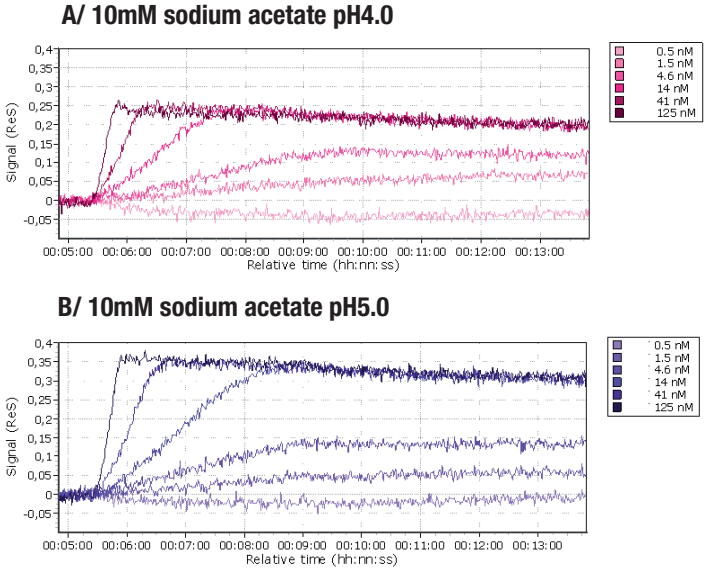


Figure 4: Averaged and reference-subtracted kinetic curves obtained for the antibody of interest immobilized using two different immobilization buffers on the functionalized SPRi-Biochip™ CMD-200MD after the injections of the hormone at 0.5, 1.5, 4.6, 14, 41 and 125nM. A/ immobilization buffer is 10mM sodium acetate at pH4.0 (left); B/ immobilization buffer is 10mM sodium acetate at pH5.0 (right).

Specific binding monitoring was observed for the antibody of interest while injecting the hormone at different concentrations for the two immobilization buffer pH levels.

Specific hormone binding responses retained on the spots of the antibody of interest are represented in Figure 5. For each injected concentration, the specific binding responses were measured during the dissociation phase of hormone injections at the same time point. Values are reference-subtracted and spot-averaged.

Validation of Antibody Specificity

The unique binding of a monoclonal antibody characterizes its specificity. Figure 6 compares averaged and reference-subtracted kinetic curves obtained for the antibody of interest immobilized using 10mM sodium acetate buffer at pH5.0 after the injections of the hormone and a structural analogue. Both molecules were injected at the same concentrations following a three-fold dilution series (from 125 to 0.5nM).

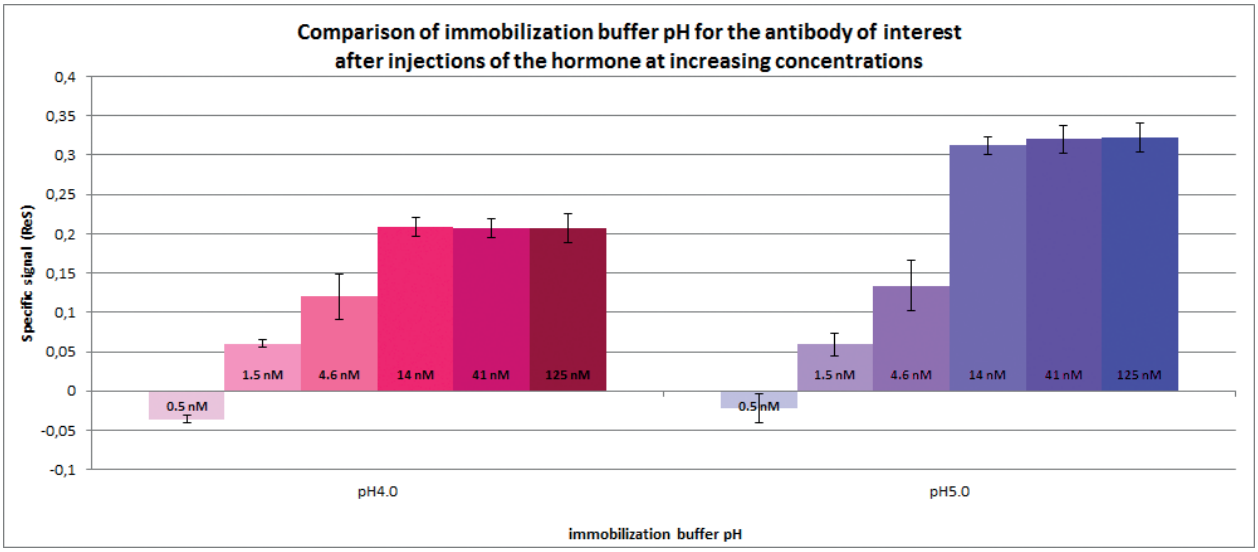


Figure 5: Specific responses retained for the antibody of interest immobilized at two different pH levels after the injections of the hormone at 0.5, 1.5, 4.6, 14, 41 and 125nM.

For the highest concentrations of the hormone, binding responses obtained were higher for the immobilization buffer at pH5.0 than for the immobilization buffer at pH4.0. According to the data analysis, we can conclude that:

- ✓ The optimal immobilization buffer pH for the antibody of interest is pH5.0.
- ✓ The limit of detection of the hormone is 1.5nM (~ 0.45ng/mL).
- ✓ The saturation level is reached at 14nM (same signal observed for higher concentrations).

A regeneration step was performed between each molecule injection using 0.1M glycine-HCl pH2.0 solution with a contact time of 30 seconds.

Specific binding responses were observed for the antibody of interest while injecting the hormone at different concentrations, whereas no binding response was observed while injecting the analogue at the same concentrations. From this, it can be concluded that the antibody of interest is specific to the 290 Daltons steroid hormone.

A/ hormone injections

B/ analogue injections

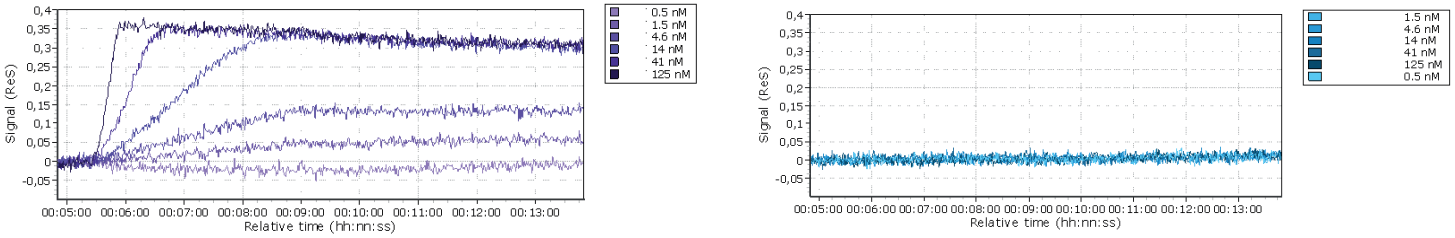


Figure 6: Averaged and reference-subtracted kinetic curves obtained for the antibody of interest immobilized at pH5.0 on the functionalized SPRi-Biochip™ CMD-200MD. A/ injections of the hormone at 0.5, 1.5, 4.6, 14, 41 and 125nM (left); B/ injections of the analogue at 0.5, 1.5, 4.6, 14, 41 and 125nM (right).

Kinetic analysis of the antibody / hormone interactions

The kinetic curves were analyzed using the EzFit software. This software is suitable for processing multiplexed data intuitively. The SPRi signal obtained on reference spots (i.e. Diaclone negative control antibody) were used for referencing. Then, the data was fitted locally (i.e. Rmax (maximum of reflectivity) different for each curve) using a 1:1 interaction model (see Figure 7; orange curves correspond to the 1:1 model fits).

To avoid mass transport effect, the ligand density can be reduced, or the flow rate can be increased.

The affinity between the antibody and the hormone is estimated around 0.2nM and has a high affinity interaction model.

This model was also tested by using a contact spotting system to immobilize the antibody of interest on a CH-LD surface chemistry to evaluate the performance of this combination “SPRi-Arrayer™ / 2D chemistry”.

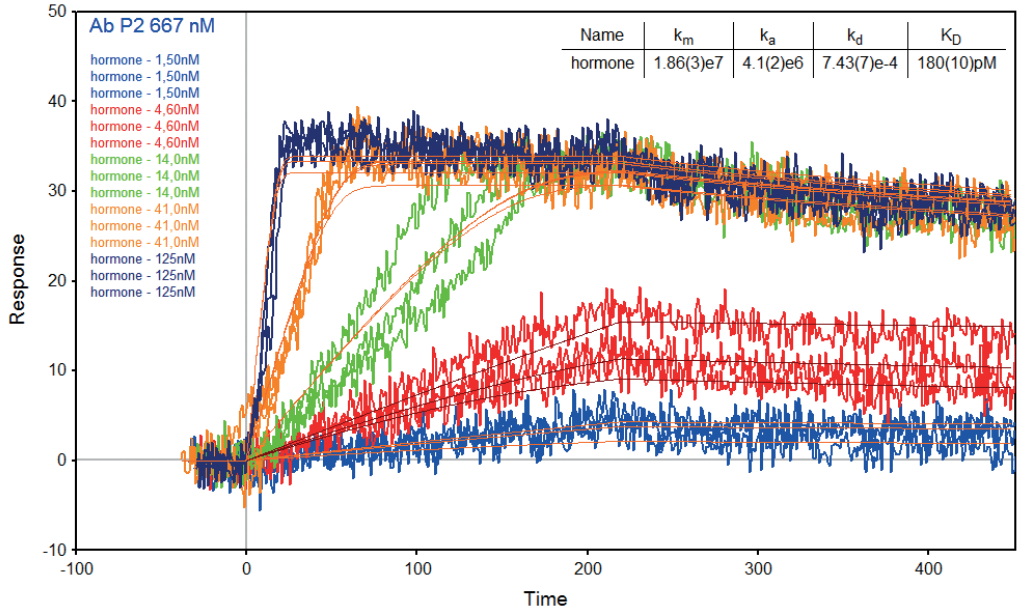


Figure 7: Kinetic analysis of the antibody / hormone interactions
(local fits using a 1:1 interaction model; hormone concentration of 0.5 nM is excluded in the analysis)

The kinetics curves obtained showed a more linear binding profile rather than an exponential one as used in the standard fitting model. This corresponds to mass transport limited kinetics. Directly after the analyte injection starts, the binding of the analyte to the ligand is faster than diffusion, creating a shortage of analyte at the surface. This mass transport limitation was integrated in the fitting model by the addition of a km constant in the results table of Figure 7.

Antibodies immobilization comparison between flow printing on 3D chemistry and contact spotting on 2D surface chemistry

Figure 8 compares averaged and reference-subtracted kinetic curves obtained for the antibody of interest immobilized using the SPRi-CFM on a SPRi-Biochip™ CMD-200MD and the SPRi-Arrayer™ on a SPRi-Biochip™ CH-LD.

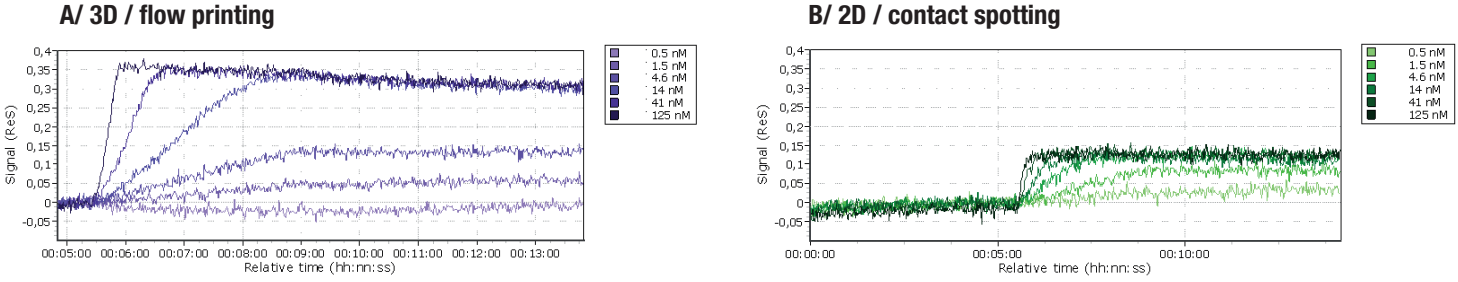


Figure 8: Averaged and reference-subtracted kinetic curves obtained for the antibody of interest, immobilized in two different ways, after the injections of the hormone at 0.5, 1.5, 4.6, 14, 41 and 125 nM. A/ immobilization system is the SPRi-CFM on a SPRi-Biochip™ CMD-200MD (left); B/ immobilization system is the SPRi-Arrayer™ on a SPRi-Biochip™ CH-LD (right).

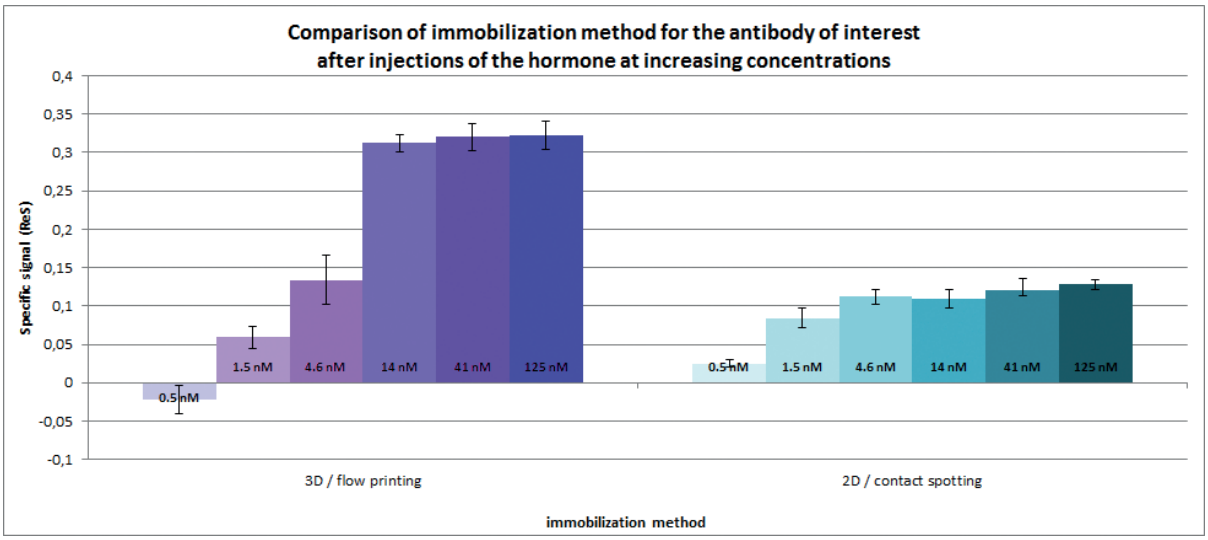


Figure 9: Specific responses retained for the antibody of interest immobilized using two different immobilization methods after the injection of the hormone at 0.5, 1.5, 4.6, 14, 41 and 125nM.

Specific binding monitoring was observed for the antibody of interest while injecting the hormone at different concentrations for the two immobilization methods. Specific hormone binding responses retained on the spots of the antibody of interest are represented in Figure 9. For each injected concentration, the specific binding responses were measured during the dissociation phase of hormone injections at the same time point. Values are reference-subtracted and spot-averaged.

Binding responses obtained were about 3 times higher for the flow printing on 3D chemistry than for the contact spotting on 2D surface chemistry for the 3 highest concentrations of the hormone injected.

However, using contact spotting and 2D surface chemistry, the limit of detection of the hormone is 1.5nM (~ 0.45 ng/mL). The saturation level in the case of the contact spotting on 2D surface chemistry is reached at 4.6nM. These results are correlated with the features of 3D chemistry since a hydrogel consists of a network of reactive groups, increasing the immobilization amount capacity and consequently the binding amount capacity without any steric hindrance issue.

The kinetic curves obtained in the case of contact spotting on 2D surface chemistry were analyzed using the EzFit software. The curves profile was linear, similar to the flow printing on 3D chemistry. However, a successful fit wasn't possible by integrating the mass transport limitation option. This is probably because of the low binding responses obtained in these conditions.

Conclusion

A recombinant monoclonal antibody rescued by Diaclone using molecular biology was evaluated using the new label-free interaction analysis platform from HORIBA Scientific, the XelPleX™ system. This antibody binds specifically to a 290 Daltons steroid, a low molecular weight molecule.

The limit of detection of the hormone was determined to be ~ 1.5nM (~ 0.45ng/mL). This limit of detection was obtained with a flow printing of the antibody on 3D chemistry, as well as with a contact spotting technique on 2D surface. A low molecular weight molecule is very challenging to analyze with "contact spotting / 2D surface chemistry", but the antibody of interest specific to the hormone showed that the detection of a 290 Daltons molecule is possible with this combination. This confirms that the produced antibody is of high quality.

The specificity of the antibody was also verified by flowing an analogue hormone at the same concentrations. No binding response was observed with the antibody of interest while injecting the analogue hormone in the XelPleX™ system.

The kinetic curves profile showed mass transport limited kinetics. An affinity of 0.2nM for the hormone / antibody interaction was calculated with this mass transport effect taken into account in the EzFit software by the addition of a km constant.

The XelPleX™ system performed well and it allowed for a complete analysis of the antibody of interest. Thanks to the array-based format of the SPRi sensor chips, the XelPleX™ system can easily extend its performance to multiple interactions and quickly integrate into biomolecule production processes such as Diaclone's monoclonal antibodies production process at the validation step.

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