Fidabio Clone Selection assay: Protein expression level and affinity directly in fermentation media without purification

- Include protein binding affinity in the early stage of selecting your optimal clones
- Quantify protein expression and target K_D simultaneously in crude cell matrices
- Work with native protein without the need for expressing His- or other tags

VERSION 01

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Introduction

Clone selection is an important step in the production of proteins. Key factors include protein expression level, target binding (K_D) and protein integrity (R_h).

In this application note we look at the selection of optimal clones to produce nanobodies, also known as VHHs.

VHHs are fragments of antibodies from Camelid animals and are gaining in popularity for protein research, diagnostics, and as therapeutical antibodies, given their simplicity in terms of development and production.

The VHH clones can be characterized by ELISA or, following purification, SDS-PAGE. However, these methodologies are hampered by low accuracy and low information content as well as the requirement for sample purification.

Here, we introduce a new method offering the advantage of including protein affinity as a selection criterion together with the assessment of expression levels directly in cell matrices without need for purification or expression of tags on the VHH.

In general, the challenge for assessing K_D early in the clone selection process is that the expression levels of the various candidates are unknown. In FIDA, binding interactions are monitored by measuring an absolute readout of hydrodynamic radius (R_h) in nanometers. Increased R_h is a measurement of increased binding. Binding can be the result of high expression level and/or high binding affinity.

The absolute readout in nanometers offers a correlation between the measured size and the amount of free-antigen versus bound-antigen. Therefore, the FIDA technology enables a first selection of top clone/protein candidates based on size measurements, followed by a direct assessment of K_D. The K_D is assessed by binding a known amount of fluorescently labelled antigen to the VHH content of a fixed volume of cell supernatant/cell lysate and thereafter titrating the mixture with known concentrations of un-labelled antigen. This competitive assay provides a correlation between measured size and K_D. Resolving the two correlations provides both K_D and expression level without prior purification being required.

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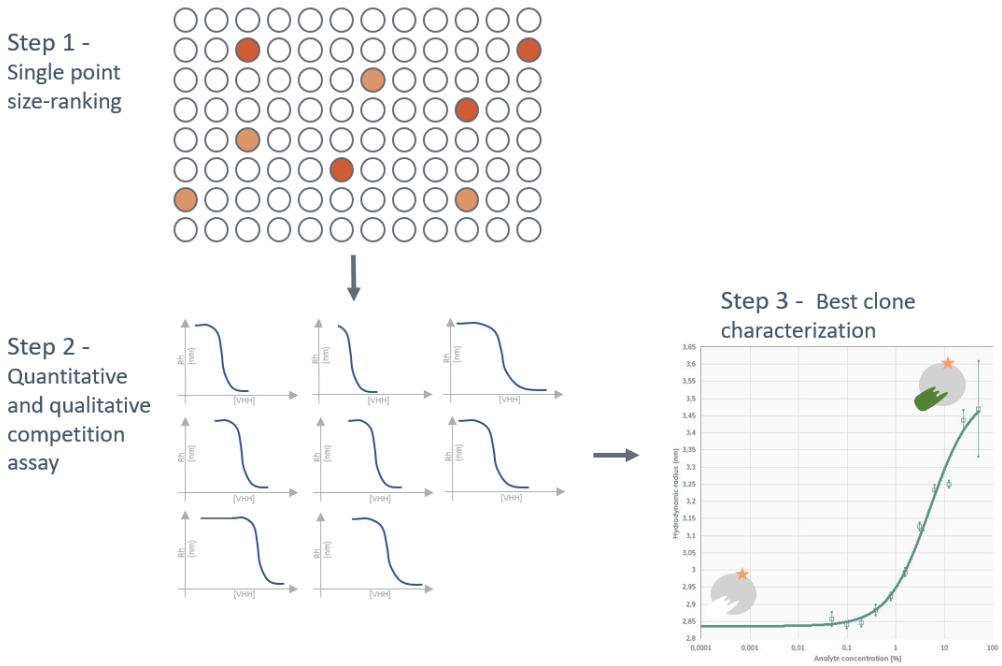

Figure 1. Schematic workflow of the Fidabio Clone Selection assay.

Material & Methods

FIDA measuring principle: Flow Induced Dispersion Analysis is a capillary-based microfluidic method, exploiting that flow rate in the center of the capillary is faster than the one at the edges of the capillary. The resulting radial concentration gradients at the front and the tail of the dispersion results in diffusion of your chosen indicator, which enables a "first principle" biophysical measurement of size. The FIDA measurement of size is broadly applicable for studying biomolecular stability, interactions, etc. To learn more, visit fidabio.com. The assay principle is illustrated in Figure 2.

Instrument: Fida 1 with 480 nm LED fluorescence detection. Fidabio standard capillary (i.d.: 75 µm, LT: 100 cm, Leff: 84 cm). The sample format was Fida 96 well microtiter plates.

Indicator: The *indicator* is a mixture containing the labelled Antigen (**Ag**-Alexa) and increasing amounts of unlabeled Antigen (**Ag**), diluted in blank (without VHH) 10% fermentation media.

Analyte: The *analyte* is 10% VHH-containing E.coli non-purified fermentation media.

Each VHH clone was titrated with increasing amount of unlabeled Antigen: 15, 30, 60, 125, 250, 350, 500, 1000, 2000 nM.

Fidabio Clone Selection assays were performed by filling the capillary with 6µL of 10% non-purified E.coli fermentation media containing the expressed VHH (**Analyte**), followed by injection of 39nL of a mixture of labelled Antigen + unlabeled Antigen (**Indicator**), where the unlabeled Antigen was titrant and labelled Antigen was kept constant.

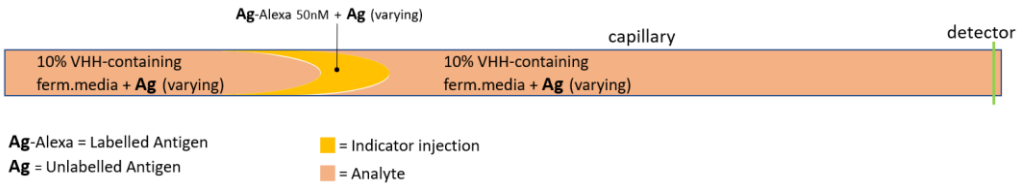

Figure 2. Assay principle adjusted to the experimental conditions of the present work.

Results

The Fidabio Clone Selection assay is divided into three steps:
Step 1 – Single point size-based ranking of 96 clones
Step 2 – Competitive assay on 8 selected clones
Step 3 – Accurate characterization of expression level and VHH affinity

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Step 1 – Single point size-ranking of 96 VHH clones

One of the advantages of the Fida 1 is that it provides an absolute measure of R_h in nanometers (Y-axis) which reveals the fraction of your protein of interest that is bound. It is possible to separately measure the size of the labeled Antigen alone (2.8 nm in the present example) as well as the size of the Antigen in complex with the VHH (3.6 nm - all VHH clones in this study had the same size) (Figure 3). The hydrodynamic radius also serves as a QC check of the proteins, since degradation results in changes in size and/or loss of binding. If a single-point size measurement of a clone is 3.6 nm, it means that the binding is saturated, either because of high expression levels or due to high affinity or, finally, a combination of the two.

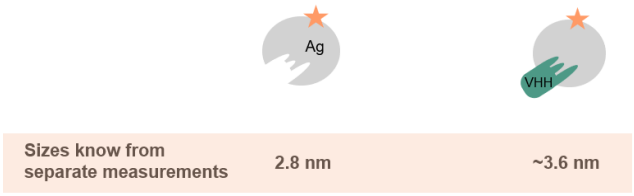

Figure 3. Size limits of the assay. Labeled Antigen 2 nm (left), and Antigen:VHH complex 3.7 nm (right).

Figure 4 shows the single point size-ranking of the 96 clones, where those in red represent the 8 highest read-outs. The process is fully automated accepting 96 well plates as sample format. The wells marked in red are selected for Step 2.

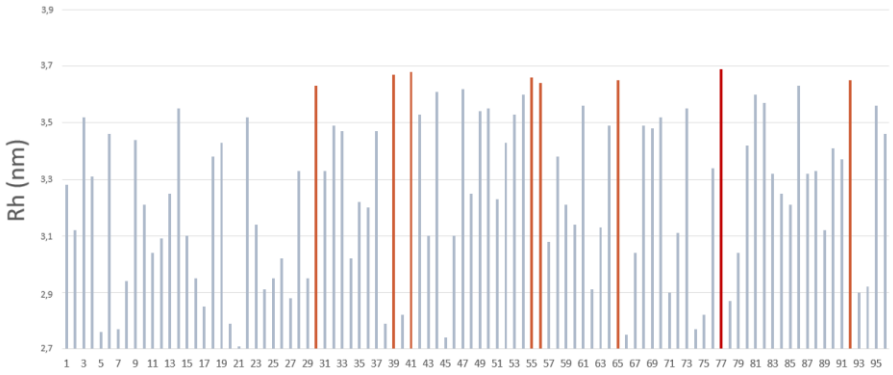

Figure 4. Single point size-ranking. In this case, sample mixing was done automatically in the capillary.

Step 2 – Competitive Fidabio assay on 8 selected clones

For each selected clone, a competition assay is performed in which increasing amounts of unlabelled Antigen is titrated into 10% clone-fermentation media (Figure 2).

When excess amount of unlabelled Antigen is present it competes with the labelled antigen resulting into a decreasing size (Figure 5B).

Figure 5A shows the reverse binding curves obtained by conducting the competitive Fidabio assay on the 8 selected clones. The reverse binding curves are fitted using the Fidabio Clone Selection model. A real-life example of a binding curve is shown in Figure 5B. Since the apparent size (R_h) is linked to the fraction bound, the affinity and the VHH-expression level (concentration in the fermentation media) of the specific clones can be assessed simultaneously.

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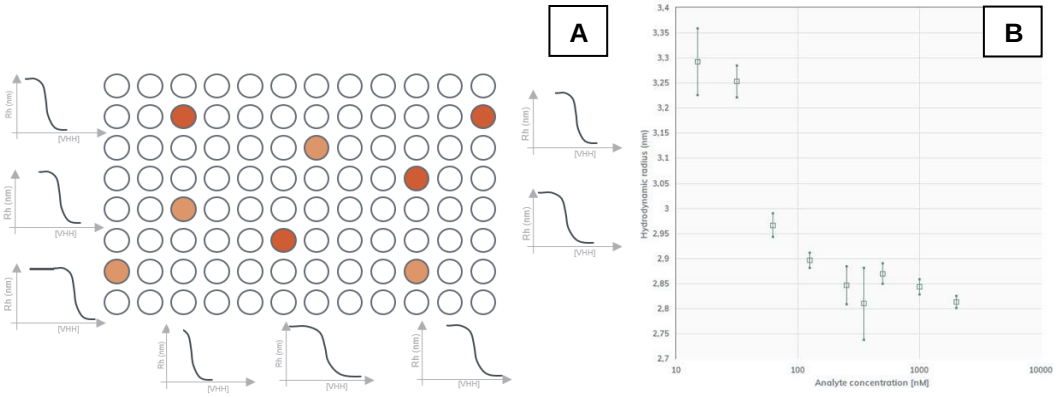

Figure 5. Representation of the Fidabio Clone Selection assay performed on 8 selected clones (A), and an example of a Fidabio Clone Selection binding curve (B). In the present example the assay is designed to quantify K_D in the interval 1 nM – 500 nM.

Step 3 – Best VHH clone accurate characterization

In Step 2, the best clone has been identified based on affinity and expression level. It will be subsequently produced in larger scale and purified. It is possible to use Fida 1 for accurate affinity characterization using the purified VHH as analyte. In this step, the experiments are no longer run in 10% fermentation broth, but in 20nM NaHPO4, pH7.4, 140µM NaCl, 0.03% Pluronic F127. Figure 6 presents the results for the best VHH clone selected in Step 2. Here, the binding characterization was performed as a standard FIDA experiment, where the fluorescently labelled Antigen was constant at 20nM and pure VHH was titrated from 0.125 nM to 100 nM. The selected clone was identified to have an affinity of 2.2 nM. The size of the complex can be compared with structural data from the Protein Data Bank using the R_h predictor in the Fida 1 data analysis software.

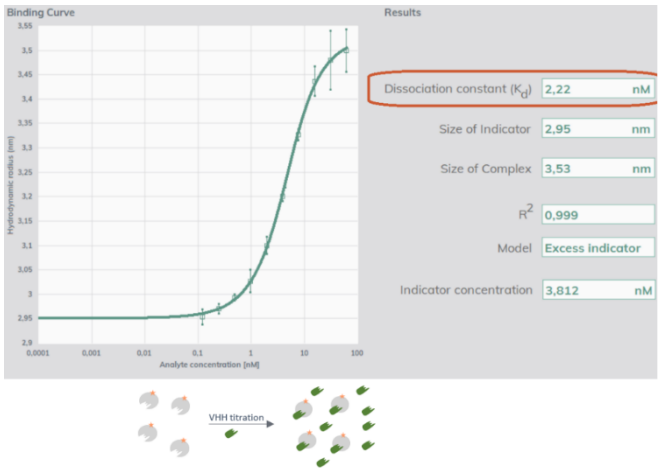

Figure 6. Step 3: standard titration of labelled Antigen (constant at 20nM) with increasing concentration of pure VHH (0.125 nM to 100 nM).

Conclusions

With the Fidabio Clone Selection assay, it is possible to take advantage of the absolute size readout in nanometers and thereby,

i) Increase the likelihood of selecting your best protein candidates by including their functional performance (affinity) early in the selection process, as alternative to only relying on expression levels.
ii) Eliminate the need for purification steps during your selection process.
iii) Work with your native protein without having to express it with His- or other tags.

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