

# WP5009: Protein quality control in SPR and BLI high-throughput screening studies

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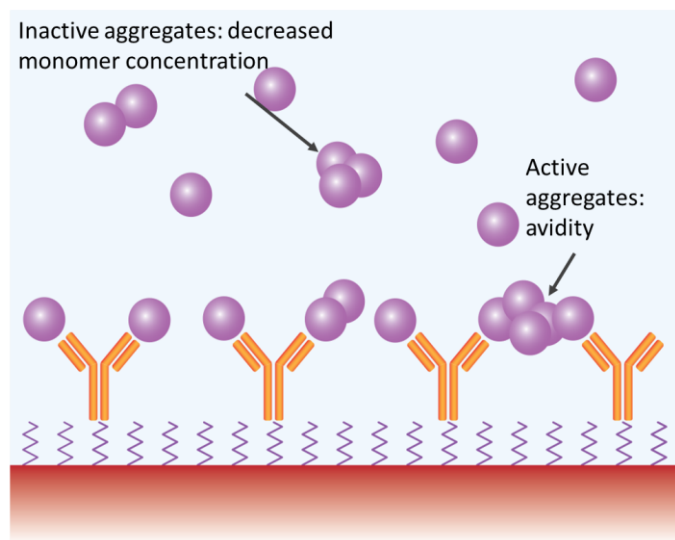
## Summary

While surface plasmon resonance and bio-layer interferometry are widely accepted and highly valued tools for screening studies of drug candidate molecules, the data they provide are only as good as the solutions loaded onto the instruments. Preliminary assessment of the quality of the sample proteins and solutions is imperative for reliable binding results.

Dynamic light scattering is commonly used to evaluate protein aggregation, degradation and solution quality. However, in the context of high-throughput screening, conventional dynamic light scattering detection is just not feasible, since these instruments work with single-sample microcuvettes and the amount of labor required would be quite extensive. The [DynaPro® Plate Reader](#) overcomes this obstacle as it measures dynamic light scattering *in situ* in industry-standard microwell plates, performing automated, non-perturbative quality assessments with minimal time and effort.

After analysis on the DynaPro, a 'heat map' created by the software offers a quick visual scan of the aggregation state in each well. Detailed particle size distributions may be examined more closely. The operator can readily determine which solutions are suitable for binding assays in order to ensure confidence in the results.

The plates can then simply be transferred to the SPR or BLI instrument with no intermediate fluid handling or perturbation. This seamless workflow greatly enhances productivity in drug discovery.



Aggregates may impact SPR and BLI measurements in several ways.



## Introduction

Surface plasmon resonance (SPR) and bio-layer interferometry (BLI) are powerful and widely used techniques for high-throughput screening and discovery of candidate biotherapeutics. In high-throughput SPR, molecules identified as key targets for treatment are immobilized on a chip, and solutions of potential binding partners injected at one or more concentrations in order to assess affinity and kinetics of interaction. In BLI, the targets or candidates are immobilized on fiber optic probes and dipped into microwells containing solutions of the opposing molecules. The outcome of the screen is the selection of one or more candidate therapeutic molecules with advantageous properties, such as high affinity and rapid binding kinetics.

In a typical screen, dozens to hundreds of candidates may be tested, so many high-throughput SPR instruments are designed to draw sample from standard microwell plates and inject into microfluidic channels. The analyte flows over the immobilized target molecule, where good candidates settle rapidly onto the chip surface via specific association with the target epitope. A similar process occurs in BLI, except no microfluidics are involved. An optical probe then provides a signal proportional to the increase in surface-bound mass, and the analysis of these signals over multiple analyte concentrations yields affinity and kinetics.

An oft-overlooked obstacle to effective candidate selection is sample quality. The purity and solution properties of molecules employed in SPR and BLI screening may impact the measurements adversely in two ways: 1) data quality and 2) microfluidic integrity. Fortunately, a valuable tool is readily available to address sample quality analysis. High-throughput dynamic light scattering (HT-DLS) with Wyatt's DynaPro Plate Reader illuminates protein quality without perturbing the solution, performing measurements in the same microwell plates used by high-throughput discovery screening platforms. HT-DLS enables rapid evaluation of sample solutions, prior to their loading onto the SPR or BLI instrument, catching material of poor quality before it has the chance to plug the microfluidics or lead to wasting of valuable time and resources on meaningless measurements. As an added benefit, DLS can determine the analyte's diffusion coefficient, an important property in SPR experiments for

identifying mass transfer limitations. More information regarding dynamic light scattering may be found at [www.wyatt.com/DLS](http://www.wyatt.com/DLS).



Figure 1: The DynaPro Plate Reader assesses solution quality in standard 96, 384 or 1536 well plates, without perturbing the samples.

### The importance of analyte quality I: Impurities

Like most techniques, SPR and BLI are subject to the unavoidable, fundamental law of experimental science succinctly put as 'garbage in, garbage out'. Analyte quality, so often ignored, is actually quite critical to obtaining accurate and meaningful measurements that ultimately lead to selection of the best therapeutic candidates—and hence the best clinical results. Impurities of low molecular weight, such as extractables and leachables, usually have relatively low impact on SPR and BLI measurements. However, large impurities, such as aggregates and foreign particles can wreak havoc on measurements by both techniques. Large impurities contribute to four basic types of experimental uncertainty in SPR and BLI measurements: noisy signals, spurious signals, inaccurate concentrations, and skewed kinetics.

The evanescent optical fields that probe binding do not extend very far into the solution, typically a few hundreds of nanometers. And yet, any nanoparticle or aggregate passing within that distance from the surface of the chip or fiber probe will result in a signal spike approximately proportional to its mass. Consider a  $100 \times 100 \mu\text{m}^2$  surface immobilized with bound ligand and illuminated by the SPR beam. Exposing this surface to a concentration of analyte  $\gg K_d$  results in full coverage and a maximum binding signal. Now, consider a single contaminating nanoparticle of  $\sim 5 \mu\text{m}$  diameter in that analyte solution. Since the contaminant contains the same volume as the

bound analyte, upon passing very close to the chip or probe surface, this particle can generate a noise spike in the binding signal equivalent to all of the bound analyte! Smaller nanoparticles and aggregates, that may be present in larger numbers in a sample of low quality, will produce a steady stream of small signal fluctuations leading to a degraded optical response. Both effects are represented in the simulated sensorgrams shown in Figure 2.

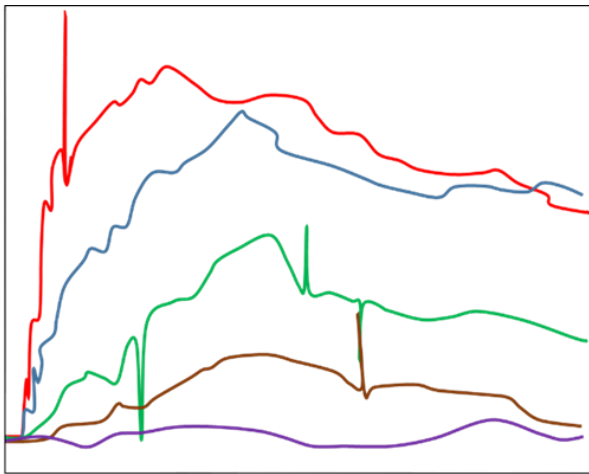


Figure 2: Sensorgram obtained in the presence of aggregated analyte (simulated).

Analyte aggregates may or may not be active. If active and present in appreciable quantities (e.g., > 5% total analyte protein mass), the aggregates will bind to the immobilized ligand and generate an SPR or BLI signal larger than that of the expected monomeric interaction, skewing the binding response towards a higher estimated affinity and on rate. Aggregates presenting multiple binding sites may exhibit ‘avidity’ effects – interacting simultaneously with multiple immobilized molecules or exhibiting a reduced dissociation rate by hopping along the surface of the chip, spuriously leading to an extreme overestimate of affinity. If analyte aggregates are inactive, the effective concentration will be lower than the measured total concentration, leading to decreased binding and an apparent decrease in affinity. Either way, aggregates lead to incorrect quantification of candidate binding properties (Figure 3).

The addition of active non-monomeric species with different diffusion properties and binding kinetics than the monomer may also adversely impact the time-dependent sensorgrams. Since the analysis assumes a single binding species with unique on and off rates, the presence of

multiple binding species will create binding curves that cannot be fit correctly under the standard assumptions.

### The importance of analyte quality II: Self-associating analyte

Standard SPR and BLI analyses require that the analyte be monomeric in solution at the concentrations employed in the experiment. The previous section addressed the adverse impact of irreversibly aggregated material on the analysis. Poorly formulated or otherwise ‘sticky’ analytes may self-associate reversibly as well as irreversibly. When this is true, the analyte monomer is in dynamic equilibrium with small oligomers, such as dimers or tetramers, and the actual concentration of monomer varies with protein concentration. Once again the effect on the final measurement depends on the activity and presentation of binding sites, where active oligomers lead to overestimates of affinity and inactive aggregates lead to underestimates (Figure 4). Moreover, presentation of multiple binding sites may lead to avidity effects and gross overestimates of affinity.

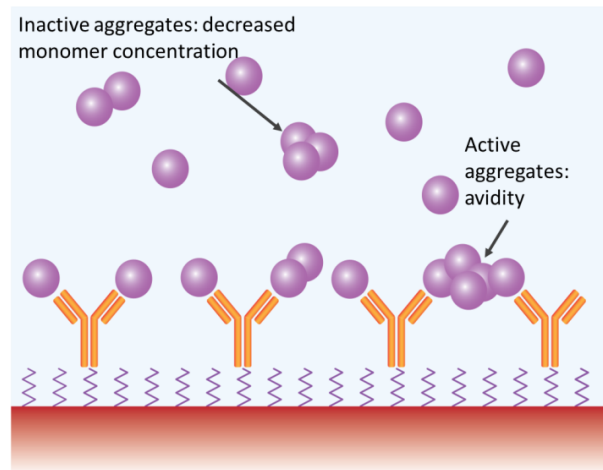


Figure 3: Aggregates may impact SPR and BLI measurements in several ways.

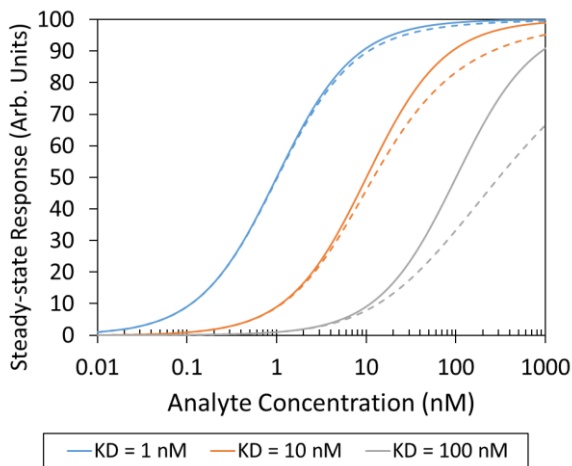


Figure 4: Analyte self-association with  $K_d = 100$  nM can shift the apparent analyte-ligand equilibrium isotherm from the expected curve (solid line) to the measured curve (dashed line). In this analysis the dimer was assumed to be inactive.

### The importance of substrate protein quality

Much as aggregated analytes can lead to experimental errors, so too can aggregated immobilized proteins. In particular, the presence of protein aggregates on the chip or fiber probe surface will most likely lead to a decrease in active material or in the average number of exposed epitopes per immobilized mass. Consequentially aggregated substrate proteins decrease the apparent affinity.

### Where's the drain opener?

Severely aggregated or otherwise impure material bearing large particulates can lead to another highly detrimental effect: clogged microfluidics in multichannel SPR. These fluidic channels tend to be narrow but long and are prone to plugging by agglomerated proteins or other 'nanocrud.' The occurrence of clogging events in the middle of a screen of dozens or hundreds of candidates can ruin the efforts of weeks, if not months, of protein expression, purification and preparation – including all the work devoted to analytical method development and assessments of SPR immobilization protocols.

Recovery of plugged microfluidics might be as simple as replacing a chip or involve lengthy system cleaning and maintenance. In any case, the damages can amount to many thousands of dollars, even before accounting for lost productivity.

### Dynamic light scattering to the rescue!

Dynamic light scattering (DLS) is a non-invasive, non-perturbative optical technique that measures the size

distribution of nanoparticles in solution/suspension, from less than 1 nm up to several micrometers. DLS relies on the principles of Brownian motion to determine diffusion rates of particles in solution. The information is transformed by **DYNAMICS**<sup>®</sup> software into a particle size distribution which can be evaluated to determine whether or not the solution may safely be injected into SPR microfluidics and whether or not the SPR or BLI measurements will produce reliable results.

DYNAMICS provides automated analysis and visualization of DLS results as a heat map indicating good, intermediate, and poor protein quality. The entire process may be completed rapidly prior to loading onto the interaction apparatus simply by transferring the microwell plate into the DynaPro HT-DLS system, running the sample screen, and then (when not contra-indicated) loading the same microwell plate onto the SPR or BLI instrument. Microwells that show low-quality material can then be deselected in the interaction screening protocol.

Molecules and nanoparticles in solution or suspension 'jitter' due to Brownian motion, a consequence of the thermal energy of solvent molecules and the momentum imparted to the nanoparticles by collisions. In DLS, a laser beam impinges on the nanoparticles and is partially scattered in all directions. The light waves scattered by different nanoparticles reach the detectors at different phases and so interfere constructively or destructively at the detector depending on the specific phase difference between them, as shown in Figure 5.

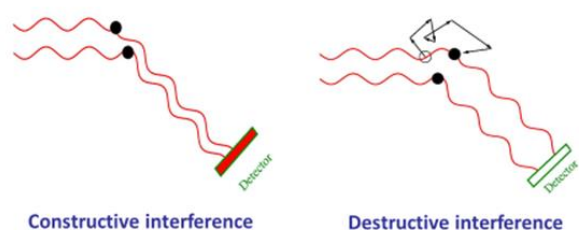


Figure 5: As molecules move through Brownian motion, the light scattered from each molecule traverses a different path. This leads to constructive or destructive interference at the light scattering detector. When scattered waves interfere constructively, the DLS detector records high light intensity; conversely when waves interfere destructively, the detector records low light intensity.

The measured intensity of scattered light fluctuates over the time scale characteristic of diffusion. DLS captures the rates of fluctuation to determine translational diffusion

coefficients  $D_t$ . As described in Figure 6, diffusion coefficients are transformed to particle sizes via the Stokes-Einstein equation:  $R_h = k_B T / 6\pi\eta D_t$ , where  $R_h$  is the particle's hydrodynamic radius,  $k_B$  is Boltzmann's constant,  $T$  the absolute temperature, and  $\eta$  the solvent viscosity.

Figure 7 shows three typical %-intensity size distribution determined by DLS. The red and blue curves arise from monomodal populations of BSA ( $R_h = 3.8$  nm) and polystyrene spheres of radius 50 nm, respectively. The green

curve arises from a solution contains a high proportion of monomeric protein,  $R_h \sim 4$  nm, as well as some large aggregates with  $R_h \sim 50$  nm. Because the scattered intensity is proportional to molar mass, the %-intensity curve is heavily weighted toward large particulates, and in fact, the total mass of polystyrene beads is much less than that of the BSA. The actual amount of aggregate vs. monomeric protein may be estimated via the %-mass size distribution graph (not shown).

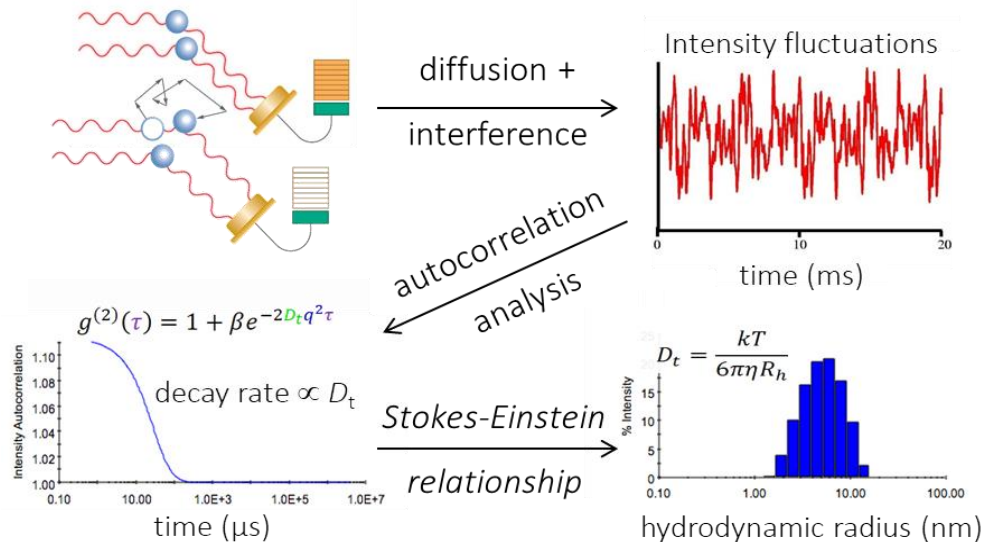


Figure 6: Autocorrelation analysis is the mathematical transformation linking light intensity fluctuations to the diffusion coefficient. Following autocorrelation, DYNAMICS converts the diffusion coefficients to size.

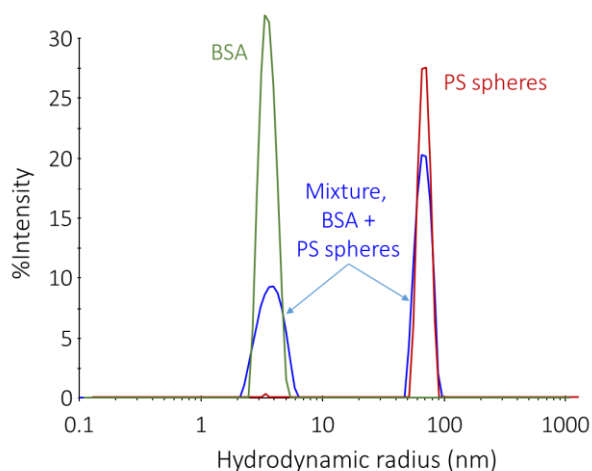


Figure 7: Representative size distributions determined by DLS.

DLS does not have sufficient resolution to discriminate monomers from dimers or other small oligomers; in general, it can only resolve populations of nanoparticles

that differ in size by 3-5x in radius (equivalent to about 100x in mass). However, the presence of small aggregates is inferred via the width of the peak (known as polydispersity) or shifts in the average value of  $R_h$  for the population.

### HT-DLS does the job, quickly and easily

Traditional DLS takes place in a microcuvette, manually, one sample at a time. It would not be feasible to test all the hundreds of candidates to be screened in this manner, though cuvette-based DLS could still be valuable for other quality assays. On the other hand, the DynaPro Plate Reader brings the power of HT-DLS to bear on quality assessment for the target and *all* the candidates, thanks to its microwell-plate based format with *in situ*, non-perturbative measurements.

With no fluidics, the DynaPro presents no concern for potential carryover of samples between the wells. Measurements may be completely rapidly, typically requiring

10-30 seconds per well including transition time between wells. The entire screen is set up to proceed unattended in the DYNAMICS software package

In HTS-DLS applications, DYNAMICS is usually configured to bin the data as a heat map based on poor, intermediate and high-quality size distributions according to bin definitions specified by the user. For example, a sample which shows a single, narrow peak at a size corresponding to that of the analyte may be classified as high quality and

allowed to proceed to the binding assay with high confidence (Figure 8, red wells). An adjoining sample which shows a broadened monomeric peak, indicative of some oligomers and perhaps low levels of additional particulates tens of nanometers in size may be classified as intermediate quality and allowed to proceed but with a warning flag as to confidence in the results (Figure 8, blue wells). A sample exhibiting significant particulate content in the micron-size range can be assumed to be either contaminated or highly prone to aggregation and prevented from continuing on to the binding assay (Figure 8, black wells).

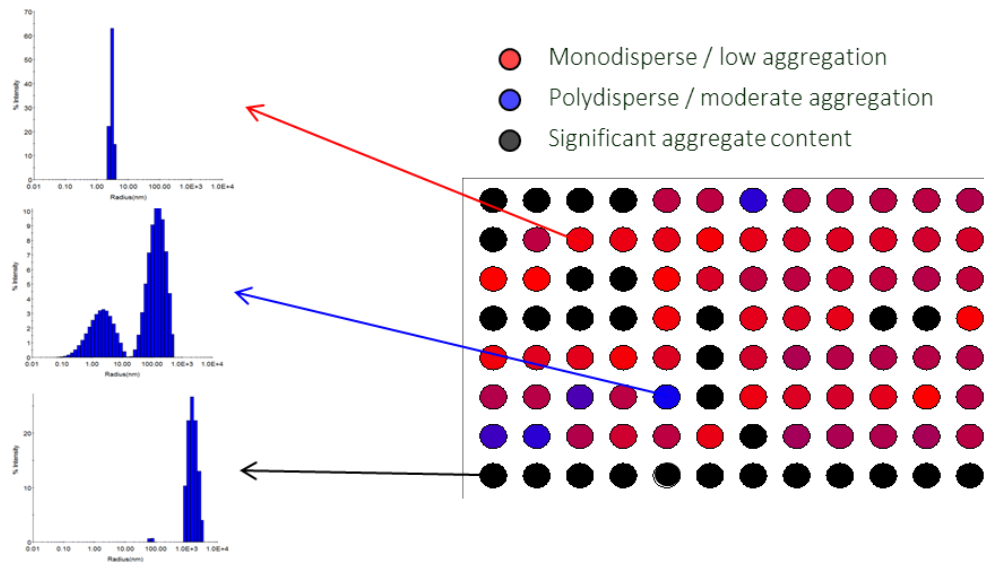


Figure 8: Visualization of protein quality via heat map in DYNAMICS. Total data acquisition time for 96 wells was < 45 minutes. Data courtesy of Sabin Vaccine Institute and Texas Children’s Hospital Center at Baylor College of Medicine

### Additional benefits

As an added bonus, DLS inherently determines diffusion coefficients which are helpful in assessing mass transfer effects: the mass-transfer-limited reaction rate in SPR is  $k_m = 0.98(D/h)^{2/3}(f/bx)^{1/3}$ , and the diffusion layer thickness is  $d = D/k_m$ , where  $D$  is the diffusion coefficient,  $h$  and  $b$  the height and width of the SPR flow cell, respectively,  $x$  the distance from the flow cell entrance and  $f$  the flow rate<sup>1</sup>.

Another unique feature of the DynaPro Plate Reader is the built-in, high-magnification camera which snaps a picture of each well after taking a DLS measurement. These images, stored and shown with the associated DLS data, are especially helpful as diagnostics. A review of the images is useful for determining why the black-classified data in Figure 8 look bad: has the sample precipitated, or perhaps the well inadvertently was not actually loaded

with sample? Figure 9 presents additional examples of sources of poor data identified by camera images.

Finally, the same microwell plates utilized in the DynaPro may be transferred to a spectroscopic plate reader for additional confirmation of content and quality.

### Sensitivity

Yes, every instrument and technique has its limitations on sensitivity. The lower limit of robust detection for the DynaPro is 0.125 mg/mL lysozyme ( $M = 14.4$  kDa). Since the intensity of light scattered by macromolecules is proportional to molar mass, the sensitivity is inversely proportional to molar mass, translating to a lower limit of 0.0125 mg/mL of a 150 kDa IgG. Sensitivity to aggregates follows the same trend, i.e., a 100 nm  $R_h$  aggregate consisting of approximately 5000 IgG monomers will be indicated at a concentration of  $\sim 2$  ng/mL.

Even if the primary sample concentration is below the limit of detection, DLS is still a useful test of solution quality since it will indicate with excellent sensitivity the 'nanocrud' content – sub-micron particulates and large

protein aggregates that are detrimental to the binding analysis and microfluidic system. The same analysis should also be used to assess dilution buffers employed in SPR to create a series of ligand concentrations.

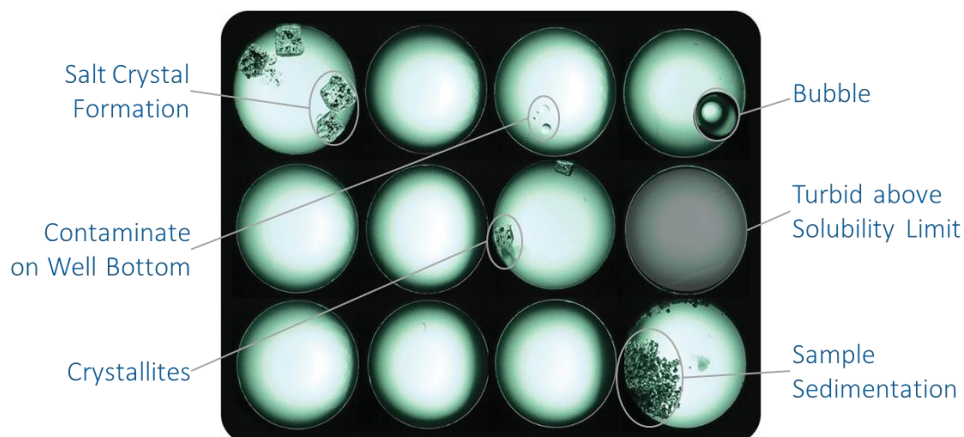


Figure 9: The DynaPro's on-board camera helps identify wells containing clean solution and those where sample has precipitated or otherwise can be expected to provide poor data quality.

## Conclusions

The selection of candidate molecules with the potential for optimal therapeutic effect and patient benefit depends on a reliable target binding screen, as performed with SPR or BLI. This requires, in turn, assuring that the solutions utilized in the analysis are of good quality. Poor quality samples impact the data quality adversely and hold the potential for fouling flow cells and microfluidic channels.

High-throughput dynamic light scattering with the DynaPro Plate Reader is readily implemented in the screening work flow to classifying solutions as 1) high quality, offering maximal confidence in the interaction analysis; 2) intermediate quality, suitable for measurement with caution in relying on the results; and 3) low quality, not suitable for analysis and potentially fouling the measurement device. Adding an HT-DLS pre-screen can prevent much of the uncertainty and productivity loss

associated with variable ligand quality, leading to more reliable binding data and confidence on the final candidate selection. After identification of the most promising candidates, the DynaPro is also widely used in pre-formulation and candidate developability studies to assess aggregation, conformational and colloidal stability<sup>2,3</sup>.

To learn more, see [www.wyatt.com/HT-DLS](http://www.wyatt.com/HT-DLS).

## References

1. Karlsson, R., Roos, H., Fägerstam, L. & Persson, B. Kinetic and Concentration Analysis Using BIA Technology. *Methods* **6**, 99–110 (1994).
2. Saito, S. *et al.* Effects of ionic strength and sugars on the aggregation propensity of monoclonal antibodies: influence of colloidal and conformational stabilities. *Pharm. Res.* **30**, 1263–1280 (2013).
3. I. Razinkov, V., J. Treuheit, M. & W. Becker, G. Methods of High Throughput Biophysical Characterization in Biopharmaceutical Development. *Curr. Drug Discov. Technol.* **10**, 59–70 (2013).



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