MEDICAL INJECTIONS

Why it is crucial to pay attention to particles

Every year at least 16 billion medical injections are administered worldwide. The majority (90%) are given for therapeutic purposes and around 5% for immunization purposes (1). Therefore, safety and efficacy of injectable medicines are essential factors not only for patients, but also for caregivers, manufacturers and regulators. Many liquid medicines contain particles which can both affect product stability and represent a potential critical risk for patient safety. In fact, those particles may cause blood vessel occlusions (2) and induce immune response to the administered drug or autoimmune responses (3,4). For these reasons, particle identification, quantification and characterization are essential control strategies to ensure quality and safety in biotherapeutic development.

Particle formation: a process with multiple causes

There are multiple factors that can cause particle formation during manufacturing, storage or transportation of liquid or reconstituted medicines. In complex protein-based therapeutics such as monoclonal antibodies, recombinant proteins, fusion proteins or antibody-drug conjugates, these particles can be extrinsic, intrinsic, or inherent. Extrinsic particles are foreign contaminants such as cellulose fibers, glass, rubber, plastic or metal as well as particles generated from interaction of formulation components and primary packaging (e.g., barium sulfate) (5). Intrinsic particles, such as protein aggregates, are associated with impurities derived from the drug product itself. Parent active components may also be prone to self-aggregation (6). Those aggregates may form due to prolonged storage, container properties, mechanical agitation during shipping and handling, changes in air, light and temperature, or changes in state from solid to liquid, which occur during thawing. Sometimes, in innovative therapeutic formulations, particles can also represent the active pharmaceutical ingredient or the drug delivery vehicle. Here, aggregate formation can hamper the effectiveness of the treatment, enhance immunogenicity or cause immunotoxicity (7).

Sub-visible particles: a critical quality attribute of biopharmaceutical products

Particles come in a variety of sizes: Visible particles are approximately 100-150µm and larger and can be observed with the naked eye, whereas sub-visible particles are approximately 100nm-100µm and are too small to be visible without the use of magnification (7). At the same time, parenterals should be virtually free of sub-visible particles, which could be harmful to patients. Therefore, regulatory authorities such as the United States Pharmacopeia (USP), the European Pharmacopeia (EP), the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) and the Japanese Pharmacopeia (JP) define limit values for sub-visible particles in parenterals.

The USP defines particulate matter as "mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions" (8). Chapters <788> and <789> of the USP define standard, validated methods for analyzing the count and size of undissolved foreign particles within injectable and ophthalmic pharmaceutical products, respectively. Both chapters provide an overview of the two standard procedures for particle analysis: light obscuration and membrane microscopy (8,9). Similar standards are defined in the EP (chapter 2.9.19) and the JP (6.07), and the chapters have been harmonized in the International Council for Harmonization (ICH) guidelines (10) (see Table 1). In addition, USP Chapter <787> deals with the analysis of sub-visible particles in therapeutic proteinaceous formulations and allows smaller sample volumes as protein formulations are often only available in very small dosages.

	Small Volume Parenterals	Large Volume Parenterals
USP, EP, JP	<6000/container @ ≥10µm <600/container @ ≥25µm	<25/ml @ ≥10µm <3/ml @ ≥25µm

Table 1: Meeting the requirements: Regulatory agencies worldwide set strict limits for particle content in parenterals.

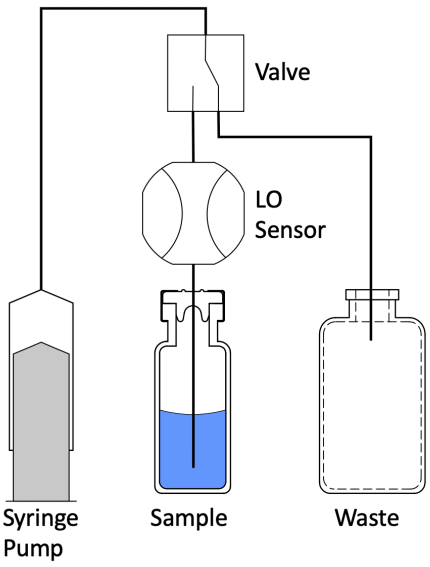
In May 2021 a revised version of the USP chapter <1788> on "Sub-visible Particulate Matter in Therapeutic Protein Injections" was made official. The new version expands the guidelines outlined in the chapters <787>, <788> and <789> with regard to sample handling, training of inspectors and individual testing methods. For the first time it also includes the micro-flow imaging method, which supports light obscuration and membrane microscopy tests providing additional orthogonal information (11).

In addition to the compendial requirements, testing systems should also be compliant with FDA 21 CFR Part 11 when used in a GMP environment. This regulation provides guidance for electronic documentation and electronic signatures and outlines the requirements for good data integrity practice. In a system with data integrity, records must be attributable to the technician carrying out the testing, legible, created contemporaneously, original and accurate (ALCOA principles) (13).

Light obscuration: from aerospace to the pharmaceutical industry

As particles are considered obligatory critical quality attributes of biopharmaceutical parenteral preparations, sub-visible particle testing is mandatory for those preparations; thus, it is used for stability testing, release testing, drug formulation and process development, as well as in-process control testing during manufacturing. The standard procedure that is used to fulfill compendial requirements for sub-visible particle quantification is light obscuration. This technology was originally designed to check hydraulic oil used in aerospace, so it was not initially tailored to the needs of the pharmaceutical industry. Scientist Leon Carver created the HIAC (or High Accuracy Products) optical particle counter in 1962. His invention marked the birth of the light obscuration method and of the optical particle counting industry (13).

Initially, this method was used mainly by the US Air Force and NASA for counting contaminating particles in hydraulic fluids (14). Within the decade, particle counting instruments evolved from light bulbs and photomultiplier tubes to laser beams and photodiodes; these instruments were also used for monitoring other fluids such as glycols, cleaning solvents, fuels, lubricants, compressed gasses and water. However, the essential counting principle remains the same, and particle counters are now widely used in the pharmaceutical industry to monitor injectable solutions.



The measuring setup for light obscuration: The destruction of the sample by the current technology is due to mixing the cleaning medium with the sample.

Sub-visible particle measurement methods

Light Obscuration

Today, the primary pharmacopeial method used to quantify sub-visible particles in parenterally administered medicines is light obscuration. This method is based upon the amount of light a particle blocks when passing through the detection window of the particle counter. The light source is a laser diode, which creates a light beam. When a particle goes through the beam, a "shadow" or obscuration is generated. This "shadow" is detected by an optical sensor which is calibrated using particles of certified sizes and transferred into a particle (15). As a measurement method, light obscuration offers a mature and robust platform for routine particle characterization as it quickly detects individual particles and provides highly accurate counts and sizes of sub-visible particles between 2 and 150 µm.

Although light obscuration is the preferred method for quantifying sub-visible particles, it also has some limitations, such as:

- A lower sensitivity in detecting some types of translucent, proteinaceous particles, resulting in lower counts;
- Information only on particle size and quantity;
- Lower accuracy of analytical results when the difference between the refractive index of the particles and that of the medium is small (16);
- Strong dependence of sizing accuracy on the properties of the reference material used for calibration and on the properties of the sensor;
- Sensitivity to air bubbles and to some degassing procedures such as sonication as those can change sample properties (17);
- Loss of the sample as it is diluted during the measuring process and cannot be reused;
- Inability to detect particles smaller than 1µm (18).

An innovative particle testing worflow

Particle analysis is also a requirement for quality control of finished medical products and for investigations based on complaints about pharmaceutical products on the market. In order to overcome some of the challenges of current light obscuration methods, Bionter focused on developing a new, non-destructive, automated light obscuration approach.

Microscopy

Manual microscopy and membrane microscopy are also compendial methods used to detect and characterize number and shape of sub-visible particles. For this purpose, particulate matter is retained on a membrane filter and then examined using a binocular microscope. The number of particles that are equal to or greater than 10 µm and the number of particles that are equal to or greater than 25 µm is determined. For estimating the particle size, a circular diameter graticule is used, and particles are compared with the 10 and 25 µm reference circles on the graticule (8). While this method allows the opportunity to filter and analyze the complete sample, it extremely time consuming. Moreover, proteinaceous particles have the tendency to be elastic and may slip through the filter mesh (19).

Micro Flow Imaging (MFI)

The MFI method was developed to overcome the limitations of light obscuration and microscopic methods regarding the underestimation of size and quantity of small transparent particles and the inability to distinguish between particle populations. MFI is also included in the modified USP chapter <1788>. In MFI, particles are digitally imaged with high resolution as they move through a flow cell (20). Sub-visible particles in the size range of 2 to 80 µm can be detected with high sensitivity, especially when they are translucent. Moreover, MFI analysis can differentiate subpopulations based on particle size, morphology and optical density. Even though MFI has some advantages compared to light obscuration, it cannot replace it, and while it is mentioned in the USP, MFI is not accepted in the European Pharmacopeia. This technology also shows some limitations: Sizing accuracy may be lower in case of particles with a refractive index close to that of the medium. Moreover, if used quantitatively, MFI may create data that is difficult to interpret and cannot be directly comparable with data from light obscuration (18).

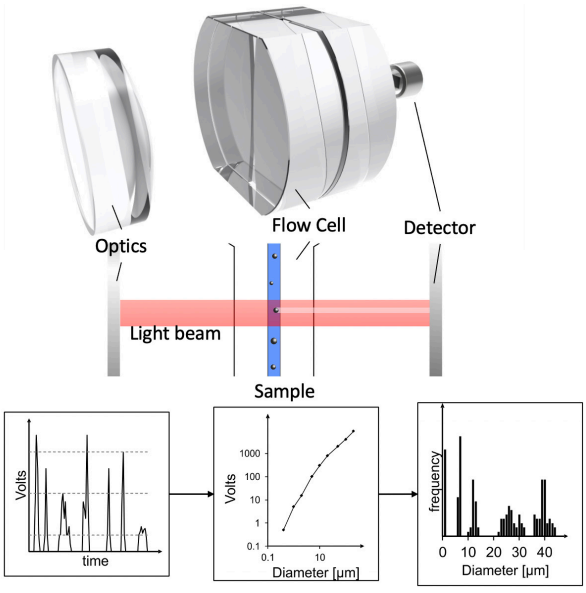
Background Membrane Imaging (BMI)

BMI is an automated, 96-well plate-based microscopic approach for particles in the size range, starting from approximately 2µm. BMI is based on the acquisition of images of filter plates before and after sample application. The before and after images are aligned and subtracted from each other by image analysis software. The resulting image shows the isolated actual sub-visible particles. Overall, BMI methods shows several promising features, such as low required sample volume, high throughput, and ease of handling (24). However, BMI remains a novel, experimental - and therefore non-compendial - test method, whose counting accuracy is limited due to probabilistic effects. Therefore, BMI may be better suitable for early formulation screening, though it will likely not replace microscopy or light obscuration.

Other Orthogonal Methods

Several novel orthogonal methods have been developed to detect sub-visible particles in protein formulations which can be missed by compendial methods such as light obscuration. All of these methods, however, are not compendial, and they are destructive. Further, they often create data which is difficult to interpret.

The Coulter principle – namely, the fact that objects placed in an electric field modify the current flow in that field - can be applied for counting and sizing particles. This approach has several advantages, such as high sensitivity, ability to detect very small particles, excellent reproducibility and high-resolution size information. However, measurements depend on conductivity of the sample, and, therefore, not all samples can be measured without dilution or change of the matrix (22).



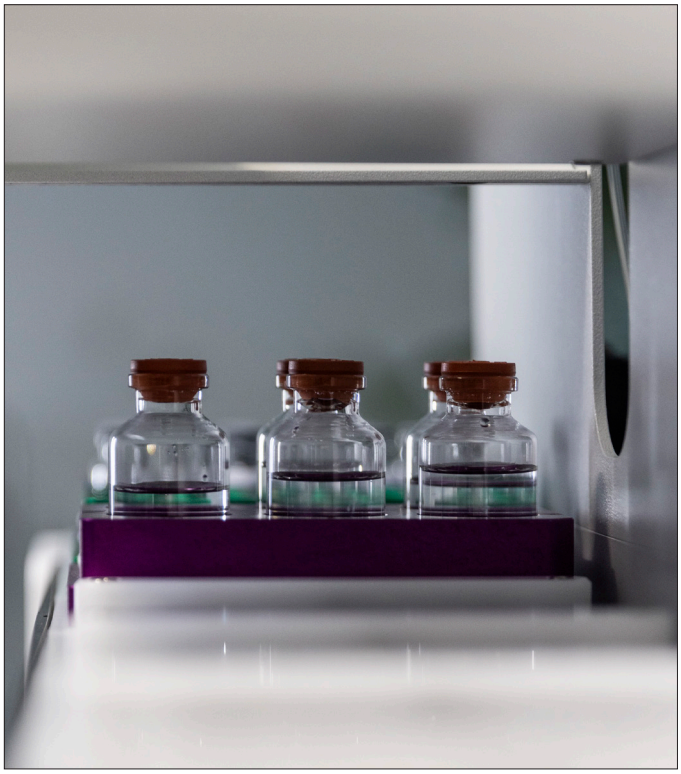
Light obscuration is based upon the amount of light a particle blocks when passing through the detection window of the particle counter. The light source is a laser diode, which creates a light beam. When a particle goes through the beam, a "shadow" or obscuration is generated. This "shadow" is detected by a calibrated optical sensor. Light obscuration detects sub-visible particles between 2 and 150 µm of size. The amount of light obscured is transferred into particle size using a calibration curve.

The *Resonant Mass Measurement (RMM)* method detects 50 nm–5 µm particles and quantifies them on a particle-by-particle basis (23). Here, the sample flows through a microchannel embedded in a resonator, which resonates with a specific frequency. When a particle enters the channel, it causes a change of the resonant frequency. This change of frequency is used to measure the mass of the sub-visible particle with high precision. However, the efficiency of this method may be diminished by the fact that only a small volume can be analyzed, that heterogeneous particles are not sized accurately and that no nanometer count standards are commercially available (24).

Nanoparticle Tracking Analysis allows the high-resolution tracking of individual nanoparticles and the acquisition of their size based on the determination of their diffusion coefficient. Even though this approach allows the observation of small changes in particle size distribution, its counting accuracy is limited due to probabilistic effects and is only acceptable for high particle concentrations (>100M/ml). Users also need to be experienced as well as able to correctly adjust the video recording settings, which are the main source of variability and inaccuracy when comparing samples (25). Nanoparticle Tracking Analysis allows the high-resolution tracking of individual nanoparticles and the acquisition of their size based on the determination of their diffusion coefficient. Even though this approach allows the observation of small changes in particle size distribution, its counting accuracy is limited due to probabilistic effects and is only acceptable for high particle concentrations (>100M/ml). Users also need to be experienced as well as able to correctly adjust the video recording settings, which are the main source of variability and inaccuracy when comparing samples (25).

Applications of sub-visible particle measurement in the pharmaceutical industry

As mentioned, accurate quantification and sizing of sub-visible particles in biopharmaceutical products are crucial aspects in drug and process development. In fact, sub-visible particles can significantly impact the safety, efficacy and immunogenicity of complex biological drug substances such as insulin, interferons, vaccines, monoclonal antibodies, recombinant proteins, fusion proteins, antibody-drug conjugates, and viral- and cell-based therapies. Particle analysis is also an essential requirement in stability studies, for product release and for the characterization of the final therapeutic product.



Bionter's EVE is based on an innovative working principle: it is automated and runs unattended. These features allow pharmaceutical labs (or industries) to make a major step towards a lean analytical workflow. Bionter's light obscuration device consists of racks for primary packaging, a conveyor belt that transports the samples to an analysis area, bottles for cleaning media and waste, a fluidic system including the particle sensor, as well as single-use consumables that allow an efficient process. Users just need to load samples onto the conveyor belt, refill the cleaning medium when necessary, empty the waste or refill the stack of single-use consumables. Right: The conveyor belt of Bionter's particle counter transports the samples to the analyzing area.

Moreover, a precise particle characterization is the first step for their effective control, which is an important factor as the industry is aiming at manufacturing "zero defect" and "essentially particle-free" drugs (26).

Analytical requirements may vary at every step of the bioprocessing workflow. During early formulation development, the availability of sample material is often very limited. Therefore, scientists would greatly benefit by being able to use low sample volumes for testing and to run several tests with the same sample, as this would allow them to maximize efficiency and minimize costs. In contrast, during late-stage formulation development, scientists usually have to screen many samples for larger studies. In this case, process automation plays an important role. For example, when handling highly concentrated formulations, a dilution step may be necessary before analysis as light obscuration instruments may show a reduction of flow accuracy with highly viscous samples (27). This dilution step results in an additional manual handling of the sample and in an increased risk of contamination. In fact, minimal human interaction for sample preparation and handling reduces the chance of contamination and, thus, the percentage of false-positive test results. Automation also increases process efficiency and reliability, which ultimately result in increased safety for the patients. Further, for GMP manufacturing, compliance to data integrity and pharmacopeial guidelines are critical. Regulatory authorities have de facto introduced more and more stringent requirements over the past few years regarding sub-visible particles, reflecting a growing concern for the presence of such particles in parenterals (6).

Particle analysis is also a requirement for quality control of finished products, for investigations in response of complaints from the market and for exploring the effects of temperature excursions on sensitive pharmaceutical products. Here, non-destructive analysis methods would allow scientists to first assess the particle load using a compendial method and subsequently use orthogonal methods on the same sample for root-cause investigation.

Detection methods for subvisible particles

Detection Method	Information on	Size Range	Advantages	Critical Points
Light Obscuration	particle size, detection of individual particles	1-100µm	standard compendial method, remarkable counting accuracy and reproducibility at concentrations below 100 particles/ml	destructive method, labor intensive, one-measurement-at-a-time, risk of contamination through manual sample preparation, risk of false positive results
Microscopy	particle size, surface characteristics	2-150µm	whole sample analysis	loss of liquid / semisolid particles, long analysis time
Micro Flow Imaging (MFI)	images of single particles	2-300µm	detection of particles that are overlooked by compendial methods, automated workflow, low sample volume needed	low sizing accuracy (oversizing at lower size and undersizing at upper size range), destructive measurement, non compendial, lack of standard instrument configuration
Background Membrane Imaging (BMI)	imaging of single particles	2-400µm	high sensitivity, ability to detect very small particles, excellent reproducibility, high-resolution size information	non compendial, counting ccuracy limited due to probalistic effects, oversizing at lower size and undersizing at upper size range, destructive measurement, non compendial, data are difficult to interpret, no nanometer count standards
Coulter Principle	particle count, particle size	0,4-250µm	high sensitivity, ability to detect very small particles, excellent reproducibility, high-resolution size information	non compendial, counting accuracy limited due to probalistic effects, oversizing at lower size and undersizing at upper size range, destructive measurements depend on the conductivity of the sample
Resonant Mass Measurement (RMM)	particle mass distribution, particle size distribution, particle buoyancy	0,05-5µm	detection of particles that are overlooked by compendial methods, compatible with high viscosity or high concentrated samples	severe undersizing effect at all size ranges and concentration levels, counting accuracy limited due to probalistic effects and the very low sample volumen, poor linearity, destructive measurement, data are difficult to interpret, non nanometer count standards avalaible
Nanoparticle Tracking Analysis (NTA)	particle size distribution, particle concentration	0,03-0,3µm	detection of particles overlooked by compendial methods, high resolution tracking of individual nanoparticles	counting accuracy limited due to probalistic effects (counting only acceptable for highly concentrated particle solutions), destructive measurement, non compendial, challenges in video recording setting, video recording may cause high variability and low precision, data can be difficult to interpret, very small sample volume

A new approach to light obscuration

Like all other technologies for sub-visible particle counting, current light obscuration methods include many manual steps and consume the entire sample during testing. The compendial sub-visible particle testing method usually requires 25 ml of total sample volume. Therefore, scientists who usually struggle with limited sample material often need to pool several smaller volume units to reach the required sample volume. However, this pooling increases the risk of sample contamination and false-positive results, a critical issue for high concentration formulations. Moreover, the loss of a considerable sample volume for testing purposes is very expensive, especially in the biotechnology sector, where the costs of a single test run typically range between $300 to more than $1,000.

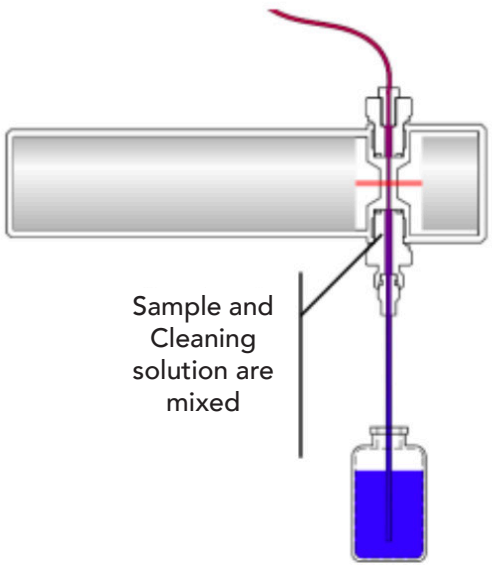
The destructive nature of the current technology is due to the mixing of the cleaning medium, usually deionized filtered water, with the sample liquid. This mixing phase "destroys" the sample and also means that the first sample aliquot needs to be discarded, since it is a mixture of sample and cleaning medium.

A non-destructive workflow would allow repeated testing of the same sample, which would be helpful to assess particle evolution over time on a deterministic basis, or to perform consecutive investigative analyses in case of particulate findings. In fact, due to the destructive particle counting procedure, scientists can assess particle concentration evolution only on a statistical basis; they need additional sample material for particle characterization beyond compendial application, such as by flow imaging, RMM or infrared microscopy.

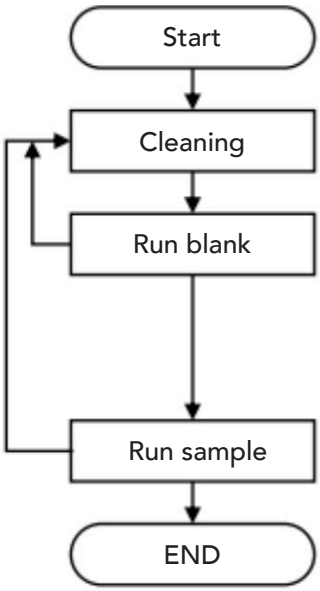
The current light obscuration process is also very labor-intensive and is interrupted by manual handling steps during both sample preparation and testing. This prevents users from working in a time-efficient manner as a significant amount of work is required to analyze each individual sample. Thus, the introduction of an automated workflow with "on-line" testing would result in significant savings both in time and money.

Biggest pain points of current light obscuration workflow

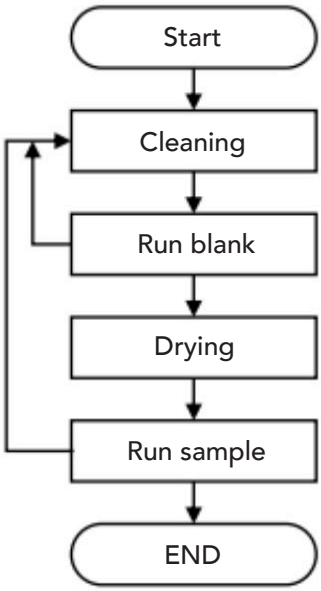
- Destructive measurement (samples are lost during testing);
- Inability to repeating tests with the same sample;
- Inability to perform consecutive or additional analyses with the same sample;
- Underestimation of sub-visible particle concentrations in samples with elevated viscosities;
- Low grade of automation;
- Expensive;
- One measurement at a time.



Current Light Obscuration (destructive process)



Bionter EVE (non-destructive)



The destructive nature of the existing light obscuration devices is due to the fact that the sample and the cleaning solution are mixed. Therefore, Bionter integrates a drying step to prevent the sample contamination. Unlike other systems, the Bionter particle counter uses pressurized filtered air to move the liquid sample and not a syringe.

Top: Single use consumables serve as temporary storage units for pharmaceutical samples prior and after particle counting. They reduce the volume of the fluid path, and ensure process efficiency.

Middle: The Single use consumable is particle free and comes as prefilled package with 72 containers

Bottom: The optical sensors for particle counting in Bionter's EVE meet the highest standards.

The Bionter approach: A non-destructive, fully automated approach to sub-visible particle counting

In order to overcome some of the challenges of the current light obscuration methods, Bionter focused on developing a new, non-destructive, automated light obscuration approach. Unlike the current instruments on the market, the Bionter particle counter does not consume or destroy the sample during testing; the workflow includes a drying step after ensuring the fluid path is clean. Therefore, the sample is not contaminated or diluted by the cleaning medium and can be used for further testing, minimizing sample loss. Also, there is no need to discard the first "diluted" sample aliquot, and the entire sample can be measured, which results in a reduction of the total sample volume needed in medicinal product testing.

In fact, the non-destructive nature of the Bionter particle counter allows pharmaceutical companies to repeat testing on the same sample. This offers the possibility to monitor particle population evolutions over time on a single sample. In case of a particulate finding, it also allows consecutive investigative analyses or the execution of orthogonal tests on the identical sample. Moreover, saving samples results in significant financial savings for biopharmaceutical companies focusing on developing new therapeutics. This innovative workflow is fully automated and runs unattended, which allows companies to make a major step towards a lean analytical workstream.

The light obscuration instrument consists of racks for primary packaging, a conveyor belt that transports the samples to an analysis area, bottles for cleaning media and waste, a fluidic system including the particle sensor, as well as single-use consumables that allow an efficient process. Users just need to load the samples onto the conveyor belt, refill the cleaning medium when necessary, empty the waste or refill the stack of single-use consumables. Unlike other systems, the Bionter particle counter uses pressurized filtered air to move the liquid sample and not a syringe pump.

Further, it adapts the pressure to the viscosity of each individual sample ensuring an accurate and precise flow. This revolutionary analytical sub-visible testing device is fully compliant with current United States Pharmacopeia, European Pharmacopeia, and Japanese Pharmacopeia requirements as it is based on the compendial light obscuration technology. Therefore, the analytical equipment can be used for development and quality control of both medical substance and medical product across the whole pharmaceutical product lifecycle.

Biggest challenges of the existing LO counters	**Current issues**	**Bionter's EVE**	**Impact**
Destructive method	Samples are lost during testing	Non-destructive testing	Minimize sample consumption; monitor particle evolution on a single vial; in case of particle findings, use orthogonal methods on identical sample
Manual handling steps	Time consumption of highly trained professinals; sample contamination	Smart automated workflow	Leave system unattended; avoid sample contamination due to pooling, transfer or dilution
Sample viscosity	Undercounting particles; false positive particle counts	Uses time/pressure for fluid flow; automatically determines viscosity level; multiple flow rates calibrated	Ability to accurately measure in a viscosity range >60cp
Large sample volume required	Requires pooling of units with smaller volume; risk of contamination and false positive results; higher costs	Minimal sample volume	Lower costs; no volume loss

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