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Amnis® Protein Aggregate and Silicone Oil Detection Kit

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Application

Characterization of particles within drug formulations is an important step in the development of safe and effective therapeutics. For protein therapeutics stored in pre-filled siliconized syringes, detection and discrimination of protein aggregates and silicone oil droplets is often necessary as aggregates may be potentially immunogenic while silicone oil is considered inert.

Combined with the Amnis® imaging cytometry platforms, this kit provides detection and discrimination of silicone oil droplets and protein aggregates using a convenient mix-and-read fluorescence assay. ProteoStat® Detection Reagent is used to detect aggregated protein based on 20–90 fold fluorescent enhancement upon binding cross-beta quaternary structure of aggregated proteins. Compared to conventional dyes such as Thioflavin T and Nile Red, ProteoStat detection reagent detects aggregates from a broader range of proteins, yield a much brighter signal, and provide superior performance in a broad range of buffer and pH (4–10) conditions. PMPBF2 (1,3,5,7,8-Pentamethyl-4,4-difluorro-4-bora-3a,4a-diaza-s-indacene) Silicone Oil Detection Reagent is a lipophilic green fluorescent dye that brightly labels silicone droplets.

Test Principle

This is a unique assay on Amnis® FlowSight® and ImageStream® platforms to detect and measure protein aggregates and silicone oil using two spectrally distinct fluorescent dyes. Lyophilized aggregated and monomeric IgG are included as positive and negative controls for protein aggregation, and simple instructions are provided for preparing a silicone oil droplet control. An analysis template using IDEAS® image analysis software is available for download, and detailed instructions for collecting and analyzing data are provided in this manual.

Workflow

Prepare samples (45 µL each)

- Experimental samples
- Silicone oil control
- Positive and negative IgG control
- Buffer only control
- Single-color compensation controls

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Prepare 10X staining reagents:

- 10X ProteoStat®/ PMPBF2 Staining Buffer
- 10X ProteoStat® Single-Color Staining Buffer
- 10X PMPBF2 Single-Color Staining Buffer
  *It may be necessary to further dilute 10X buffers into 1X Assay Buffer based on titration data

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Stain samples

- Add 5 µL of appropriate 10X Single-Color Staining Buffer to single-color compensation controls
- Add 5 µL 10X ProteoStat®/ PMPBF2 Staining Buffer to remaining samples
- Mix each sample gently with a pipette, and incubate 15 minutes at room temperature

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Run samples on the ImageStream® or FlowSight®

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Analyze data files in IDEAS® using analysis template provided

Kit Components

- 1000X ProteoStat® Protein Aggregate Detection Reagent (Part Number 4700-1681, 20 µL, 400 tests/vial)
- 1000X PMPBF2 Silicone Oil Detection Reagent: (Part Number 4700-1688, 20 µL, 400 tests/vial)
- Aggregated IgG Positive Control (Part Number 4700-1685, 100 µg, 20 tests/vial)
- Monomeric IgG Negative Control (Part Number 4700-1683, 100 µg, 20 tests/vial)
- 10X Assay Buffer (Part Number 4700-1680, 5 mL/vial)
- Nuclease Free Water (Part Number 4700-1684, 5 mL/vial)

Materials Not Supplied

1. ImageStream® or FlowSight® imaging cytometer
2. SpeedBead® ImageStream® System Calibration Reagent (Catalog No. 400041) or FlowSight® Calibration Beads (Catalog No. 400300)
3. 10% Bleach
4. Samples of interest
5. Microcentrifuge tubes
6. Micropipettes
7. Disposable pre-sterilized, filtered micropipette tips
8. Optional: Silicone Oil (Sigma Aldrich, CAS: 0063148629)
9. Optional: 0.1 µm Ultrafree® – MC VV Centrifugal Filters (EMD Millipore, Catalog No. UFC30VV25)
10. Optional: Round bottom 96 well plate (Corning, product # 3879) and X-Pierce™ film plate cover (Sigma-Aldrich, Catalog No. Z722502)

Precautions

- This product is for research use only and is not intended for diagnostic purposes.
- Wear proper laboratory attire (lab coat, disposable nitrile gloves, safety glasses) when handling this product.
- The ProteoStat® and PMPBF2 detection reagents contain DMSO which is readily absorbed through the skin. DMSO is harmful if ingested or absorbed through the skin and may cause irritation to the eyes. Observe appropriate precautions when handling these reagents. Fluorescent reagents should be treated as possible mutagens and should be handled with care and disposed of properly.
- During storage and shipment, small volumes of product may become entrapped in the seal of the product vial. For maximum recovery of the product, centrifuge the vial briefly before removing the cap.
- Do not use the reagent beyond the expiration date.
- Safety Data Sheets (SDSs) for kit reagents are available from our website (www.luminexcorp.com) or by contacting Luminex Technical Support.

Storage

All reagents are shipped on dry ice. Upon receipt, the kit should be stored upright and protected from light at ≤ -20°C. When stored properly, these reagents are stable for at least twelve months. **Avoid repeated freezing and thawing**. The reagents provided in this kit are sufficient for 400 samples with a 50 µL volume each. The PMPBF2 and ProteoStat® stains are stable for several hours upon incubation of sample.

Before You Begin

Titration: Perform a titration to identify appropriate dye concentration, as dye precipitates can form or Speed-Beads® used in the ImageStream® can label at high dye concentrations. To perform a titration, compare particle concentration (objects/mL) for sample containing silicone oil and/or protein aggregates against a buffer-only sample at decreasing dye concentrations. Table 1 shows titration data measured on FlowSight®. Signal/Noise (S/N) can be used to determine optimal concentration.

Table 1: Titration of ProteoStat® andPMPBF2 using FlowSight. Counts are shown in objects/mL for "Dye+" population (See Data Analysis, Step B). S/N (Signal/Noise) is defined as counts for particle-containing sample divided by counts in water sample for each dye concentration.

		Dye Concentration					
	Sample	**1X**	**1/2X**	**1/4X**	**1/8X**	**1/16X**	**1/32X**
ProteoStat	**Aggregates**	25,766,417	18,589,052	11,397,760	6,638,449	4,191,393	1,613,629
	Water	50,764	37,173	33,976	24,596	19,617	15,620
	S/N	507.6	500.1	335.5	269.9	213.7	103.3
PMPBF2	**Silicone Oil**	5,958,676	2,583,855	725,816	412,984	352,926	312,989
	Water	5,458,694	1,445,261	96,762	19,911	21,846	6,806
	S/N	1.1	1.8	7.5	20.7	16.2	46.0

Time considerations: The process of labeling samples with this kit takes approximately 30 minutes. Acquiring data on the ImageStream or FlowSight can vary depending on the experimental goals and sample concentration. Both instruments can image at rates of up to 5,000 particles/second, and process samples at approximately 1 µL/minute with typical settings.

Methods and Procedures

- Allow all reagents to thaw at room temperature before beginning the procedures.
- Briefly centrifuge vials to gather the contents at the bottom of the tube.
- At the time of first use aliquot 2-µL volumes of 1000X ProteoStat® and 1000X PMPBF2 stocks into separate tubes to avoid repeated freeze/thawing.

- It may be necessary to dilute samples to be within linear range of the instrument where particle concentration is very high. Typical linear range is <100 million/mL
- Perform experiment in biosafety cabinet if possible to minimize contamination with dust, bacteria, etc.

Preparation of Buffers

Prepare all buffers fresh each day of your experiment. A 2 µL minimum pipette volume is recommended, therefore buffer is prepared in excess for <36 tests. To ensure there is enough buffer for your experiments requiring >36 tests, prepare N+4 tests (2 for single color compensation controls, 2 for loss to pipetting).

1. **1X Assay Buffer:** Mix the following reagents together.

Component	<36 Tests	N Tests
10X Assay Solution	25 µL	0.50 µL x (N+4)
DI H_2O	225 µL	4.50 µL x (N+4)
Total Volume	250 µL	5.00 µL x (N+4)

2. **20X ProteoStat® Buffer:** Mix the following reagents together.

Component	<36 Tests	N Tests
1000X ProteoStat	2 µL	0.05 µL x (N+4)
1X Assay Buffer	98 µL	2.45 µL x (N+4)
Total Volume	100 µL	2.50 µL x (N+4)

3. **20X PMPBF2 Buffer:** Mix the following reagents together.

Component	<36 Tests	N Tests
1000X PMPBF2	2 µL	0.05 µL x (N+4)
1X Assay Buffer	98 µL	2.45 µL x (N+4)
Total Volume	100 µL	2.50 µL x (N+4)

4. **10X ProteoStat/PMPBF2 Staining Buffer:** Mix the following reagents together.

Component	<36 Tests	N Tests
20X ProteoStat	90 µL	2.25 µL x (N+4)
20X PMPBF2	90 µL	2.25 µL x (N+4)
Total Volume	180 µL	4.50 µL x (N+4)

5. **10X ProteoStat Single-Color Staining Buffer:** Mix the following reagents together.

Component	1 Test
1X Assay Buffer	5 µL
20X ProteoStat	5 µL
Total Volume	10 µL

6. **10X PMPBF2 Single-Color Staining Buffer:** Mix the following reagents together.

Component	1 Test
1X Assay Buffer	5 µL
20X PMPBF2	5 µL
Total Volume	10 µL

Preparation of Controls

1. **Aggregated Protein Controls:** Aggregated IgG Positive Control and Monomeric IgG Negative Control are supplied as lyophilized powder (100 µg each). Add 500 µL Nuclease Free Water to each vial and gently mix. Do not vortex or cause unnecessary bubbles. Unused stock control samples may be stored in aliquots at 4°C for several weeks. Do not centrifuge the positive control, as this can pellet the aggregates. On the day of the experiment, mix 25 µL of each control with 25 µL nuclease free water or buffer of choice to generate positive and negative controls (100 µg/mL). Control standard may need to be further diluted based on high particle concentration if the lot has a small size distribution.
2. **Silicone Oil Control:** A silicone oil control can be prepared by adding 10 µL silicone oil (not included) to 50 mL deionized water or buffer of choice (0.02%), vortexing briefly, and sonicating for 10 minutes. The silicone oil positive control can be reused several times, but should be vortexed and sonicated before each experiment.
3. **Buffer Control:** Prepare a sample containing filtered sample buffer only to measure background particles.
4. **Single-Color Controls:** Prepare a sample containing protein aggregates for the ProteoStat® single-color compensation control; prepare a sample containing silicone oil droplets for the PMPBF2 single-color compensation control.

Fluorescent Staining

1. Add 45 µL of each sample to a 1.5 mL Eppendorf tube for single sample loads or a 96-well plate for the autosampler.
2. To each single-color compensation control, add 5 µL of the appropriate 10X Single-Color Staining Buffer.
3. To each remaining sample, add 5 µL 10x ProteoStat®/PMPBF2 Staining Buffer.

4. Gently mix each sample by pipetting.
5. Protect the sample from light and incubate for 15 minutes at room temperature.

Acquire data

1. Detailed instructions on using the systems are available in the INSPIRE™ User's Manual for the Amnis® brand ImageStream® or FlowSight® Instruments.
2. Use the following INSPIRE version:
   - ImageStream^X - 4.1.501 or later
   - ImageStream^X Mark II - 200.0.755 or later
   - FlowSight - 100.2.256 or later
3. Set Illumination Settings
   - Turn on the 488-nm laser to generate ProteoStat® and PMPBF2 images. PMPBF2 emission is in Ch02 and ProteoStat Ch04.
     - To optimize sensitivity for small particles, set the 488-nm laser power to maximum intensity. **Note:** this may cause saturation of fluorescence signal for larger particles.
     - To avoid signal saturation, laser powers can be set based on a labelled sample that contains the largest particles. **Note:** this will reduce detection sensitivity for small particles.
   - Set brightfield to 1 for six-channel instrument and 1 & 9 twelve-channel instrument.
   - Turn on the 785-nm laser to generate SSC images. 10 mW used for experiments in this manual. SSC into Ch12 is recommended when available.
4. Set Collection Parameters
   - Collect the 'All' population, or apply a fluorescence Intensity threshold to the PMPBF2 and ProteoStat channels (See Data Analysis, Step B) to discard fluorescence negative events and reduce file size.
   - Input the number of events to be collected
5. Acquire data files for each sample and single color compensation control. Gently resuspend sample with a pipette prior to loading it into the instrument.
   - **Note:** When Collecting single color compensation controls, it is critical that the SSC (785 nm) laser and the brightfield are OFF and that ALL channels are enabled.
   - **Note:** Before collecting each single color compensation control, briefly load a 10% bleach sample and then water sample to remove any leftover fluorescent staining in the tubing.
   - **Note:** Reduce carryover by loading a 10% bleach sample to quench fluorescence in tubing followed by a water sample to wash remaining bleach. This is especially important when following a sample with high concentration by a sample with low concentration.

Data Analysis

Below is an example analysis to identify and size silicone oil droplets and protein aggregates using IDEAS® image analysis software.

This template may be requested from Luminex Technical Support at *support@luminexcorp.com*.

Use IDEAS version 6.2.71 or later. For additional instructions refer to the *IDEAS User's Manual*.

1. First, open the data file and perform compensation.

   NOTE: The dyes used in this kit spectrally shift depending on their local environment, and consequently are difficult to compensate with high accuracy. For example, PMPBF2 fluorescence is red-shifted when bound to silicone oil compared to protein aggregates due to increasing hydrophobicity of the binding site. We recommend using Uncompensated Intensity features to avoid the challenges of compensating spectrally shifting dyes, as shown in the analysis below. While this analysis does not require a compensation matrix, it is still recommended that a compensation matrix is applied, as this is useful for visually verifying heterogeneous particle complexes, and generating compensated brightfield and SSC imagery for further morphological analysis.

   - Open IDEAS and click on '**Start Analysis**'.
   - Select **Data file** to open, click '**Next**'.
   - Click '**New Matrix**' The compensation wizard opens. Or you may select a matrix previously created.
     - **Step 1: Select the control files for compensation.** Click 'Add files' and select your single color control files. Click 'Next'.
     - **Step 2: Select/remove channels for compensation.** For this kit you will use Channels 2 and 4. Check appropriate boxes and click 'Next'.
     - **Step 3: Validate the compensation matrix.** Click 'Finish' and save the .ctm file. The Best Fit method is recommended and the best fit lines can be validated by double-clicking on the coefficient values. For more details on validating compensation, see the *IDEAS User manual*. The compensation wizard ends and the matrix is added. Click 'OK' and then Click 'Next.' The template that you requested from Technical Support may be loaded here.
     - **Step 4: Name your file.** Click 'Next' It is recommended to keep the default names

2. Second, gate populations using scatter plots and histograms (Figure 1).

   - **Step A: Gate out SpeedBeads (ImageStream® Only):** Position the "Not SpeedBeads" region to exclude SpeedBeads which have high SSC (Ch06 or Ch12) Intensity and low Ch02 Intensity.
   - **Step B: Gate PMPBF2 Or ProteoStat® positive events.** Position the "Dye+" region to include objects with Ch02 and Ch04 Uncompensated Intensities above a threshold. An appropriate threshold may be determined using a stained buffer sample. A minimum threshold of 200 was used for experiments in this manual.
   - **Step C: Discriminate Silicone Oil from Protein Aggregate.** Position the "Protein Aggregate" region to include particles shifted toward Ch04; position the "Silicone Oil" region for include particles shifted toward Ch02. Protein Aggregate Positive Control and Silicone Oil Positive Controls can be used to determine the appropriate location for each region.
   - **Step D: Measure Silicone Oil size distribution using the Diameter feature.** The template defines regions for >2 µm, >10 µm, and >25 µm size bins. Move or create regions to generate desired size bins.
   - **Step E: Measure Protein Aggregate size distribution using the Diameter feature.** The template defines regions for >2 µm, >10 µm, and >25 µm size bins. Move or create regions to generate desired size bins.

Tips

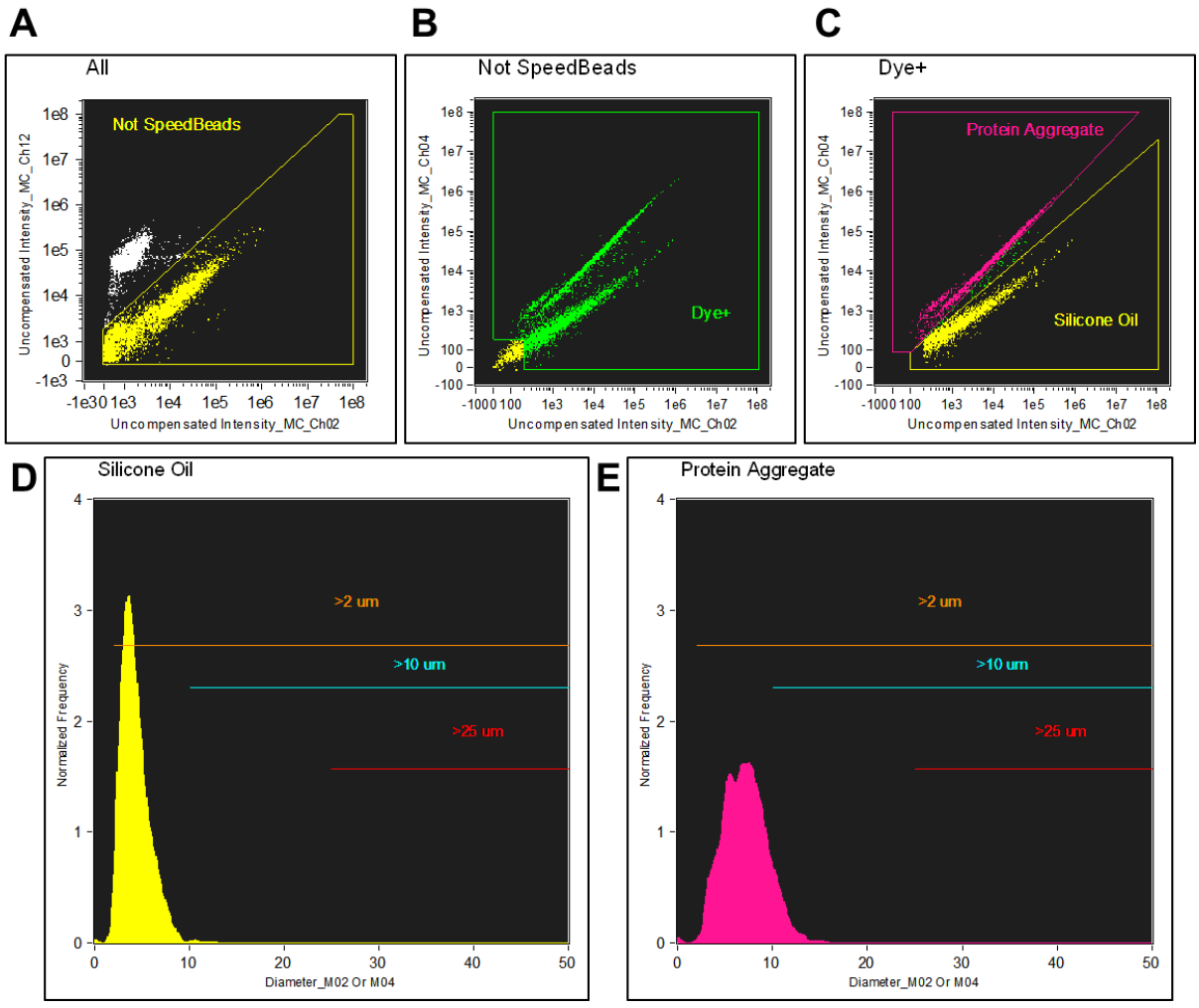
1. In this template, Diameter is measured using the mask 'M02 Or M04', which masks any pixels above background in the PMPBF2 or ProteoStat® channels. If desired, Diameter can be measured on a different mask, for

example a brightfield mask. However, note that a brightfield mask may not be accurate for very transparent particles.

2. Use the Region Manager to set exact coordinates and rename regions.

If you do not use the template you will need to use the Feature Manager to create some of the features.

Figure 1: Sequence of analysis steps for ImageStream®. **Step (A)***: Gate out SpeedBeads®, **Step (B)**: Gate events positive for PMPBF2 or ProteoStat, **Step (C)**: Identify Protein Aggregate and Silicone Oil populations, **Step (D)**: Measure Silicone Oil size distribution, **Step (E)**: Measure Protein Aggregate size distribution. *Skip Step (A) for FlowSight®.

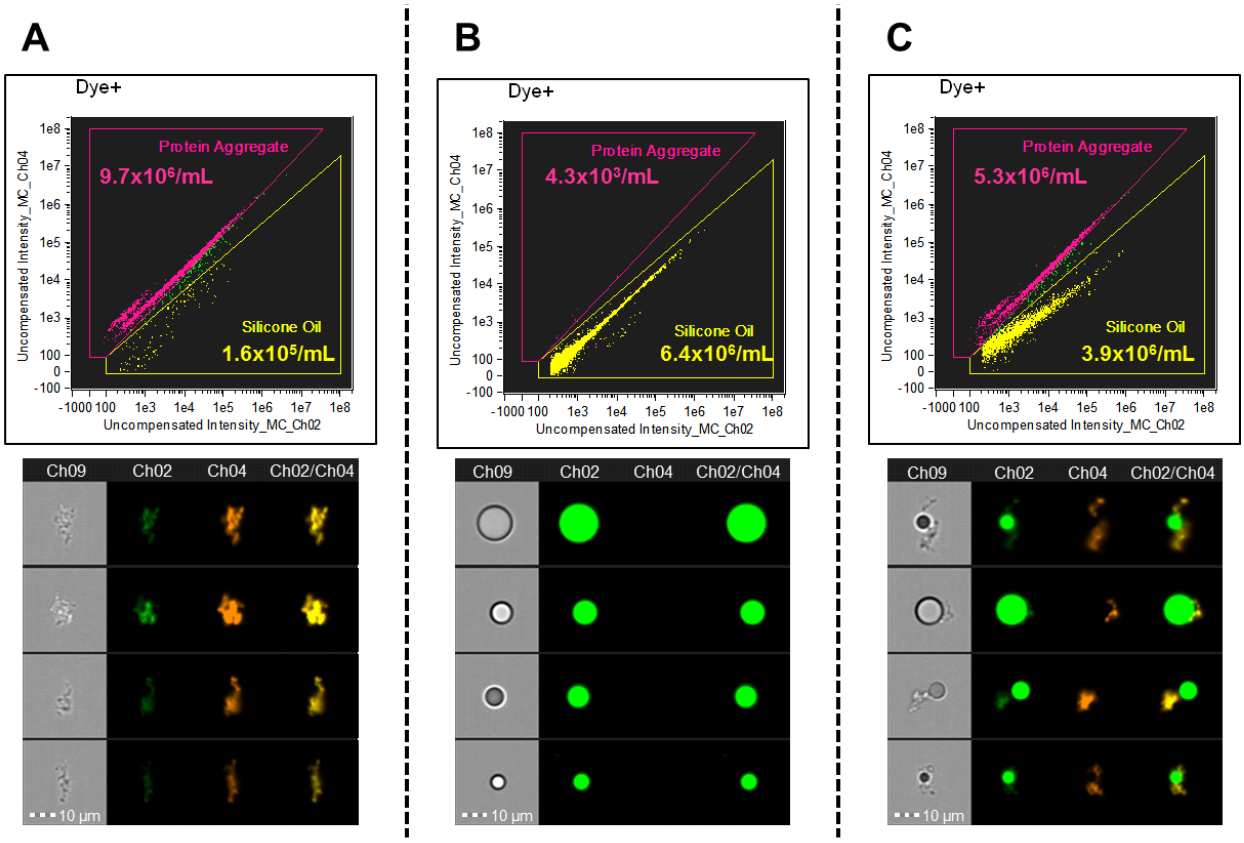


Sample Results

Figure 2: Example of ImageStream® data. Fluorescence classification of particles and representative images for (A) Protein 1 (stressed IgG, 30 µg/mL), with images of aggregates, (B) Silicone Oil, with images of silicone oil droplets (0.02%), (C) a 50:50 mixture, with images of heterogeneous complexes containing both aggregate and silicone oil droplet. (D) Size distribution analysis.

Results: For samples containing a single particle type, protein aggregate and silicone oil populations are located in

distinct regions of the scatter plot. When mixed, protein aggregates and silicone oil droplets remain in similar locations on scatter plot, indicating they are mostly homogeneous. Upon visual inspection of imagery, particles with fluorescence intensities in-between Protein Aggregate and Silicone Oil regions are heterogeneous particle complexes comprised of both particle types. Protein 1 has sufficient contrast to be visualized brightfield image. Approximately 90% of aggregates are <10 µm for Protein 1, and nearly 100% of silicone oil droplets are <10 µm for the silicone oil emulsion.



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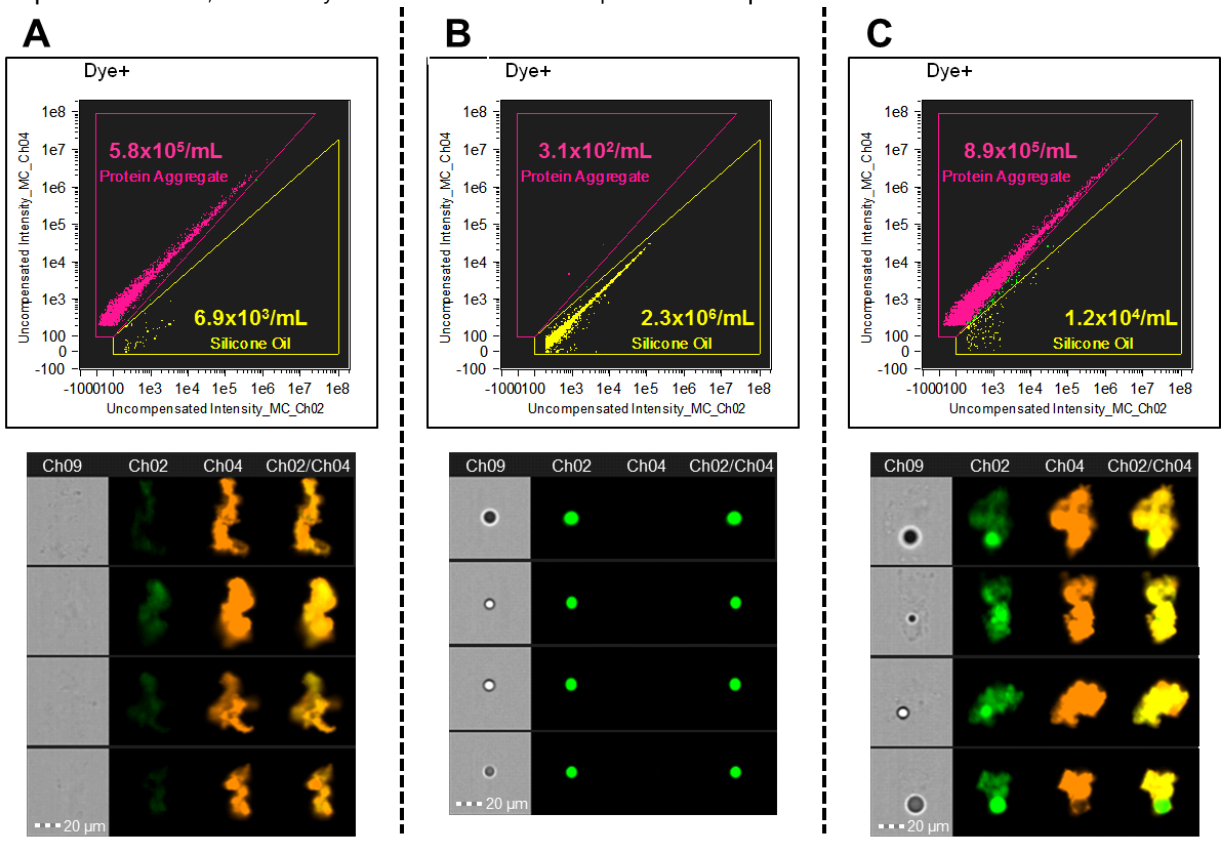
	Silicone Oil % Gated			Protein Aggregate % Gated		
Sample	**>2 µm**	**>10 µm**	**>25 µm**	**>2 µm**	**>10 µm**	**>25 µm**
Protein 1	98%	1%	0%	100%	12%	0%
Protein 1 + Silicone Oil	99%	0%	0%	100%	11%	0%
Silicone Oil	97%	0%	0%	100%	0%	0%

Figure 3: Example of FlowSight® data. Fluorescence classification of particles and representative images for (A) Protein 2 (stressed Lysozyme, 500 µg/mL), with images of aggregates, (B) Silicone Oil (0.02%), with images of silicone oil droplets, (C) a 50:50 mixture, with images of heterogeneous complexes containing both aggregate and silicone oil droplet. (D) Size distribution analysis.

Results: For samples containing a single particle type, protein aggregate and silicone oil populations are located in distinct regions of the scatter plot. When mixed together, a sharp decrease of particles in the Silicone Oil region is observed. Upon visual inspection of imagery, the silicone oil particles have adsorbed to large protein aggregates and there is significant increase in Ch02 Uncompensated Intensity. Protein 2 lacks sufficient contrast to be

visualized in brightfield, and should be sized using the fluorescent image. Approximately 82% of aggregates are <10 µm for Protein 1, and nearly 100% of silicone oil droplets are <10 µm for the silicone oil emulsion.



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	Silicone Oil % Gated			Protein Aggregate % Gated		
Sample	**>2 µm**	**>10 µm**	**>25 µm**	**>2 µm**	**>10 µm**	**>25 µm**
Protein 2	82%	0%	0%	97%	16%	2%
Protein 2 + Silicone Oil	67%	6%	0%	98%	13%	2%
Silicone Oil	99%	1%	0%	100%	0%	0%

Technical Hints

- Use positive and negative particle controls to verify that an experiment worked.
- Staining concentrations should be optimized using titration of researchers' own samples.
- Verify particle concentration is within linear range of instrument (<100 million/mL).
- Sample dilution can occur for early time points from mixing with sheath. This can be avoided by increasing the prime volume in INSPIRE™. For this assay we recommend using a 15 µL prime rather than the default 5 µL prime volume. Contact Luminex Technical Support to change these settings.
- For samples containing larger particles, sedimentation can affect concentration measured over time. Control for sedimentation by collecting each sample under similar conditions, or collecting sample until dry.
- As a default, clipped objects will be discarded with INSPIRE, which can underestimate particle concentration, especially for very large particles where clipping is more likely. Clipped objects can be retained by checking the 'Keep Clipped Objects Box' under Advanced > Acquisition.

Troubleshooting

Potential Problem	Possible Cause	Suggestion
Acquisition rate decreases dramatically	Instrument clogging	The flow cell is 250 µm wide. Ensure particles/clumps greater than 200 µm are not present.
Acquisition rate increases/decreases over time Fewer/greater particles measured than expected for concentration standard	Particle sedimentation	Collect each sample for the same time duration with the same start time (e.g., 1 minute after loading) to allow inter-sample comparison. If absolute counts are required, collecting the entire sample volume will control for sedimentation effects. If it is desirable to collect less sample than loaded, a set amount of the sample can be primed out. Consult Luminex for how to use these settings.
	Sample mixing with sheath fluid upon loading	Increase the prime volume to 15 µL to eliminate the diluted portion of the sample. Consult Luminex for how to use these settings.
No events measured for highly concentrated sample	Sample is too concentrated and individual events can no longer be identified	Dilute with the sample buffer or until linear particle counts are measured (the typical linear range is less than 1×10^8 particles/mL).
Single-color ProteoStat® compensation control has PMPBF2 in it	PMPBF2 has stained instrument tubing and is contaminating subsequent samples	Before acquiring compensation controls, sterilize the sample line or load a 10% bleach sample to quench leftover stain in the tubing, followed by a deionized water sample to remove leftover bleach.
Single-color PMPBF2 compensation control has ProteoStat in it	ProteoStat has stained instrument tubing and is contaminating subsequent samples	Before acquiring compensation controls, sterilize the sample line or load a 10% bleach sample to quench leftover stain in the tubing, followed by a deionized water sample to remove leftover bleach.

Potential Problem	Possible Cause	Suggestion
Poor fluorescent signal is observed in the positive control	Detection reagent has been exposed to strong light	Protect samples from exposure to strong light.
	Kit reagent has degraded	Verify that reagents are not past their expiration dates and have been stored according to recommendations in this manual.
	Instrument settings are not correct	Use the ImageStream® or FlowSight® to collect data. Ensure the 488-nm laser is set to the appropriate power. Channel 2 should be used for PMPBF2 fluorescence and Channel 4 should be used for Proteo-Stat fluorescence.
	Insufficient dye concentration	Perform a titration to identify optimal concentration. Validate increased concentration does not result in generation of particles or unacceptable non-specific binding.
	The aggregated protein is not in solution	Do not centrifuge the aggregated protein in samples or control. Centrifugation will cause the aggregate to precipitate.

Potential Problem	Possible Cause	Suggestion
High fluorescence-positive particle counts observed in negative control	Inappropriate dye solution	Follow the procedures in this manual. Perform a titration to identify optimal concentration. Validate increased concentration does not result in generation of particles or unacceptable non-specific binding. It is important that there are no particles in the dye. Centrifuge dye well before use.
	PMPBF2 is unstable in buffer	PMPBF2 is a lipophilic dye and maybe unstable under some aqueous buffer conditions. PMPBF2 precipitates are very red shifted, and are brightest in Ch05. PMPBF2 precipitates can be excluded from analysis using a Ch04 vs. Ch05 plot and gating out events where Ch05/Ch04 >1. Protein aggregates brightest in Ch04 will be retained using this strategy.
	Protein aggregates are present in monomeric protein control	During reconstitution and storage a small amount of protein particles can form in the monomeric solution. Use a 0.1 µm filter to remove any remaining aggregates in monomeric control.
	Carryover from previous sample	When going from sample with high-particle load to low particle load, load a 10% bleach sample to quench leftover stain in the tubing, followed by a deionized water sample to remove leftover bleach.
	The sample and/or the instrument is contaminated	Add a fluorescent stain to sample to identify bacteria in instrument as well as in sample. Luminex has an instrument decontamination protocol that is available upon request.
There appears to be no protein in the aggregated controls	Sometimes the dried protein is difficult to see	Resuspend the protein as recommended.