GENProtocols

Protocol

Detect Specific Protein Aggregates in Biologic Formulations with the Aura Immunoassay

ANALYTICAL TESTING ⭐   **DRUG DISCOVERY**   **CANCER RESEARCH**

Steven Le, Halo Labs

Aug 04, 2021

Abstract

Biologic co-formulations target multiple pharmacologically active sites to increase therapy efficacy. However, either biologic can be a source of protein aggregation that can negatively influence the product's safety, efficacy, and therapy. Aura systems are the only particle analyzer that can pinpoint exactly which biologic is aggregating, so you'll know which molecule to focus on stabilizing during formulation. It uses a primary antibody specific to one of the biologics in your co-formulation, a secondary antibody conjugated to a fluorophore, and fluorescence membrane microscopy (FMM) to detect and quantitate just the particles you are interested in. The Aura immunoassay can be multiplexed and only requires 2.5 hours to label the aggregate with both primary and secondary antibodies before 96 samples are imaged in a little under an hour. Find your best formulation with Aura, even with complex co-formulations.

Introduction

Antibodies are widely used in immunoassays to reliably target and identify a particular protein of interest. Proteins are labeled with a primary antibody specific to the protein and detected with a secondary antibody conjugated to a chemiluminescent or fluorescent molecule. Countless applications using antibody-protein interactions have been developed for PK/PD assays to identify and quantify protein content. The Aura™ aggregate analysis system utilizes Fluorescence Membrane Microscopy (FMM) to identify particles stained with a fluorescent dye. For example, proteinaceous particles stained with Thioflavin T (ThT) can be differentiated from other non-proteins, as described in Application Note 7, Rapidly Distinguish Protein from Non-Protein Particles in Biologic Formulations. Labeling protein particles with fluorescently tagged antibodies enables specific and multiplexed protein identification of sources of stability issues in co-formulated antibody therapeutics in conjunction with subvisible particle information.

Here, we introduce a two-step fluorescent antibody labeling method that identifies aggregates of a specific protein in a mixed sample with a high degree of specificity and accuracy on the Aura system. The method is robust and can be used with proteins labeled both in their native liquid state as well as post filtration.

Reagents and Equipment

Reagents

- Human IgG (HIgG)
- Hen egg-white lysozyme (HEWL)
- Rabbit monoclonal recombinant Human-IgG antibody (AbCam ab109489)
- Alexa Fluor® 488 conjugated goat anti-rabbit IgG (AbCam ab150077)
- PBS

- Bovine IgG (BIgG)

Instruments

- Aura instrument
- Black Halo Labs plates

Procedure

Human IgG (HIgG) and hen egg-white lysozyme (HEWL) proteins were antibody labeled using a solution phase or membrane phase protocol before imaging on the Aura. In both methods, ThT was applied to each well after the initial image acquisition to determine the total protein content to corroborate the antibody labeling efficiency and accuracy. ThT staining and IgG labeling were imaged using the FL1 and FL2 channel, respectively.

Solution Phase Labeling

50 µg/mL HIgG or HEWL were each incubated in a 1.5 mL Eppendorf tube with rabbit monoclonal recombinant Human-IgG antibody (AbCam ab109489) diluted 1:200 in PBS for 90 minutes at room temperature with rotation (Figure 1). After the primary incubation, Alexa Fluor® 488 conjugated goat anti-rabbit IgG (AbCam ab150077) diluted 1:100 in PBS was added to the solution and incubated at room temperature for 60 minutes with rotation. An antibody only sample was used as a negative control. 35 µL of each sample was applied to a black membrane plate well and measured in triplicate. For the mixed HIgG + HEWL sample, 35 µL of labeled HIgG was first filtered through the membrane before 35 µL of labeled HEWL was applied. All samples were washed 3x with PBS to reduce non-specific binding prior to imaging. Bovine IgG (BIgG) was used as a separate control for multiplexed solution phase labeling to assess co-formulation application feasibility.
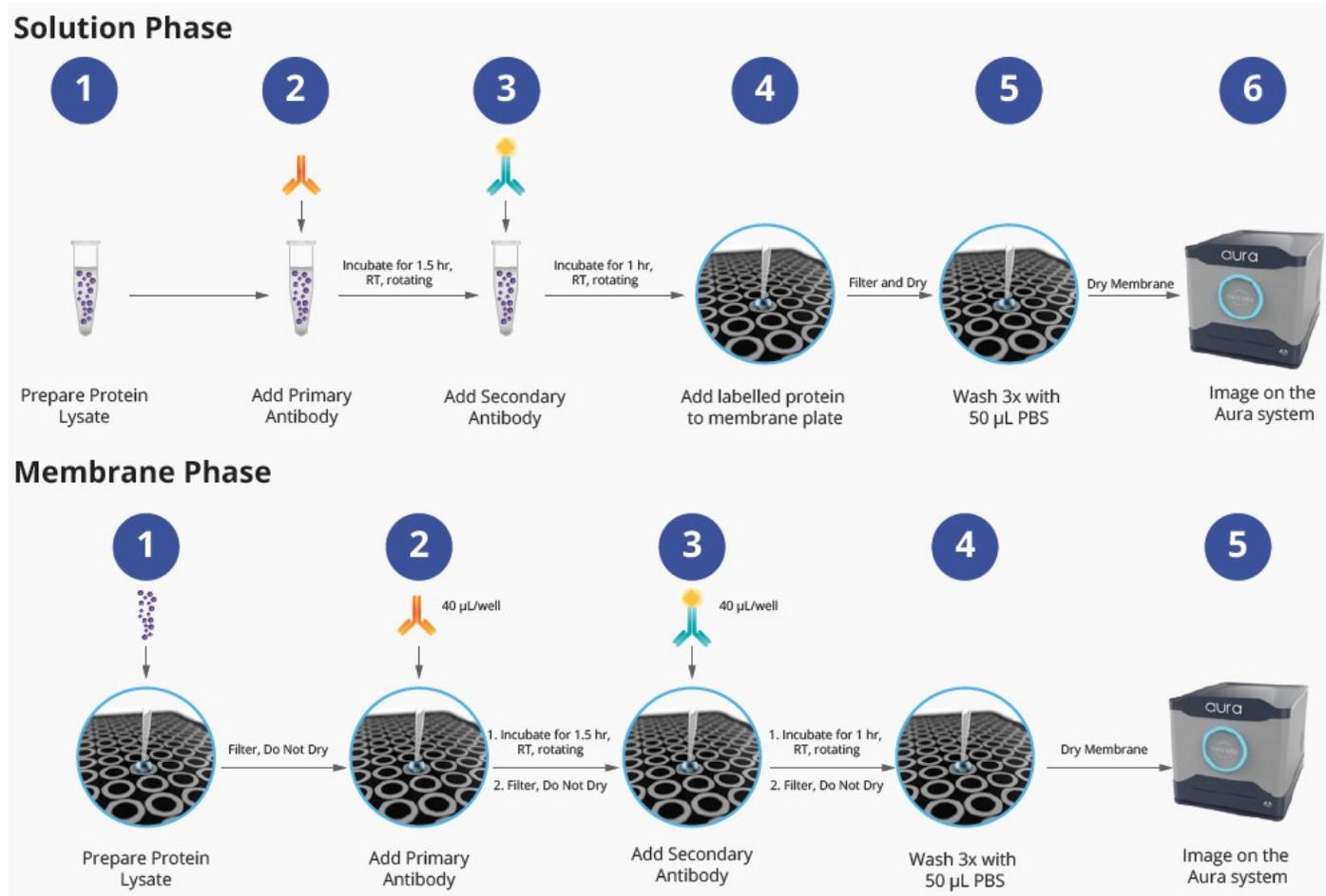

Figure 1: Schematic representation of the two antibody labeling protocols. The solution phase protocol labels the protein aggregates with antibody in PBS before applying the protein-antibody conjugate to the membrane. The membrane phase

protocol applies the protein to the membrane first and antibody labels in the plate.

Membrane Phase Labeling

35 μL of HIgG, HEWL, or 35 μL of HIgG + HEWL was applied to corresponding wells in a black membrane plate in triplicate. Antibody only was used as a negative control. 50 μL of the HIgG antibody (1:200 dilution in PBS), were applied to each well and left to sit but not vacuumed for 90 minutes. Then, 50 μL of secondary antibody (1:100 in PBS) was applied to the membrane for 60 minutes before filtering the plate. All sample wells were washed with PBS prior to imaging.

Results

Samples that were antibody labeled using the solution phase protocol were imaged on the Aura (Figure 2a). The red signal in the combined brightfield and fluorescent image represents total protein content stain by ThT, green designates proteins fluorescently labeled with antibody, and the yellow represents the combination of both signals.

Most of the signal in the HIgG well (top left) is yellow indicating these are HIgG protein particulates. In the HEWL image (top right) the protein is only stained red with ThT (total protein) revealing that while there is protein, it is not HIgG. The HIgG + HEWL mixed sample (bottom left) shows the ability of the Aura to specifically recognize HIgG protein aggregates in a solution containing HIgG (yellow) and HEWL (red). While the primary antibody only control (bottom right) does form some protein aggregates, there is no discernable fluorescent signal due to secondary antibody labeling.

To demonstrate feasibility for multiple antibody coformulation applications, samples were labeled with HIgG and Bovine IgG primary antibody. The assay discerns between two different antibodies and isn't limited to just differentiating between two different proteins (Figure 2b). Human IgG on the left displays significant staining in the FL2 channel, while Bovine IgG does not.
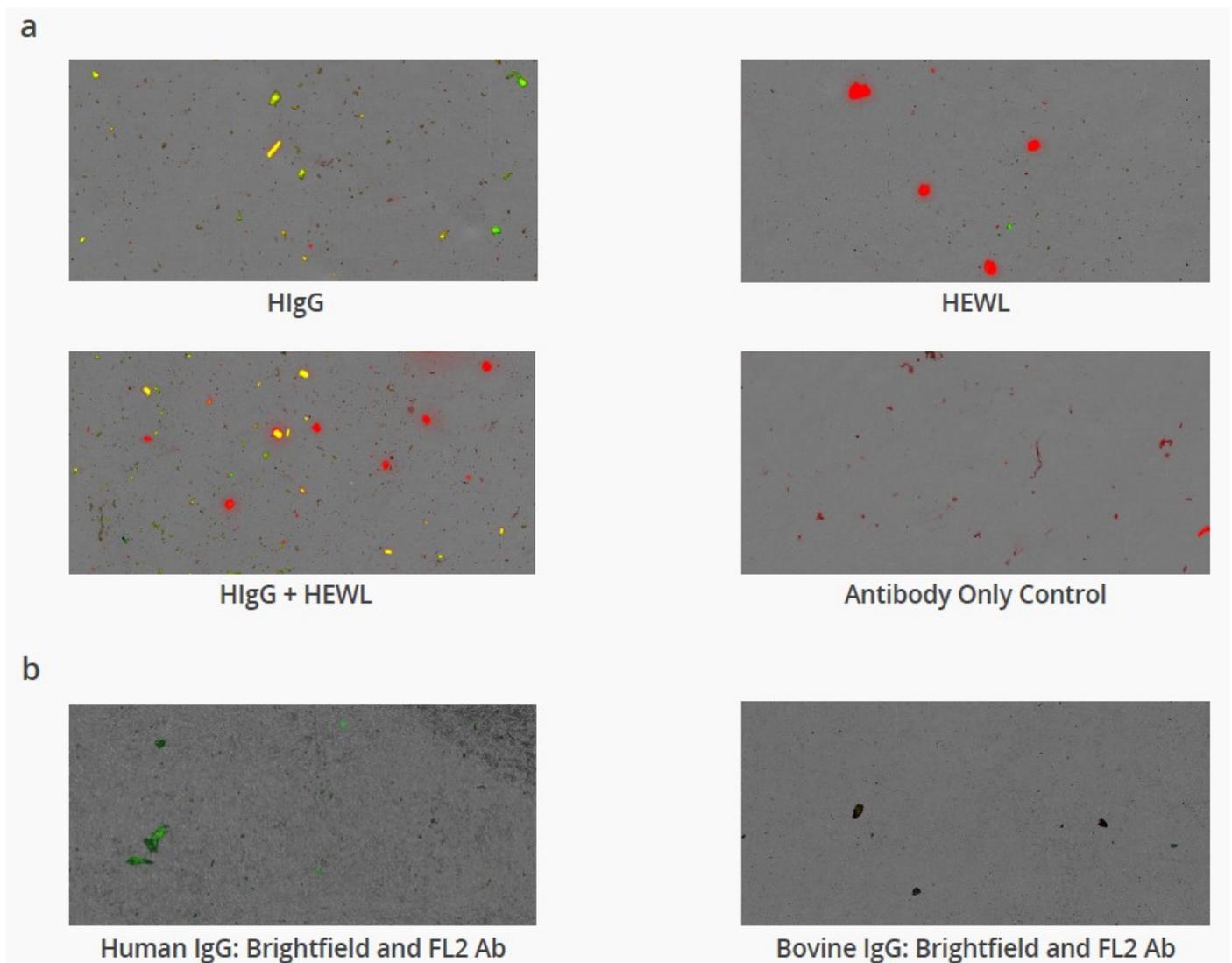
Figure 2: Combined brightfield and fluorescence images from the Aura system from solution phase antibody labeled HIgG, HEWL, and HIgG + HEWL mixed sample (a) and solution phase labeled HIgG and Bovine IgG (b).

Antibody samples labeled using the membrane phase labeling protocol were also imaged on the Aura (Figure 3). Again, the red signal in the fluorescent images represents total protein stain by ThT, green is antibody fluorescence, and yellow is the combination of both signals. Most of the signal in the HIgG well (top left) is yellow indicating that these are HIgG protein particulates, just like in solution phase stained samples. In the HEWL image (top right) the lysozyme protein is only stained red with ThT (total protein) suggesting that while it is a protein, it is not antibody labeled HIgG. The HIgG + HEWL combination (bottom left) shows how Aura can differentiate between different proteins in a lysate containing multiple proteins, as HIgG protein is dually labeled and stained with antibody and ThT, respectively (yellow) and the HEWL is only stained with ThT (red). While the antibody only control (bottom right) does form some protein aggregates, there is no discernable fluorescent signal due to secondary antibody labeling.

Quantitative Analysis and Assay Accuracy

We then quantitated total protein counts as measured in brightfield (all particles), FL1-ThT (all proteins), and FL2-Antibody (HIgG only) channels (Figure 4). Total fluorescent counts were calculated using appropriate thresholding in the Expression Engine function of Particle Vue software. The data show that the Aura immunoassay correctly detects over 80% of HIgG protein aggregates > 5 μm present on the membrane, while delivering a 13% false positive rate, likely correlating with the overall specificity of the antibody to protein aggregates vs. native state proteins. Negative controls containing ETFE, antibody only, and PBS did not display significant values for FL2-Antibody signal despite the presence of particles in the brightfield (ETFE, and Antibody only) as well as FL1-ThT (antibody only) (data not shown). Thioflavin-T is a highly sensitive and specific protein dye

which reliably stains all proteins aggregates (>99% staining efficiency for aggregates ≥5 µm). The difference in the number of FL1-ThT and FL2-Antibody positive particles in the HEWL group corresponds to the decrease in the respective groups in the HIgG + HEWL group. These data show that the Aura immunoassay is capable of accurate and precise protein labeling and these data confirm specific labeling of Human IgG protein aggregates with primary and secondary antibody, as well as their detection with the Aura system.
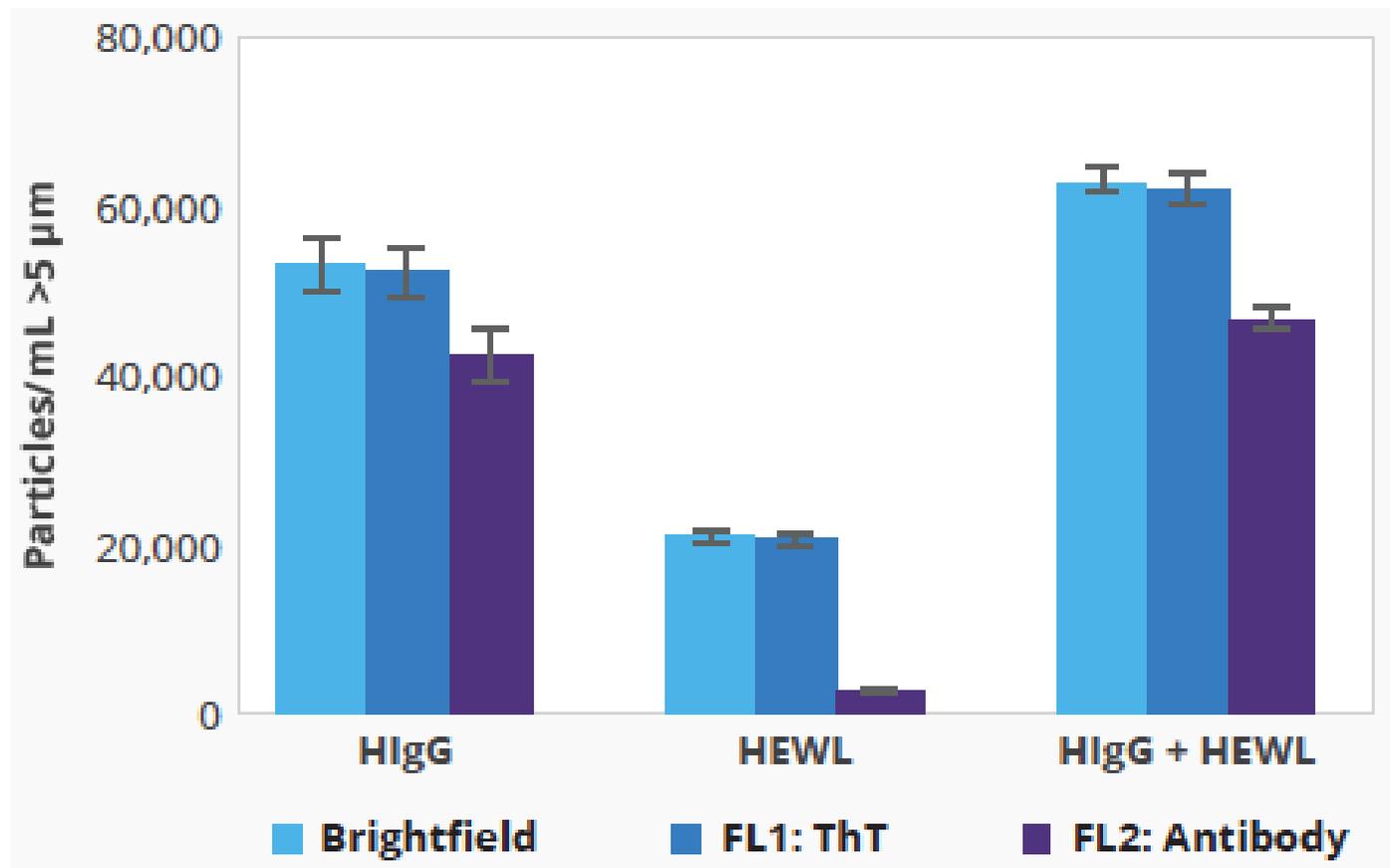

Figure 4: : Fluorescent particle quantitation analysis of antibodylabeled and ThT stained protein aggregates in HIgG, HEWL, and amixed HIgG + HEWL sample.

Notes and Comments

The Aura is capable of specifically detecting different protein aggregates on a membrane using fluorescent antibody-based labeling. Additionally, this assay discerns between two different antibodies (HIgG and BIgG), which is useful for antibody co-formulation applications and assessing drug product vs carrier protein aggregates. Powered with imaging in brightfield as well as two fluorescent channels, the Aura can help bridge the gap with common assays and subvisible particle stability. Custom fluorescence channels are optional to adapt to your fluorescent assay needs.

References

N/A