Flow Cytometry as a Tool for Potency Determination in ATMP Development

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BACKGROUND

Developing methods to determine potency for Advanced Therapeutic Medicinal Products (ATMPs) is particularly challenging. The assay(s) must strike a balance between science, compliance, and efficiency. Flow cytometry has shown significant potential as a platform for potency assays due to its selectivity, ability to generate multiple data sets from a singular readout, and ability to establish a functional relationship to the therapeutic mechanism of action. There are specific situations in which the application of flow cytometry may offer discrete advantages over traditional and/or matrix assay approaches. Here we share several case studies to demonstrate the application of flow cytometry to the phase appropriate development and/or validation of potency assays for ATMPs including mRNA vaccine (LNP), AAV, plasmid, etc. The detection targets include replacement proteins, surface expressed antigens, and others. Case studies compare flow cytometry with other detection approaches, providing insights into when to use or avoid this technique for potency assays. Finally, the creation of criteria for the qualification, validation and/or transfer of flow methods is discussed to facilitate the implementation of these methods as part of robust potency assurance strategy.

CASE STUDY #1 – mRNA LNP FOR VACCINE DELIVERY

Lipid nanoparticles are frequently used with the objective to deliver mRNA which is translated in vivo to a target antigenic protein as part of a vaccine strategy. These proteins are often then expressed in model cellular systems to create methods to determine the relative potency of the ATMP in question. Here the LNP-mRNA is transfected into HEK293T cells, and three separate expressed antigens are to be detected and quantified as a function of dosed drug, multiplicity of infection (MOI).

Two analytical techniques are used to monitor the proteins. Flow cytometry (Beckman Cytoflex) is used to detect the expressed protein using antibodies directed to them. The proteins may be cytosolic, or membrane bound. Mass Spectrometry (MS) is also used to detect the proteins after cell lysis and digestion of the released proteins to create surrogate peptides. The peptides are detected by HPLC connected to high resolution mass spectrometry (Thermo Scientific, Orbitrap). Isotopically labelled peptides are used to aide quantification as a housekeeping protein. The MS method is intended as a proof of principle evaluation of the approach for comparison to flow cytometry.

The workflow is provided in the Figure 1 below. In addition, representative data from both the Flow and MS methods are provided as a function of LNP/mRNA dosing. Both methods show a discrete dose-response in the signals. The flow cytometry provides a readout with strong biological relevance and MS provides a detection method for expressed protein that overcomes the need for targeted reagent (i.e. antibody). Flow cytometry itself overcomes the challenge of multiple or diverse protein expression (cytosolic, membrane, etc).

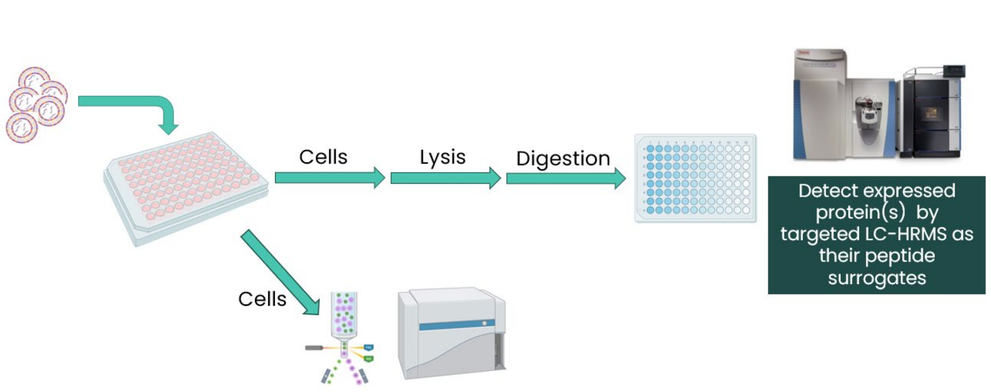

Figure 1. mRNA LNP Transfection into HEK cells and Protein Detection (process of transfecting cells and detecting expressed proteins)

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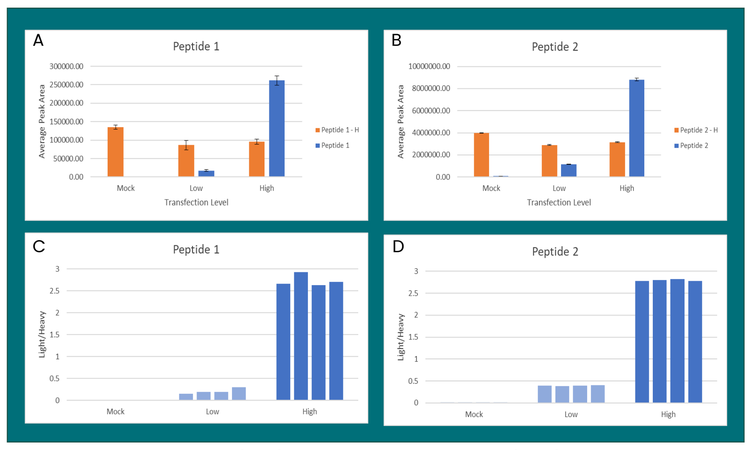

Table 1. Flow Cytometry (A, B) vs Mass Spectrometry (C, D): Key Metrics Comparison

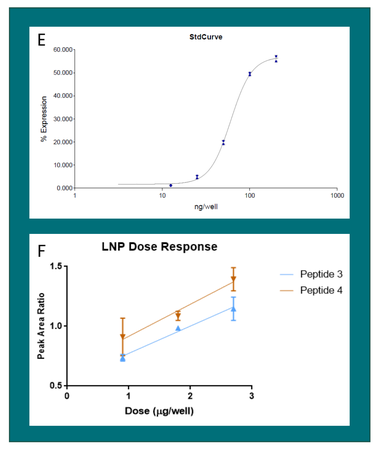

Figure 2. Comparative Analysis of Flow Cytometry (E) and Mass Spectrometry (F) (in detecting protein expression across varying does of LNP/mRNA)

CASE STUDY #2 – AAV FOR REPLACEMENT PROTEIN THERAPY IN METAL ION TRANSPORT

Flow Cytometry is a key platform for measuring intercellular functions using immunochemical, activity and chemical staining methods. In this instance an AAV is used to deliver a single-stranded DNA carrying the gene for a replacement protein used in transport of a specific metal ion. The absence of this transport mechanism creates a toxicity for the cells and results in cell death and, in humans, results in a metal ion associated disease state. The objective was to leverage a method or platform of methods that permitted the quantification of the activity of the expressed protein in a model system to provide a relative potency assay for the AAV therapeutic. Flow cytometry seemed to be an ideal candidate owing to its ability to determine multiple readouts simultaneously and to measure both expressed and functional attributes. Flow was used to determine expressed protein (in cell ELISA) and functional outcome (viability). In addition, Flow was a logical complement to PCR (not shown here) used to monitor translational processes.

Knockout/knockdown cells (Hepatoma) in which the metal ion transport protein had been genetically silenced were used. Cells were exposed to metal ion at various concentrations and then dosed with AAV containing the gene on interest. After pre-determined dosing times, the viability of the cells was determined using flow cytometry. The amount of expressed protein was determined by Western blot or in-cell ELISA. Transcription of DNA was also measured using RT-qPCR. This is all depicted in Figures 3-4. Figure 5 demonstrated that cell survival/recovery is strongly correlated with viability.

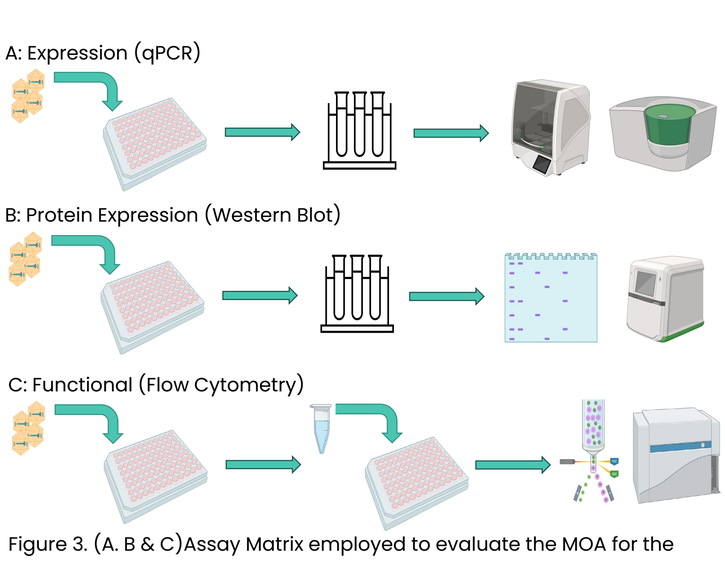

Figure 3. (A. B & C)Assay Matrix employed to evaluate the MOA for the protein replacement with delivery by an AAV

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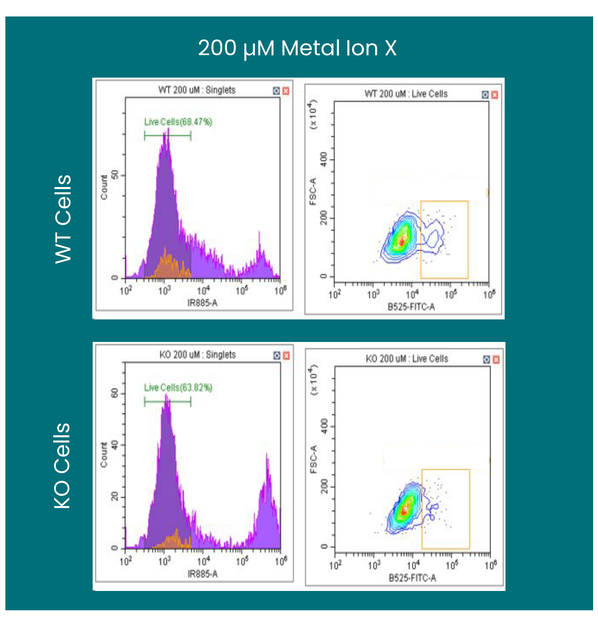

Figure 4. Demonstration using Flow of expressed protein. This is confirmed using Western, Image 1. Flow Cytometry is then used to measure improved viability of exposure to toxic levels of metal ion.

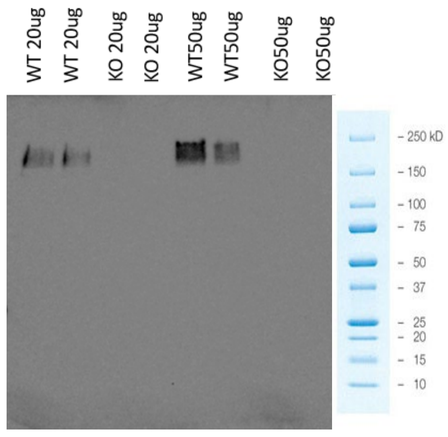

Image 1: Western Blot of Replacement Protein in KO vs WT cells

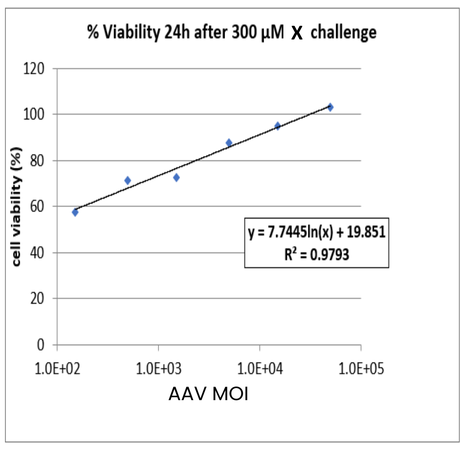

Figure 5. Measurement of cell viability as a function of AAV dose (MOI) after exposure of cells to toxic levels of metal ion.

CONCLUSIONS

The results presented here demonstrate the flexibility of Flow Cytometry in the implementation of cell-based potency assays. Potency assays are a regulatory expectation and flow cytometry offers the unique advantages of the capability for simultaneous measurement of multiple parameters (e.g., size, granularity, protein expression, viability), providing measurements at the single cell level, and the ability to identify discrete, rare target populations not readily detectable by other analytical techniques. Flow cytometry assists in overcoming the complexity of ATMPs by providing a tool to accurately analyze the expression and function of a drug of interest with high throughput in a short timeframe. The above case studies offer a few examples of how the utilization of Flow Cytometry can successfully be incorporated into a control strategy for the potency assessment of ATMPs.