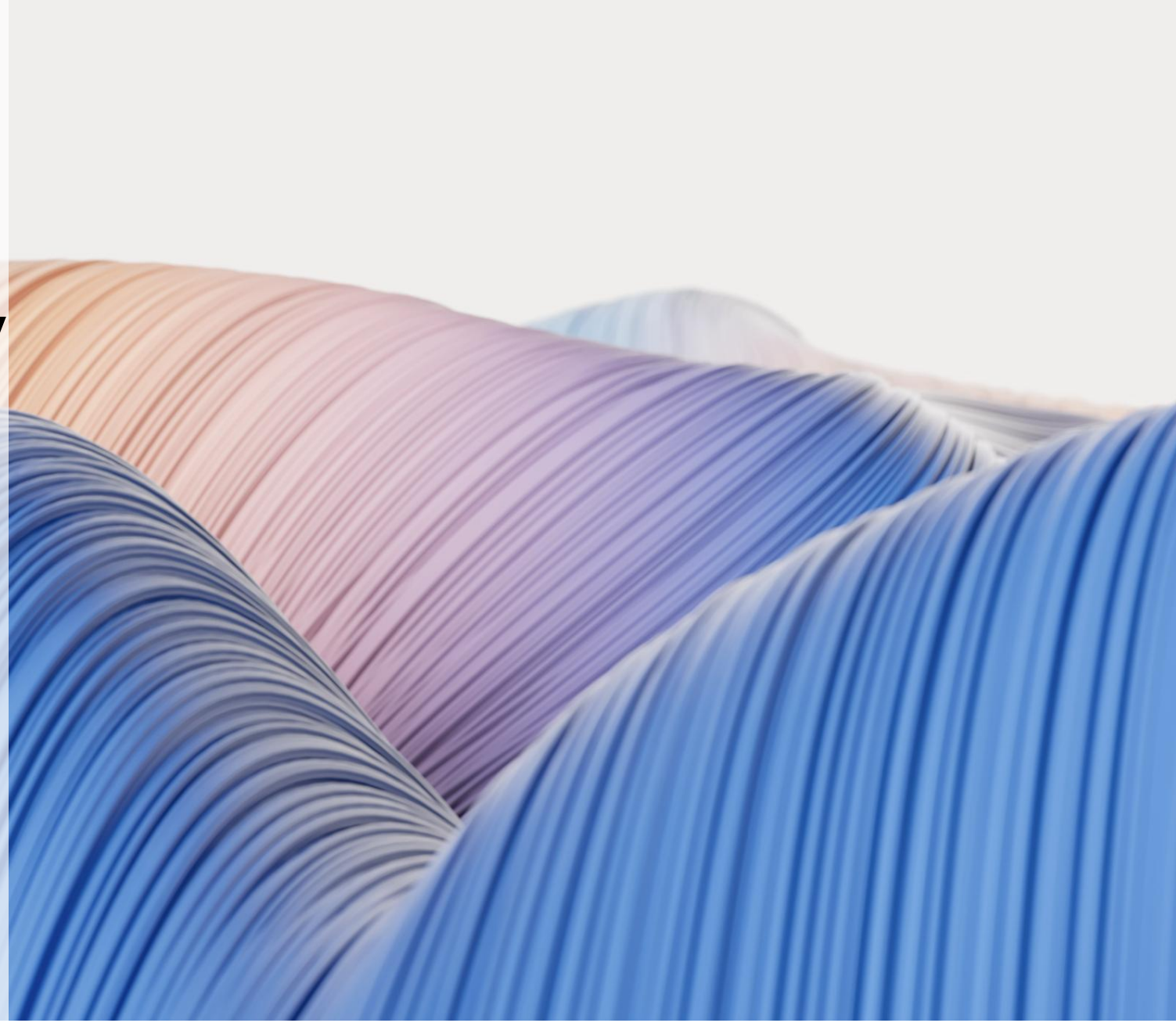
A to Z of IVE: From Single Antigen to Multiplex Flow Cytometry and LC-MS/MS

Emilia Byrne, BTxPS, Pfizer

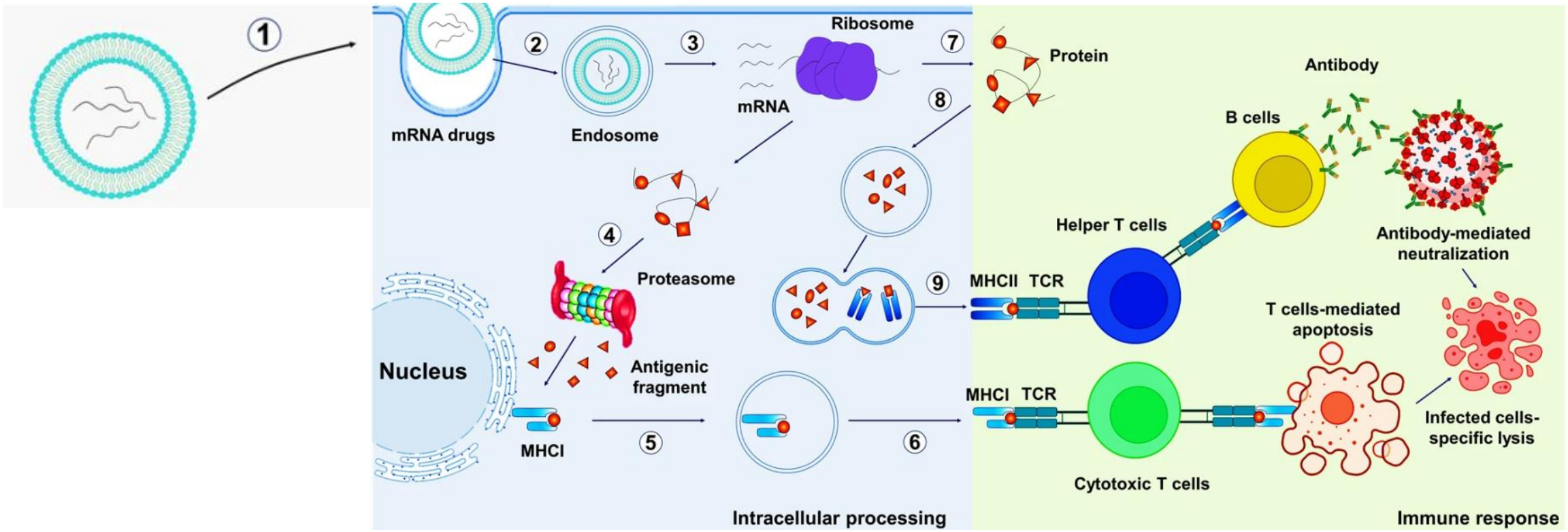
May 2025



Outline

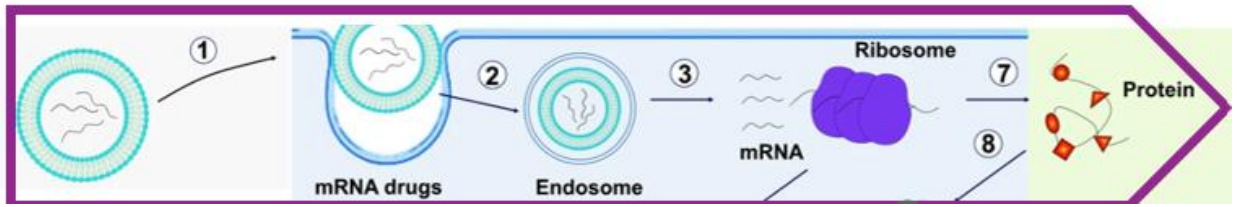
- Introduction
- Assay designs for IVE-Flow Cytometry
- Multiplex challenges:
  - Valency
  - Cross-reactivity
  - Seasonality and validation
- Orthogonal IVE: antibody-free LC-MS/MS detection
- Summary
- Acknowledgements

mRNA Vaccines Elicit Immune Response



Qin et al., 2022 Signal Transduction and Targeted Therapy Vol. 7 No 166

Functional Assays Cover Part of the MOA



USP Analytical Procedures for Quality of mRNA Vaccines and Therapeutics

Biological Activity by Cell-Based Assay

Potency testing intends to determine cellular functionality of the DP (uptake, release, translation of RNA). DP potency can be demonstrated by verifying the cellular translation of the DP-containing mRNA into the encoded protein. There are two ways to detect cellular translation, I) antibody dependent detection of the target protein (FACS, ELISA, WB etc.) or II) antibody independent detection (aptamers, MS etc.). The method described here uses ELISA as the protein detection method, but other methods may also be used.

European Pharmacopoeia: mRNA vaccines for human use (53600)

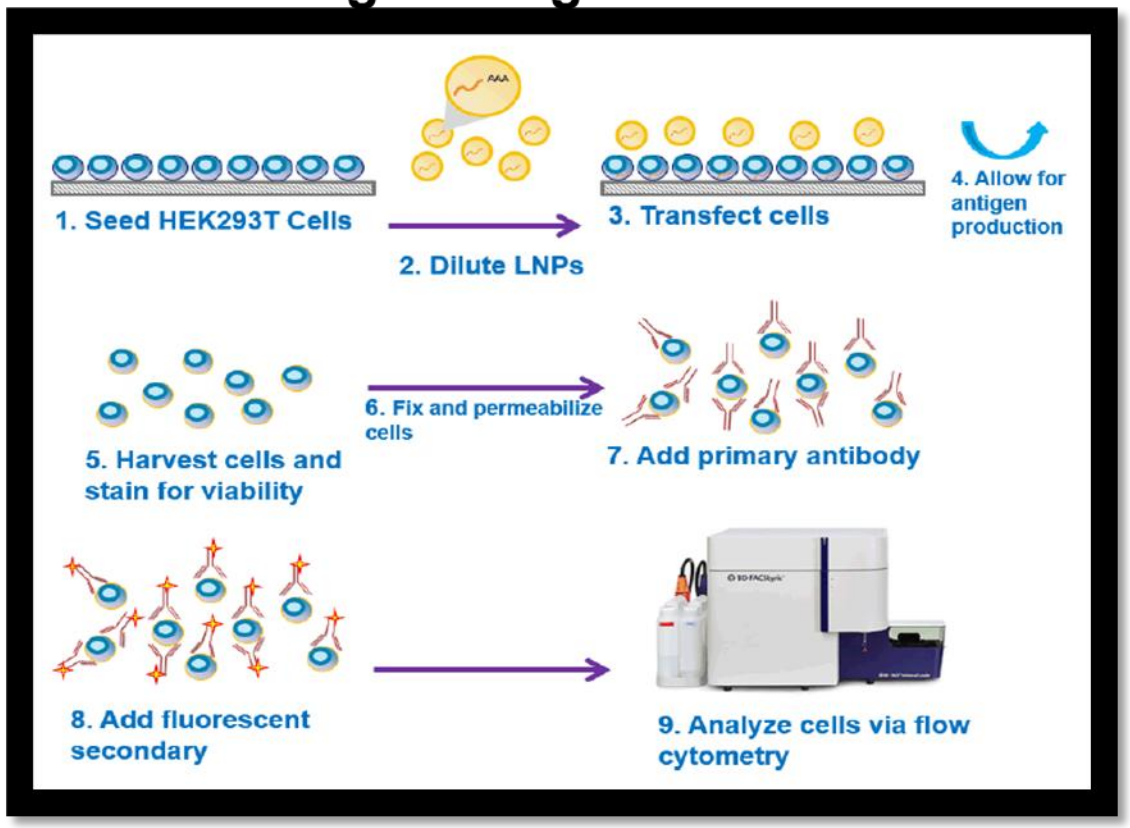
The potency of each final lot is determined with the combination of RNA Content, Encapsulation Efficiency, Integrity. In addition, a functionality assay is carried out according to:

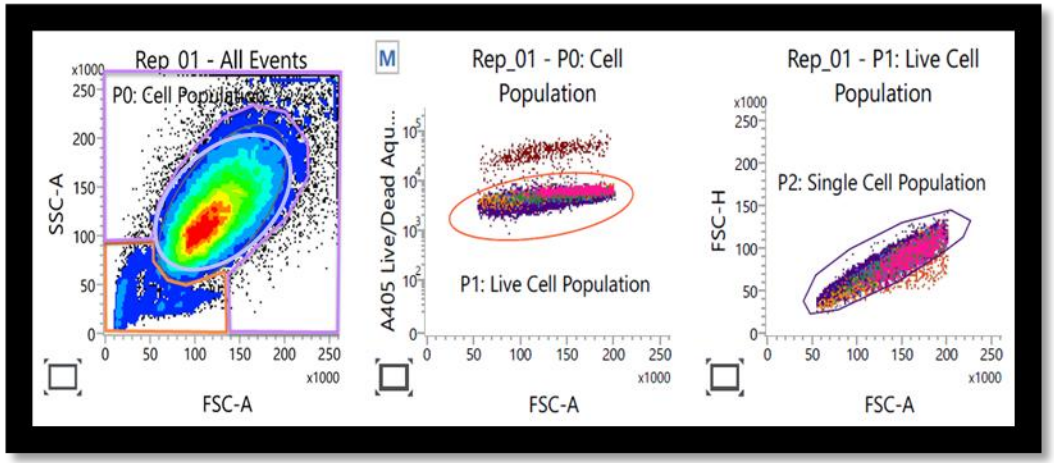
6-4. **Cell-based *in vitro* expression assay**. A cell-based *in vitro* expression assay is carried out to demonstrate that the quality attributes of the vaccine are adequately controlled to enable LNP uptake, mRNA escape from the endosome and translation of the mRNA into the encoded protein. Analytical procedures may include cell-based transfection systems with a first step consisting of transfection into cells to enable expression of the protein before detection by a suitable method, such as immunochemical assay (*2.7.1*), flow cytometry (*2.7.24*) or mass spectrometry (*2.2.43*).

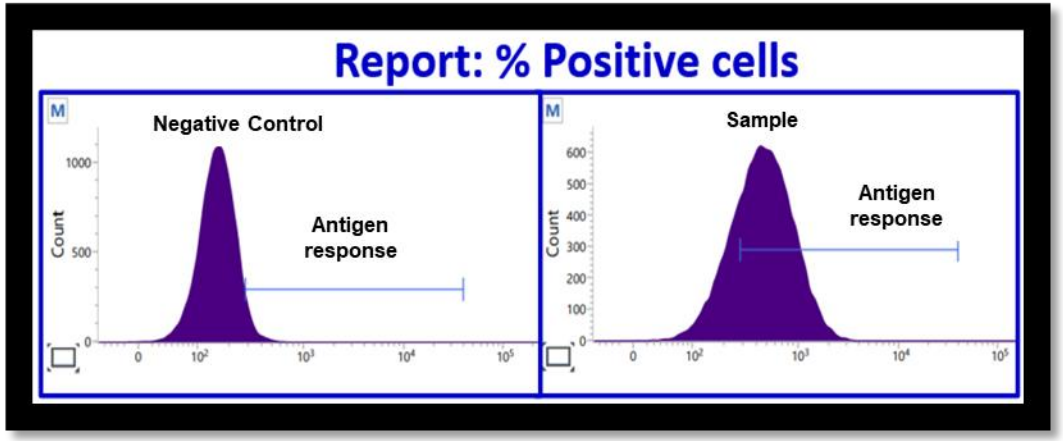
For multivalent vaccines containing more than one mRNA substance, *in vitro* expression of the antigens corresponding to each mRNA should be specifically confirmed unless otherwise justified.

In Vitro Expression (IVE) Flow Cytometry Assay

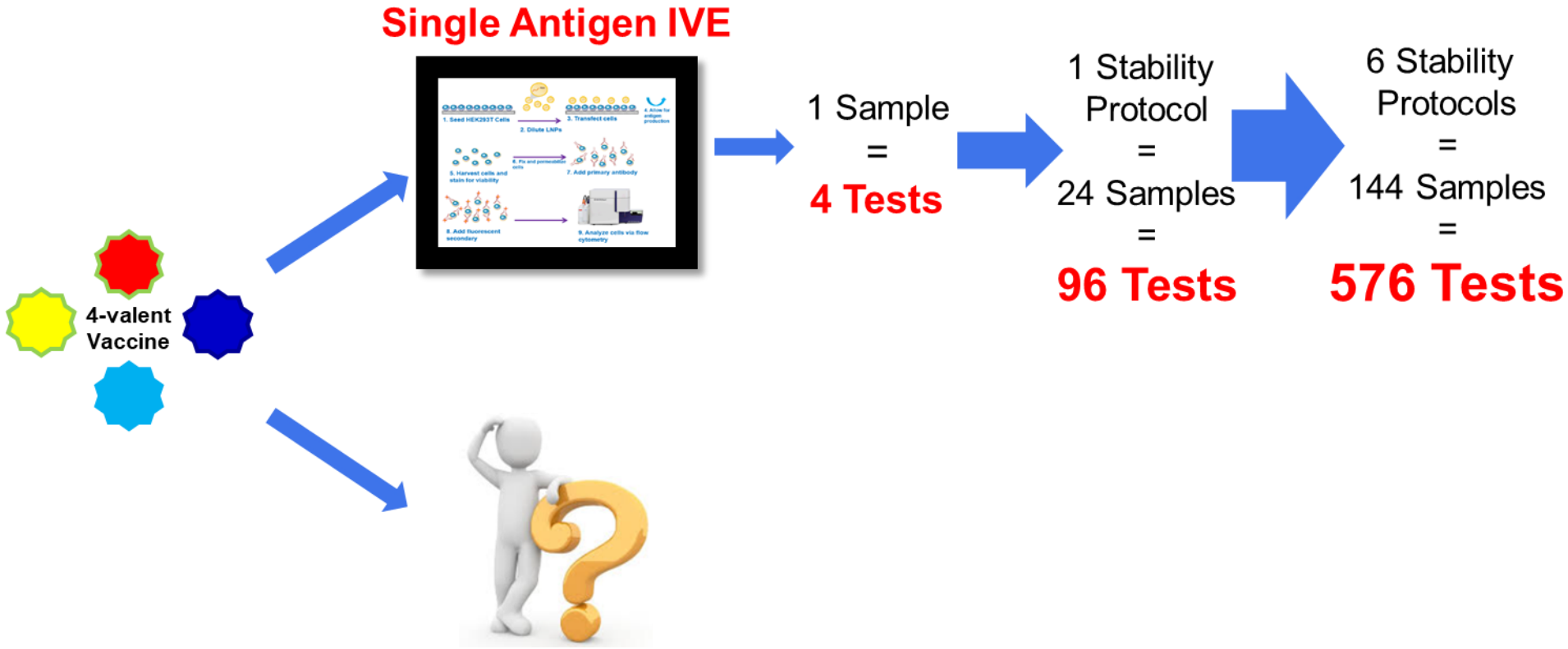
Single Antigen IVE



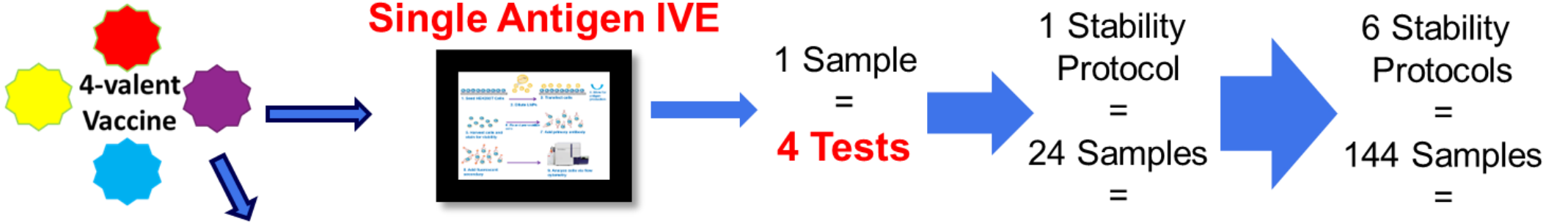


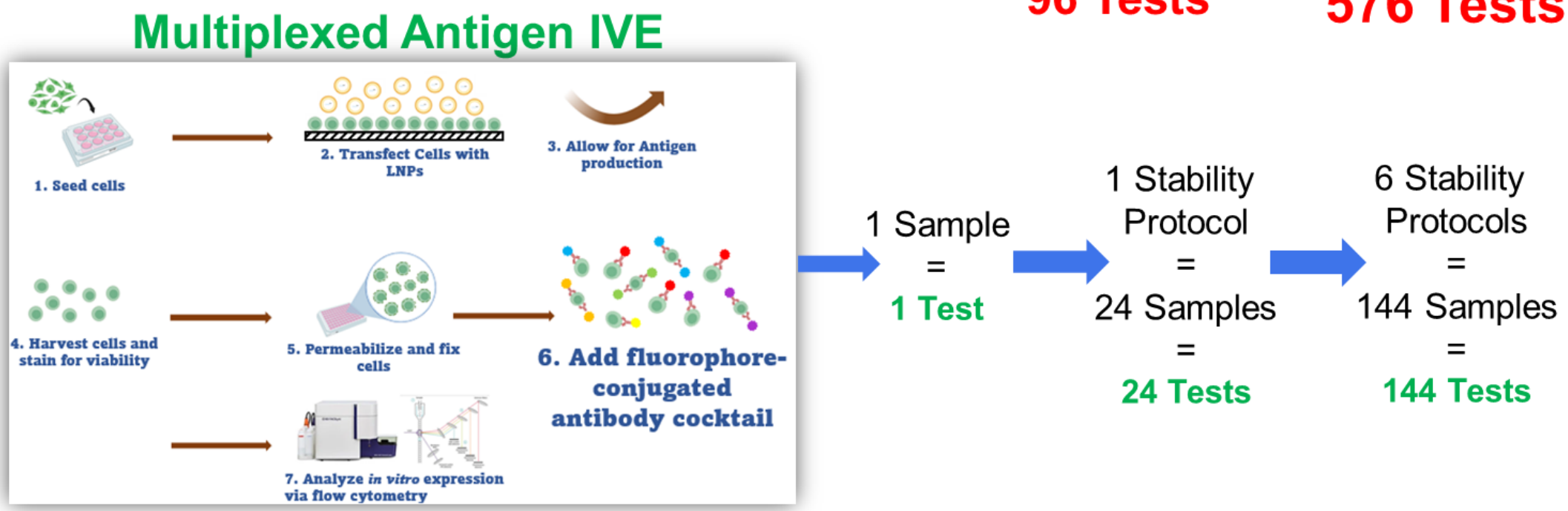


Higher Valency: Burden of Single Antigen Testing



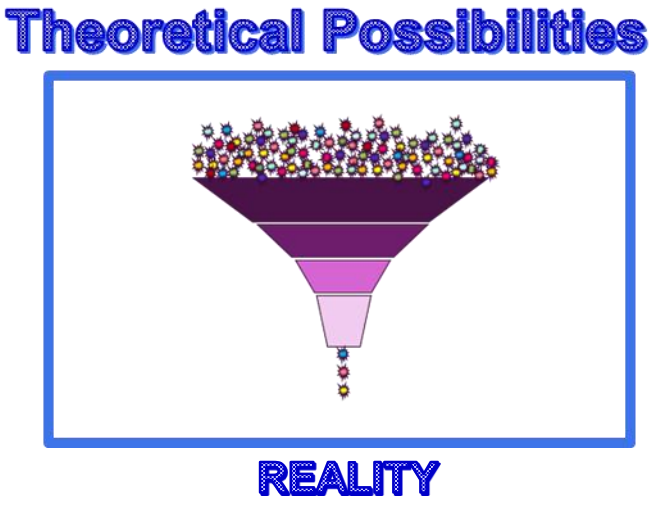
Solution: Simultaneous Detection (Multiplexing)

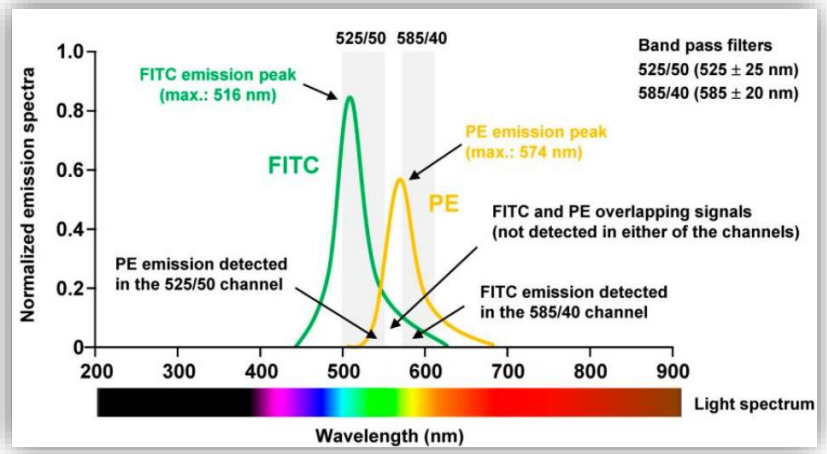




Multiplex Design Considerations & Challenges

- Equipment suitability for panel expansion:
  - Configuration - number of lasers, filters, and detectors
  - Autofluorescence subtraction
  - Spectral unmixing
- Ability to generate custom or access suitable commercial conjugated antibodies
- Potential fluorophore conjugation challenges: yield, purification, scale up, cost
- Fluorophore intensity matching to target antigen abundance
- Optimal spacing and compensation for spectral overlap
- Fluorophore stability (fixation, permeabilization, storage, light exposure)
- Layers of calibration reagents, may require combinations of custom beads, stained cell controls, equipment reference settings
- Robust gating strategy to minimize confounding events





Drescher et al., 2021 Biomedicines 9(11):1613

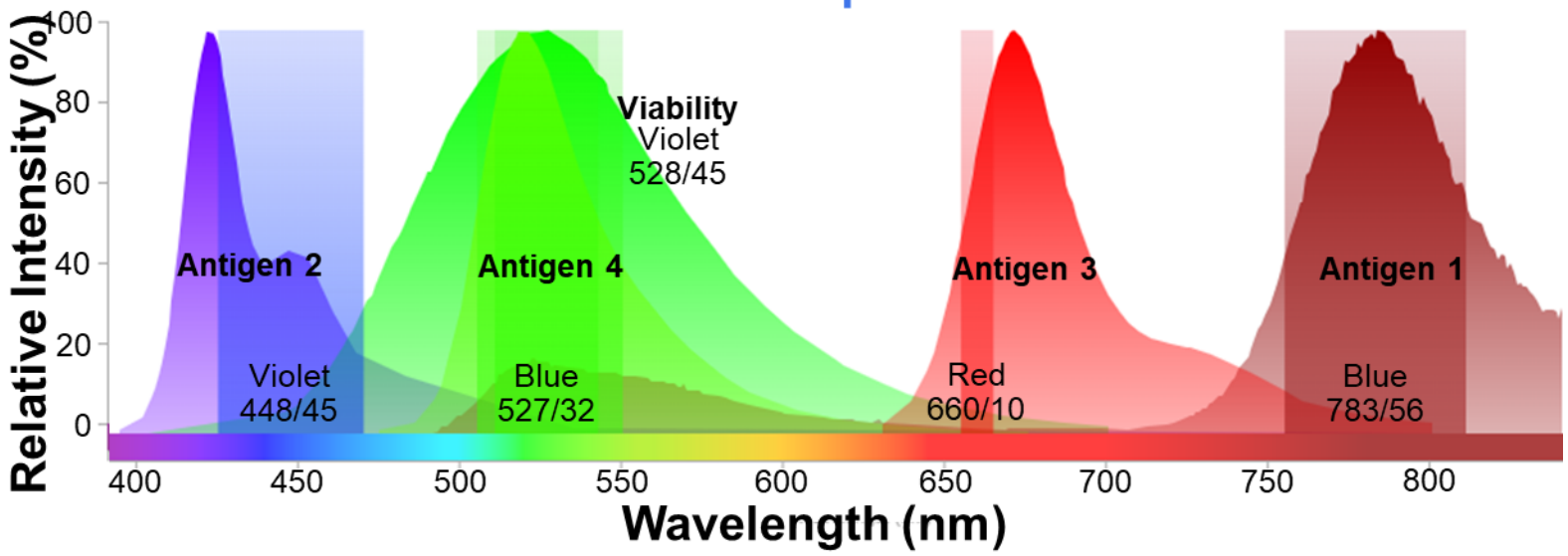
Analyte, Reagent, & Equipment Driven Panel Design

Inverse match

Maximize similar target distance

Target	Abundance	Laser & Filter Used by Fluorophore/Stain	Relative Brightness
Antigen 1	Low	Blue, 783/56	Very Bright
Antigen 2		Violet, 448/45	
Antigen 3	Moderate	Red, 660/10	Bright
Antigen 4	High	Blue, 527/32	Moderate
Viability Dye	Low	Violet, 528/45	Dim

Optimize laser use



Antibody Cross-Reactivity Considerations

- High degree of target similarity and polyclonal antibody use increase the potential for cross-reactivity

Undesirable – reduces assay specificity

Desirable – extends antibody use to additional targets of interest

- Management of undesirable cross-reactivity:
  - Titration to reduce to acceptable level
  - Panel design to minimize spectral overlap between impacted antibody fluorophores
  - Affinity purification to remove cross-reactive titer
- Extensive target similarity which makes generation of selective antibodies impractical, may require combined antigen monitoring of the related targets (Total Antigen Expression):
  - Highly comparable target recognition is key for this approach
  - Considerable target bias introduces a degree of uncertainty
- Desirable cross-reactivity supports nimble formulation changes for seasonal vaccine products

Specificity: Minimal Evaluation Matrix

- Antibodies require evaluation individually as well as in the multiplex cocktail
- Antibodies in cocktails can behave distinctly from single stains
- Nonspecific signal may arise from:
  - Rogue fluorescence in secondary detectors
  - Cell processing technique
  - Physical antibody cross-reactivity
- Likely sources of rogue fluorescence:
  - Poorly compensated spillover
  - Cell auto-fluorescence
- N-1 antigen controls become increasingly challenging with higher valency

Antibody Cocktail: Combination of 4 Antibodies				
Analyte	**Antigen 1 Signal**	**Antigen 2 Signal**	**Antigen 3 Signal**	**Antigen 4 Signal**
Antigen 1	+	-	-	-
Antigen 2	-	+	-	-
Antigen 3	-	-	+	-
Antigen 4	-	-	-	+
Antigens 1-4	+	+	+	+

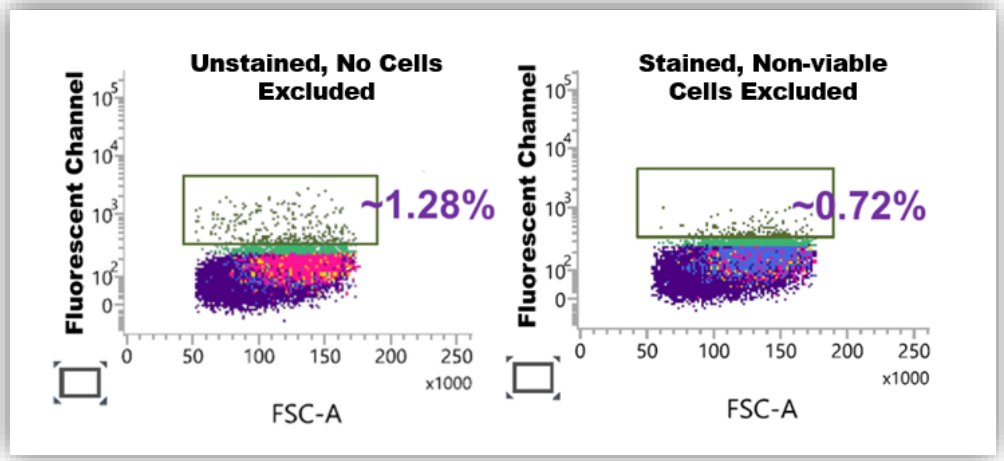
Individual Antibodies				
Conjugated Antibody	**Antigen 1 Signal**	**Antigen 2 Signal**	**Antigen 3 Signal**	**Antigen 4 Signal**
Anti-Antigen 1	+	-	-	-
Anti-Antigen 2	-	+	-	-
Anti-Antigen 3	-	-	+	-
Anti-Antigen 4	-	-	-	+

+	Specific Response
-	Non-specific (cross-reactive) Response

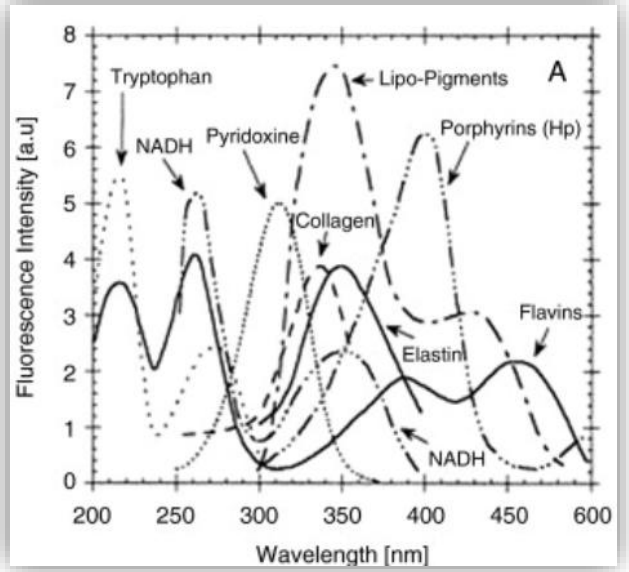
Managing Autofluorescence

What to do about it?

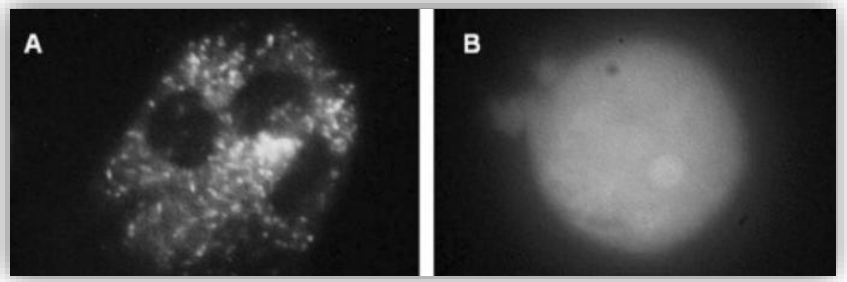
- Select equipment with autofluorescence subtraction capability
- Investigate cell types for degrees of inherent autofluorescence
- Define conditions for optimal viability / minimize toxicity
- Select fluorophores strategically to minimize channels with higher propensity for detecting autofluorescence
- Assess residual autofluorescence



Inconvenience or functional biology?



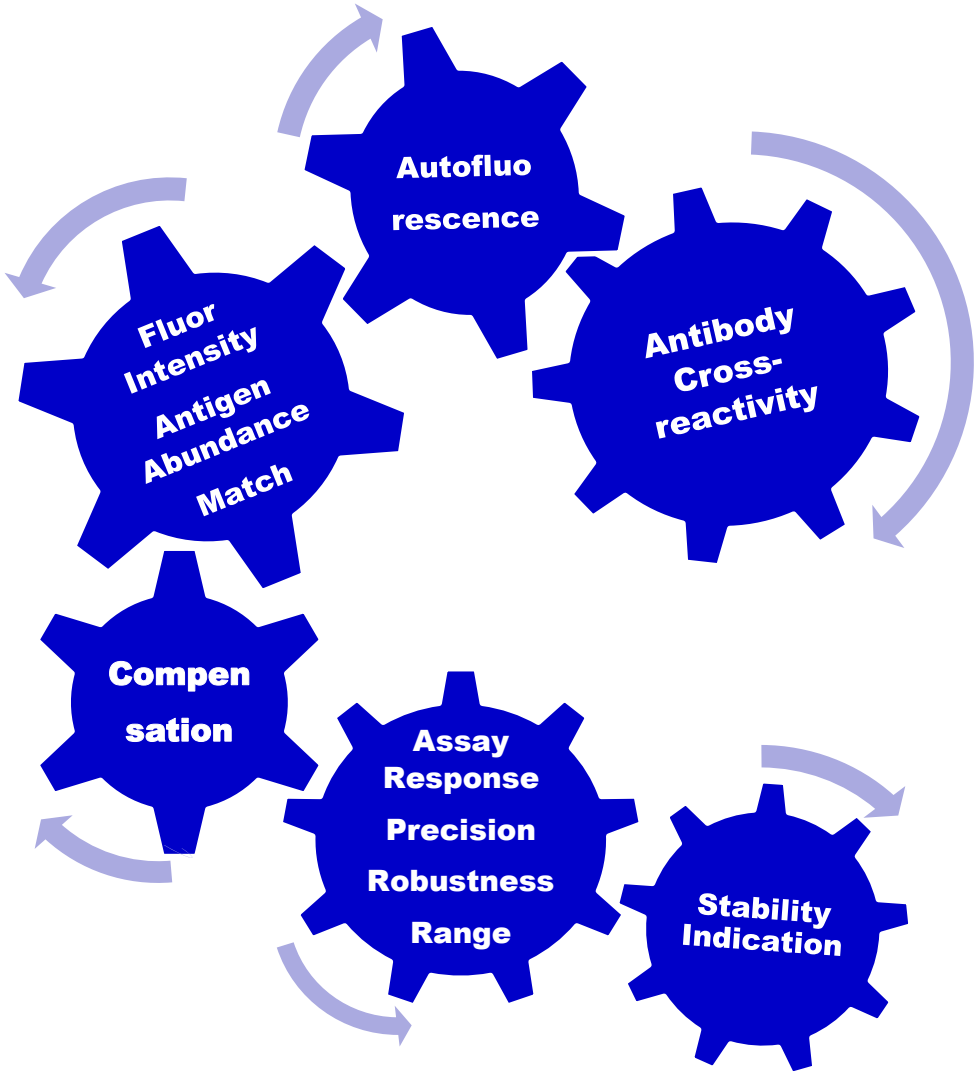
Cellular energy & metabolism

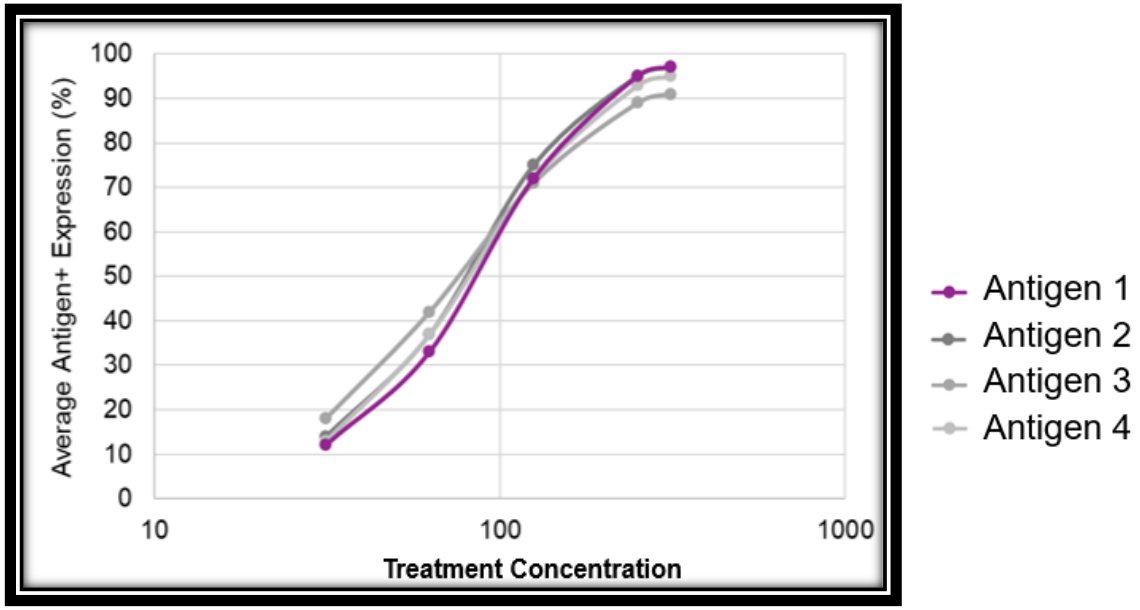


Normal granulocyte Leukaemic granulocyte

Monici et al., 2005 Biotechnology Annual Review Vol. 11 P.227

Synchronized Antigen Detection: A Balancing Act





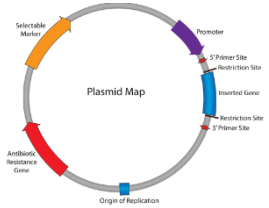
Target	Abundance	Laser & Filter Used by Fluorophore/Stain	Relative Brightness
Antigen 1	Low	Blue, 783/56	Very Bright
Antigen 2		Violet, 448/45	
Antigen 3	Moderate	Red, 660/10	Bright
Antigen 4	High	Blue, 527/32	Moderate
Viability Dye	Low	Violet, 528/45	Dim

Challenges to Platform For Seasonal mRNA Vaccines

CONSTANT:
Formulation, Strength, Backbone

VARIABLE:
Gene of Interest

DNA Starting Material



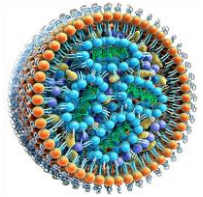
- Appearance/pH
- DNA Content
- **DNA Identity**
- DNA Topology
  - Circular: linear & supercoiled
  - Linear: linearization efficiency
- Residuals
  - Host Cell DNA, RNA & Prote
  - Antibiotics
  - Enzymes/Carrier Protein
- Bioburden/Endotoxin

mRNA Drug Substance

- Appearance/pH
- RNA Content
- **RNA Identity**
- RNA Integrity
- 5´Cap
- Poly(A) Tail
- Residuals
- DNA Template
- Double stranded (ds)RNA
- Bioburden/Endotoxin

Antigen specific **primers, probes**, **antibodies**

mRNA/LNP Drug Product



- Appearance/pH
- Subvisible Particles
- Osmolality
- Lipid Content & Identity
- LNP Size & Polydispersity
- RNA Content/Encapsulation
- **RNA Identity/Ratio**
- RNA Integrity
- **Functional Assay (IVE)**
- Container Closure/BLEF
- Volume in Container
- Sterility/Endotoxin

Approach to Validation for Seasonality

New mRNA Antigen

Applicability Assessment
- Manufacturing process
- Quality attributes
- Platform method operating parameters/differences
- Validation applicability

Extension of Validation

Method 1	Method 2	Method 3: Critical Parameter Change
Scientific Rationale	**Laboratory Verification**	**Supplemental Validation**
• Attribute measured unaffected by sequence change • Document scientific rationale	• Confirm expected result under real laboratory condition • Under protocol • Predefined acceptance criteria • Document scientific rationale & data	• **Challenge per ICH Q2** • **Under protocol** • **Predefined acceptance criteria** • **Document rationale & data**

IVE Flow Cytometry

IVE requires new combinations of antibodies and targets, necessitating supplemental validation for new seasons

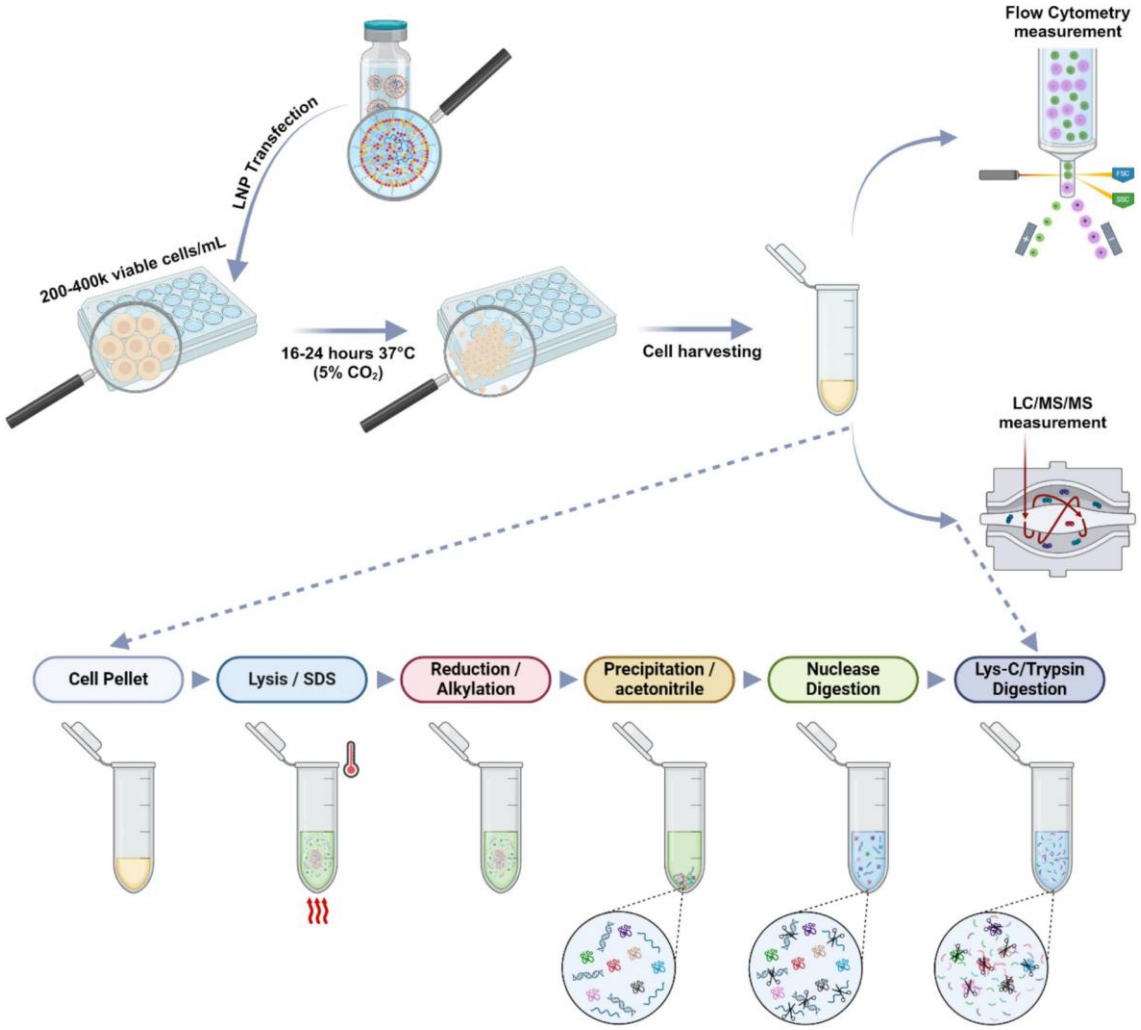
Alternative Cell Based IVE: LC-MS/MS Detection

Advantages

- Direct measurement of expressed antigens without the need for antigen-specific antibodies
- Suitable for complex, high valency mixtures
- High sensitivity and selectivity due to the nature of MS-based approaches
- Relatively rapid development for new products
- Amenable to platform, for example, to support seasonally changing formulations

Disadvantage

- Unable to measure the cell population fraction which expresses the target
- Does not account for proper antigen structure
- Cost of implementation in GMP workflows

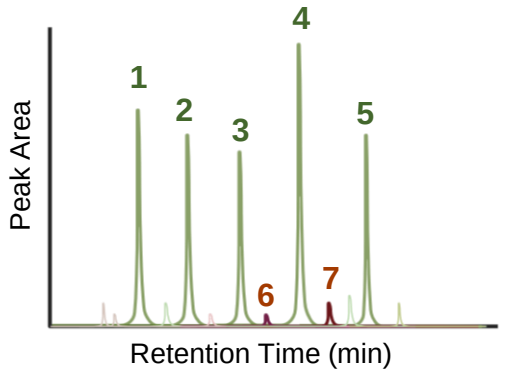


Wang et al., 2025 Sci Rep. Vol. 15 P: 10336

IVE LC - MS/MS Approaches to Quantitation

Relative Quantitation (% expressed antigen to host cellular proteins)

$$X.X\% = \frac{Average\ Peak\ Area\ of\ (2\ Antigen\ Peptides)}{Average\ Peak\ Area\ of\ (5\ Host\ Cell\ Protein\ Peptides)}\ \%$$



- ✓ Eliminates the need for cell counting
- ✓ Normalizes for instrument performance variation
- ✓ Helps with cell variability
- ✓ No need for any protein or peptide standards in routine analysis

- ❑ **x.x%** is an artificial value and **can only be compared for the same antigen protein**

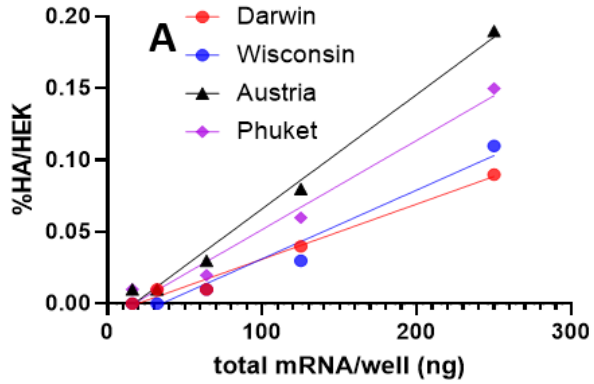
"Absolute" Quantitation: pmol of expressed antigen per cell pellet

- Convert relative x.x% to pmol of expressed antigens per cell pellet based on known amount of recombinant protein standards (pmol) added to the negative control cell pellets
- **Valuable for comparing expression between distinct antigens**

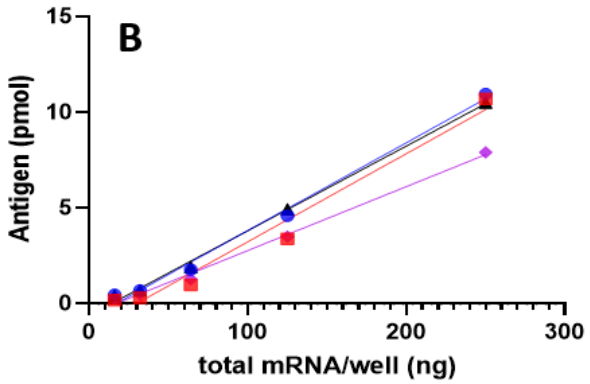
Detection of 4 Antigens in Multivalent Vaccine

IVE-LC-MS/MS:

% Antigen peptide / host

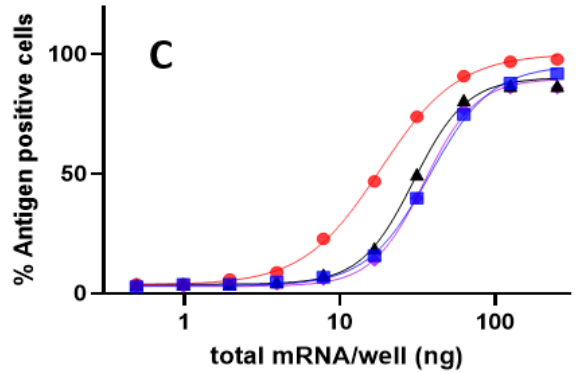


Linear Regression

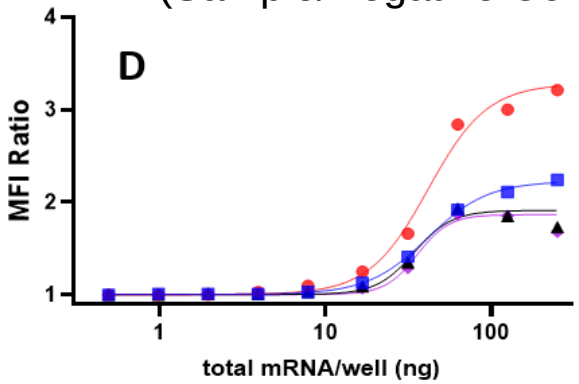


IVE-Flow Cytometry:

% Antigen positive cells

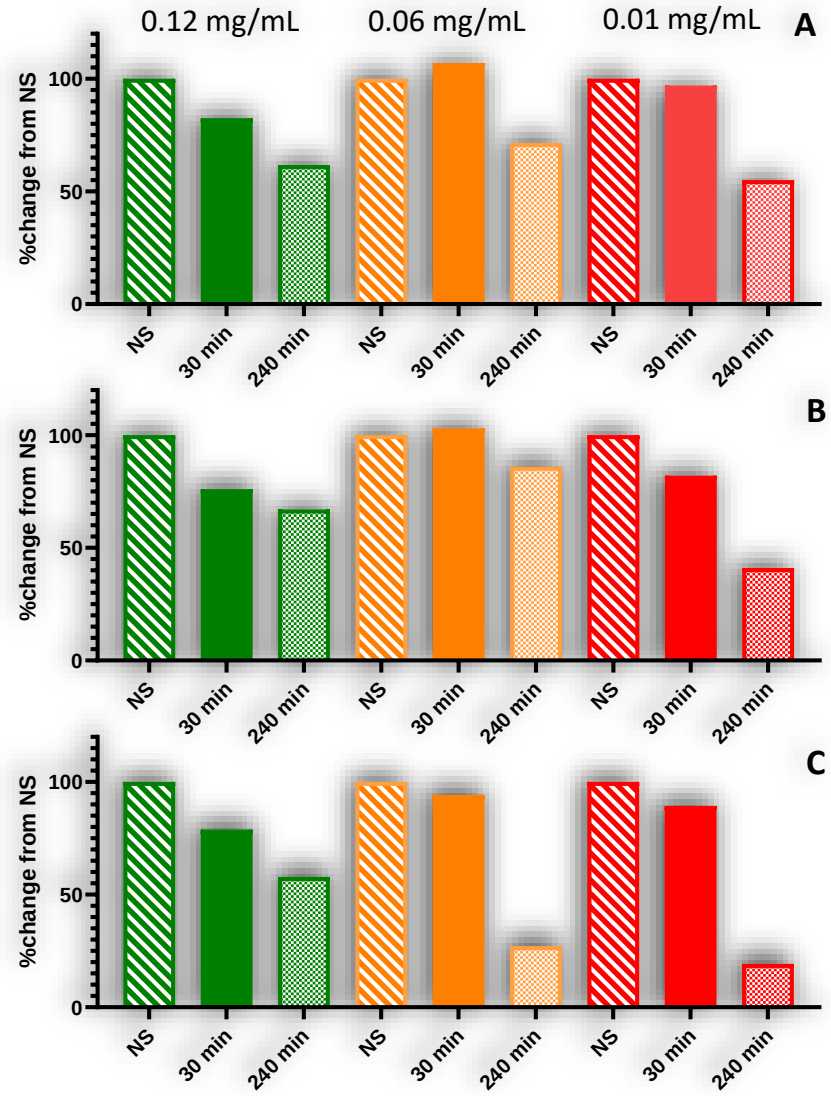


MFI (Sample/Negative Control)



Wang et al., 2025 Sci Rep. Vol. 15 P: 10336

Stability Indication: Flow Cytometry and LC-MS/MS



A **IVE-flow cytometry:**
% HA positive cells

B **IVE-flow cytometry:**
MFI

C **IVE-LC/MS/MS:**
% Antigen/HEK

- **Treatment condition**:
- Shaking for 30 or 240 mins at 0.12, 0.06, and 0.01 mg/mL mRNA concentrations

- **Graphed as:**
- % change from non-shaking
- condition (NS)

Wang et al., 2025 Sci Rep. Vol. 15 P: 10336

Summary

- Functional expression assays monitor a critical portion of the mechanism of mRNA products, from LNP uptake through cellular translation
- Multiplexing with antigen specific reagents is a complex efficiency solution for higher valency, which requires upfront investment for methodical construction and implementation
- Reagent cross-reactivity presents simultaneously, a special challenge when undesirable, and a potential solution when target extending without loss of essential specificity
- Although not readily platformable, IVE methods requiring minimal parameter change for new seasonal formulations considerably simplify supplemental validation
- LC-MS/MS in lieu of antibody dependent detection provides a sensitive, multiplex-enabling alternative. The tradeoffs, loss of ability to measure the proportion of antigen expressing population or sense higher-order antigen structure, are offset by reduced antibody dependence, and if using standards, the ability to evaluate expression across distinct antigens
- The two methodologies may be applied strategically as complementary for robust mRNA portfolio support

Thank you to the colleagues from:

- Pfizer Pharmaceutical Sciences:
  - Bioassay and Impurity Testing (Chesterfield, MO and Andover, MA)
  - Pharmaceutical Research & Development (Andover, MA)
  - Characterization and Analytical Sciences (Chesterfield, MO)
- Pfizer Vaccine Research Division
  - Early Bioprocess Development (Pearl River, NY)

Thank you!