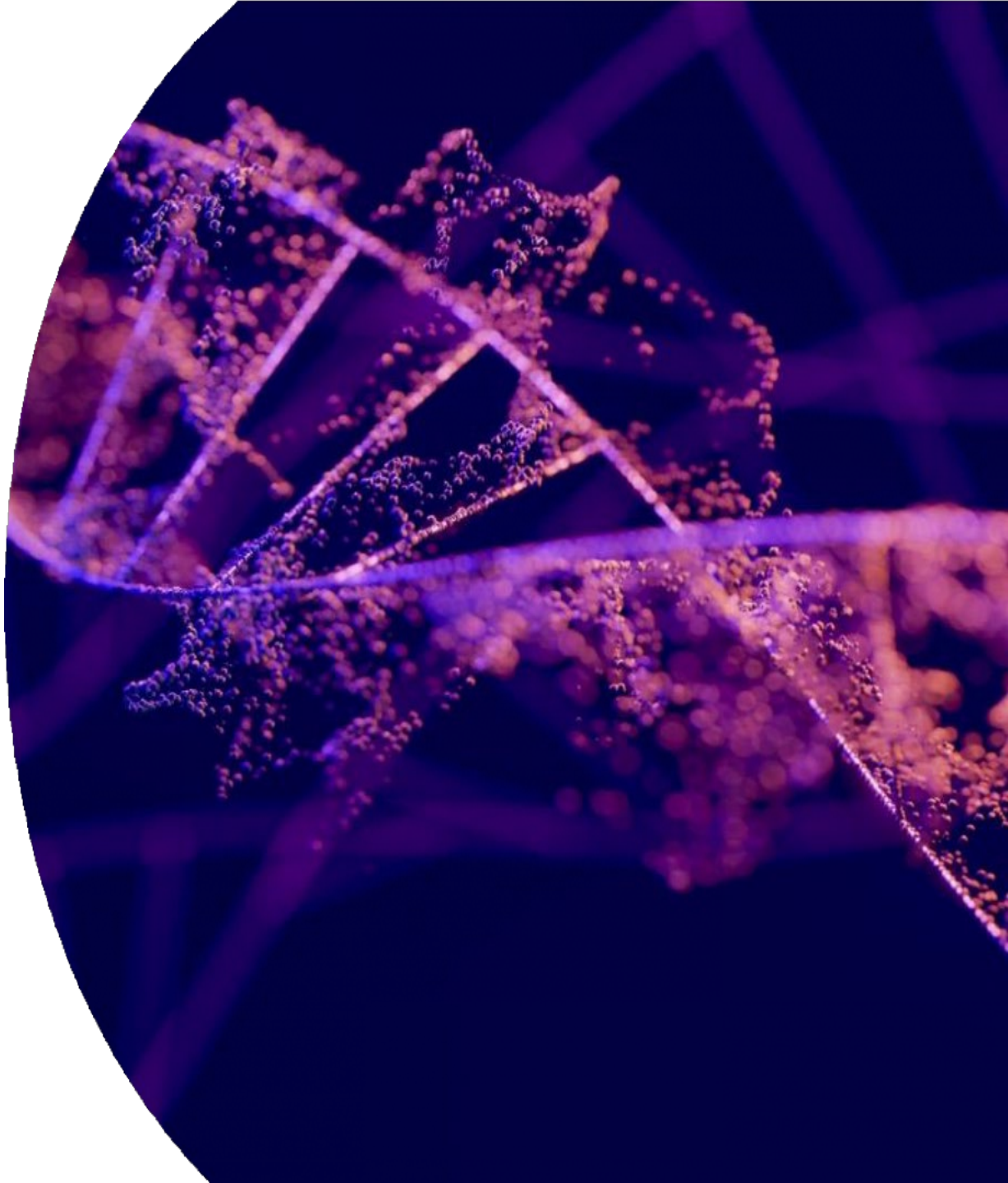
Comprehensive characterization of multivalent *mRNA vaccines functionality* by *Flow Cytometry* and *Mass Spectrometry*

Thibaut Willemin
Sanofi mRNA Center of Excellence
Unit Manager - Analytical Development Bioassay

mRNA Virtual Summit. March 11-12th, 2025



• Together, we chase the *miracles* of science •

Funding: Sanofi

Disclosures:
The author is a Sanofi employee and may hold stocks or shares in the company.

Vaccines *potency* testing

Vaccines are tested using *potency* assays that are *quantitative, stability indicating* and *conformational.*[1]

They allow to calculate a *relative potency* for dose-definition[2]

There is an increase in the number of clinical trials involving *mRNA vaccines* and in the need to analyze their *potency*

Is this "traditional" approach the most appropriate for mRNA vaccines ?

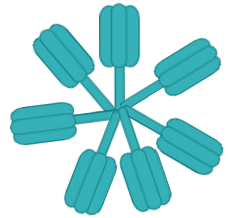
Live attenuated

Split viral inactivated
Recombinant

Virus

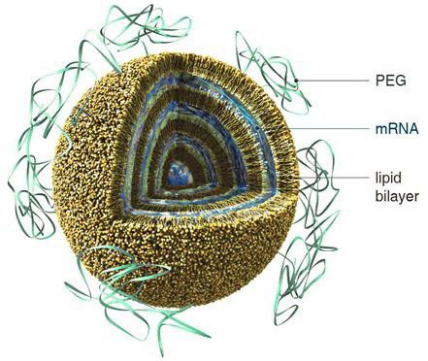
*FFA**
(Focus Forming Assay)

HA oligomer*

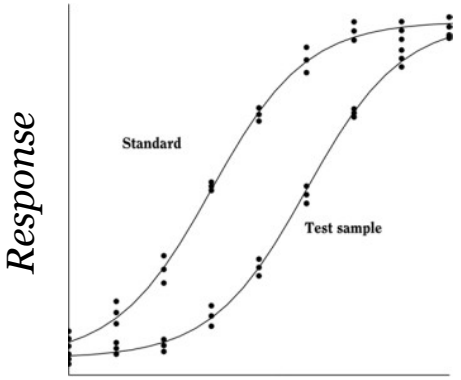


*SRID***
(Single Radio immunodiffusion Assay)

mRNA-LNP



?

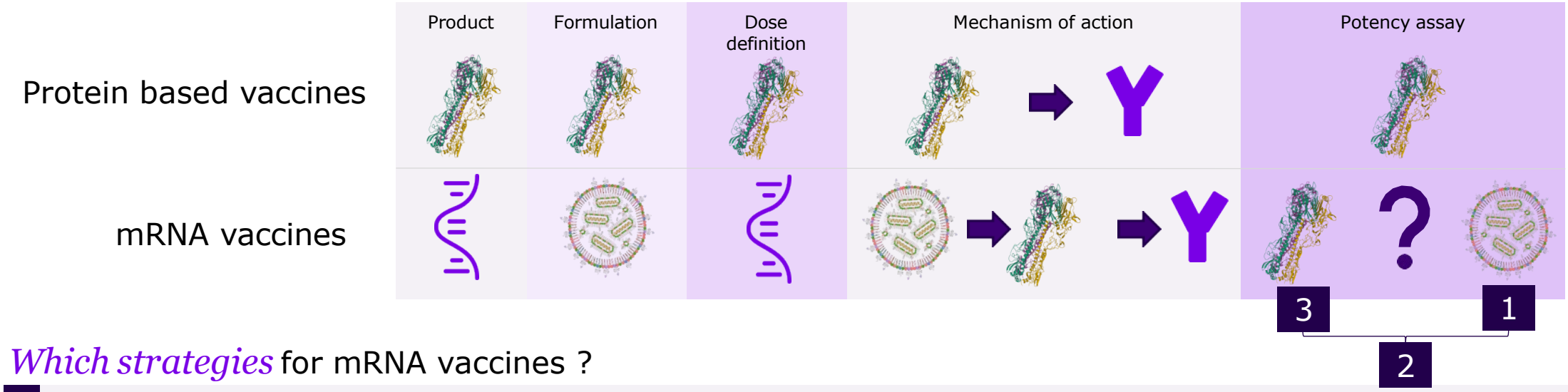


1:USP <1032> Design and Development of biological Assays
2:Eu.Ph.5.3. STATISTICAL ANALYSIS OF RESULTS OF BIOLOGICAL ASSAYS AND TESTS

*Mallory et al, Influenza Other Respi Viruses. 2018

**J. Wood, in: K. G. Nicholson and R. G. Webster (Eds.), Textbook of Influenza, 1998

mRNA vaccines *potency* testing



Which strategies for mRNA vaccines ?

1 › Potency based on *Drug Product's biophysical/biochemical parameters*
Pros: Precise assessment of the CQAs, time-efficient
Cons: Not a direct potency assay, lack of knowledge regarding link with functionality

2 › Potency based on *biophysical/biochemical parameters* and *functionality* is verified by *in-vitro protein expression*
Pros: Precise assessment of the CQAs, time-efficient, matrix of assays for potency
Cons: Complex

3 › As for other vaccines, potency based on *antigen quantity* and *conformation = quantitative functionality test*
Pros: Direct quantification of the antigen
Cons: Different parameters impact different steps of the mechanism of action; high variability; complex

Characterization of mRNA vaccines *functionality*

Some methods have been published to analyze *"mRNA vaccine potency"* [1,2]

Antibody-based methods that analyze *percentages of positive cells* after mRNA vaccine transfection *in-vitro*

- May not allow to quantify different *levels* of expression in positive cells
- The use of *specific antibodies* is not ideal from an analytical platform standpoint

mRNA platform: reduced adaptations from one disease to another

- *Analytical methods* should also be *fast to adapt*

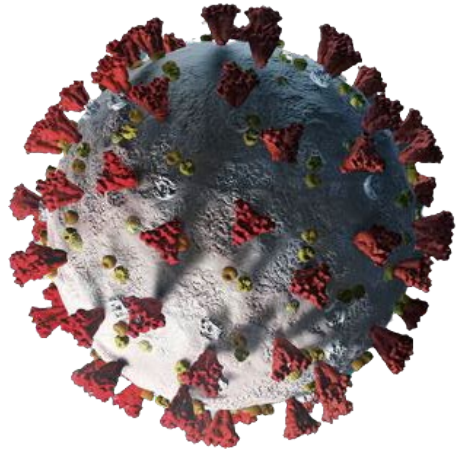
3

Goals :
Determine the most *relevant read-out* for antibody-based methods
Determine if an *antibody-independent method* could be used instead
Can *functionality* be assessed quantitatively with a *relative potency* design ?

1:Development and Characterization of an In Vitro Cell-Based Assay to Predict Potency of mRNA–LNP-Based Vaccines. Patel et al. 2023

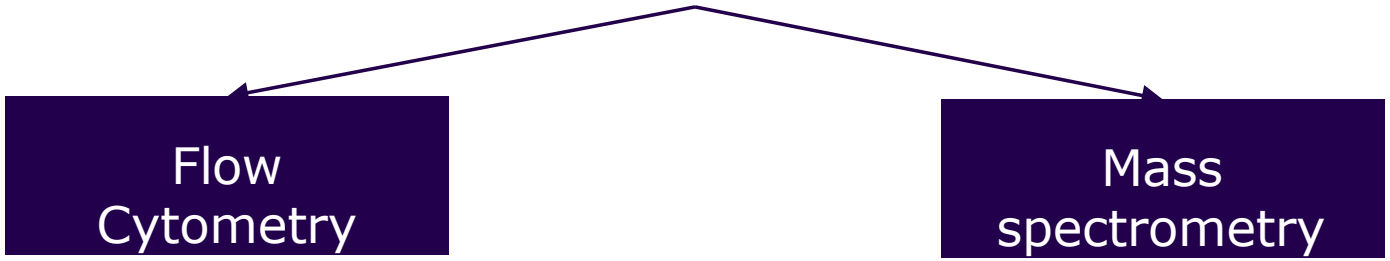
2:Development and qualification of cell-based relative potency assay for a human respiratory syncytial virus (RSV) mRNA vaccine. Hualin et al. 2023

mRNA vaccines *expression test*

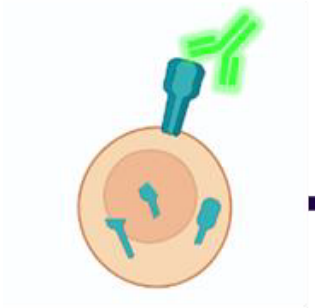


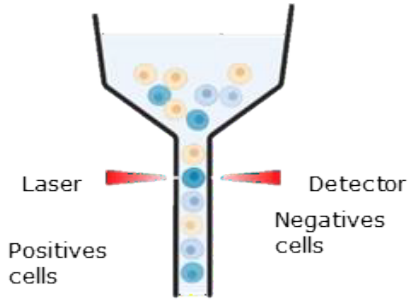
- *Transfection* of human cells with escalating doses of Drug Product (DP)
- Analysis of *protein expression* for each dose
- *Assess parallelism* by comparison of the sample dose-response to the Reference batch
- Calculation of *EC50* and *Relative Activity*

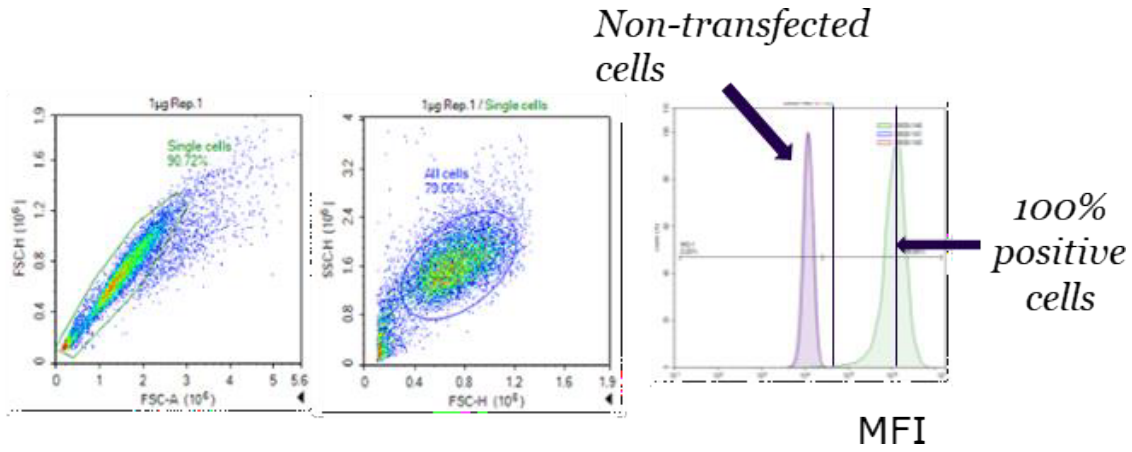
Analysis of *protein expression* by



Antibody based method: *Flow cytometry (FCM)*



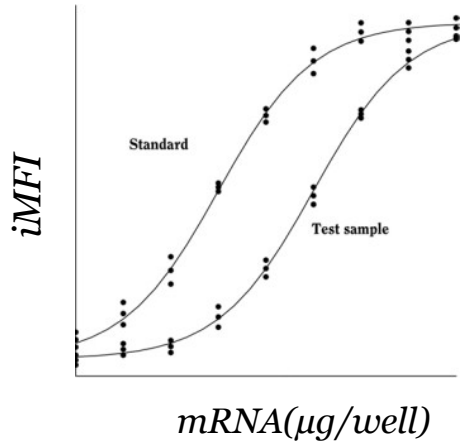




Detection of well conformed *proteins* if proper characterization of the antibody, can be selective of membrane bound proteins

- *Quantitative :*
  - *Percentage of positive cells*
  - *Median Fluorescence Intensity (MFI)*
  - *Integrated MFI (iMFI)*[1;2]
- *Conformational*
- *Stability indicating*

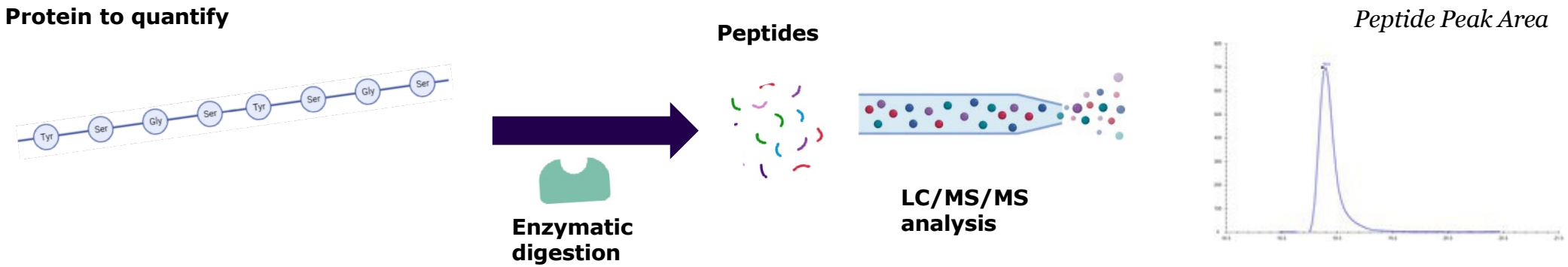
Time consuming development due to antibody use



1:Shooshtari P. Correlation analysis of intracellular and secreted cytokines via the generalized integrated mean fluorescence intensity. Cytometry A. 2010

2:Darrah PA. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat Med. 2007

Antibody independent method : *Mass spectrometry (MS)*

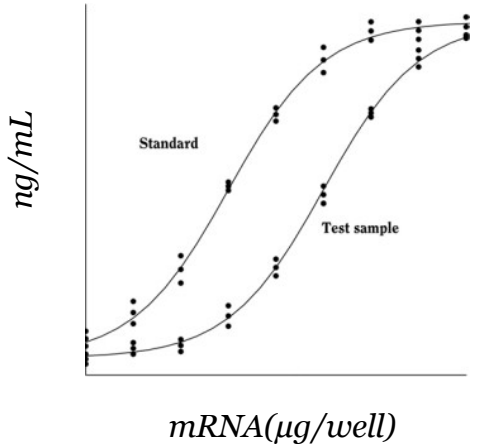


Detection of *peptides* in cell lysate

Analysis and quantification of specific peptides for each protein

- *No antibody*
- *Absolute quantification* in ng/mL with *AQUA peptides*
- Can be used for *secreted proteins*

Not conformational : might not be stability indicating if product degradation induces misfolded proteins



Characterization of mRNA vaccines *functionality*

Flow Cytometry read-outs
- *Percentage of positive cells*
- *Median fluorescence intensity*
- *Integrated Median Fluorescence intensity*

Flow Cytometry/Mass spectrometry
- *Comparison of FCM read-outs with quantity in ng/mL by mass spectrometry*

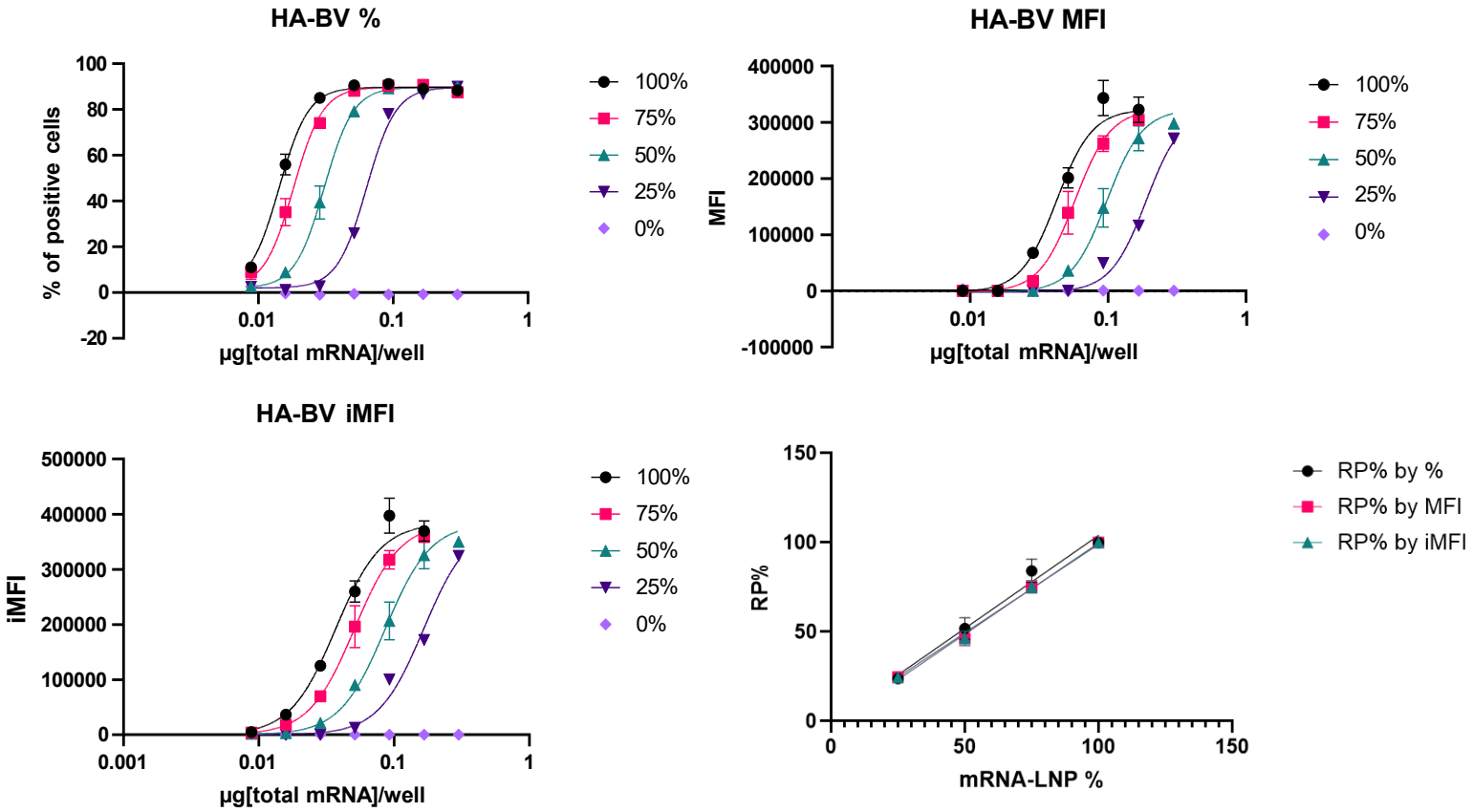
Free mRNA impact
- *Spiking of free (non encapsulated) mRNA*

mRNA ratio impact
- *Comparison between a bivalent and a quadrivalent Drug Product*

Drug Product Degradation
- *Spiking of a degraded Drug Product*

Stability studies
- *Dose-response analysis*
- *Maximum expression analysis*

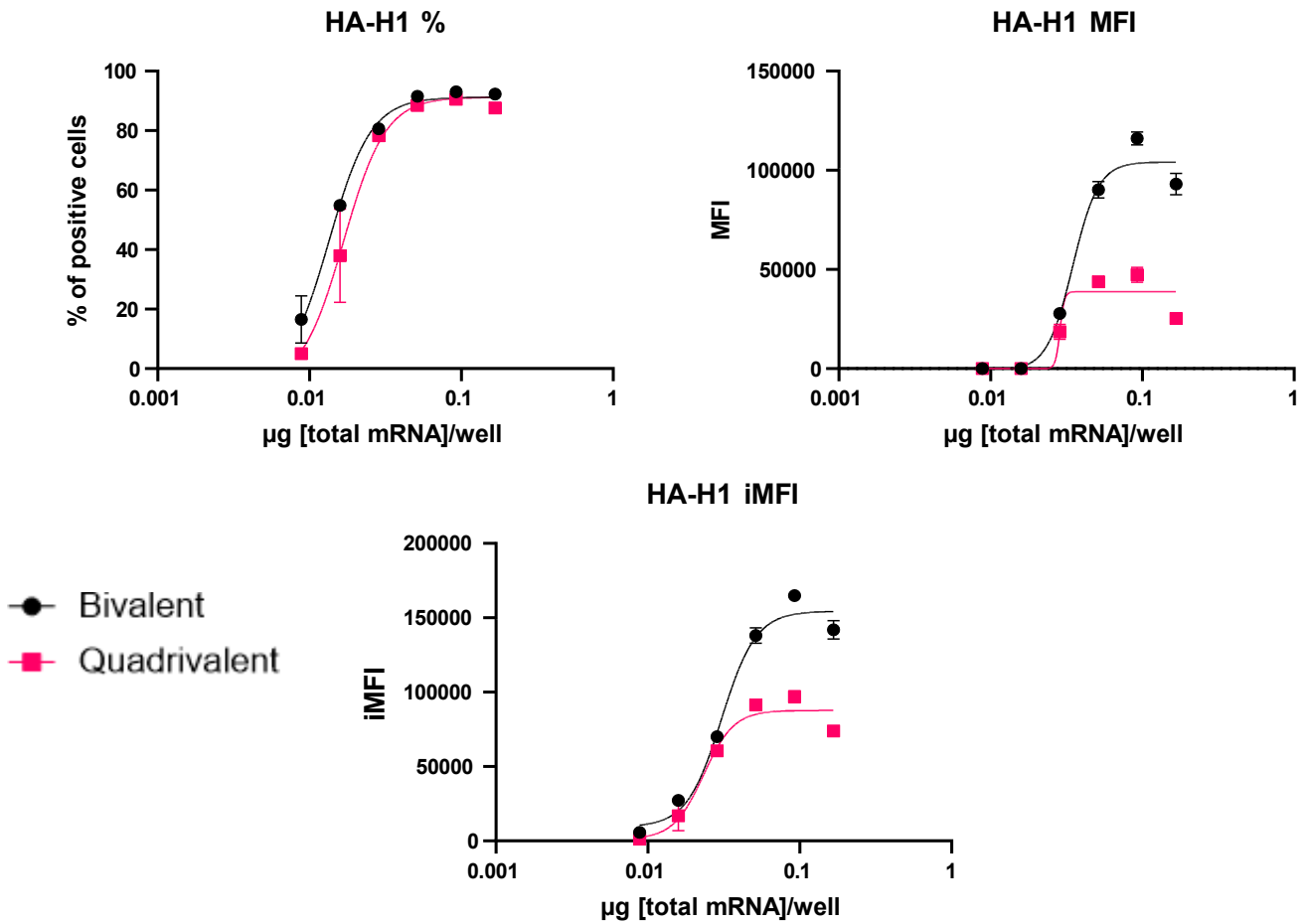
Free mRNA impact: Spiking of free mRNA



Theoretical levels of *encapsulation*: DP with mRNA for four strains of HA *spiked* with a pool of the same *unencapsulated mRNAs*

- *Free mRNA* does not impact parallelism and leads to a decrease in *relative potency*
- All read-outs *correlate* highly with the percentage of free mRNA that was spiked
- Samples with *lower encapsulation percentage* behave as *dilutions* of the fully encapsulated sample

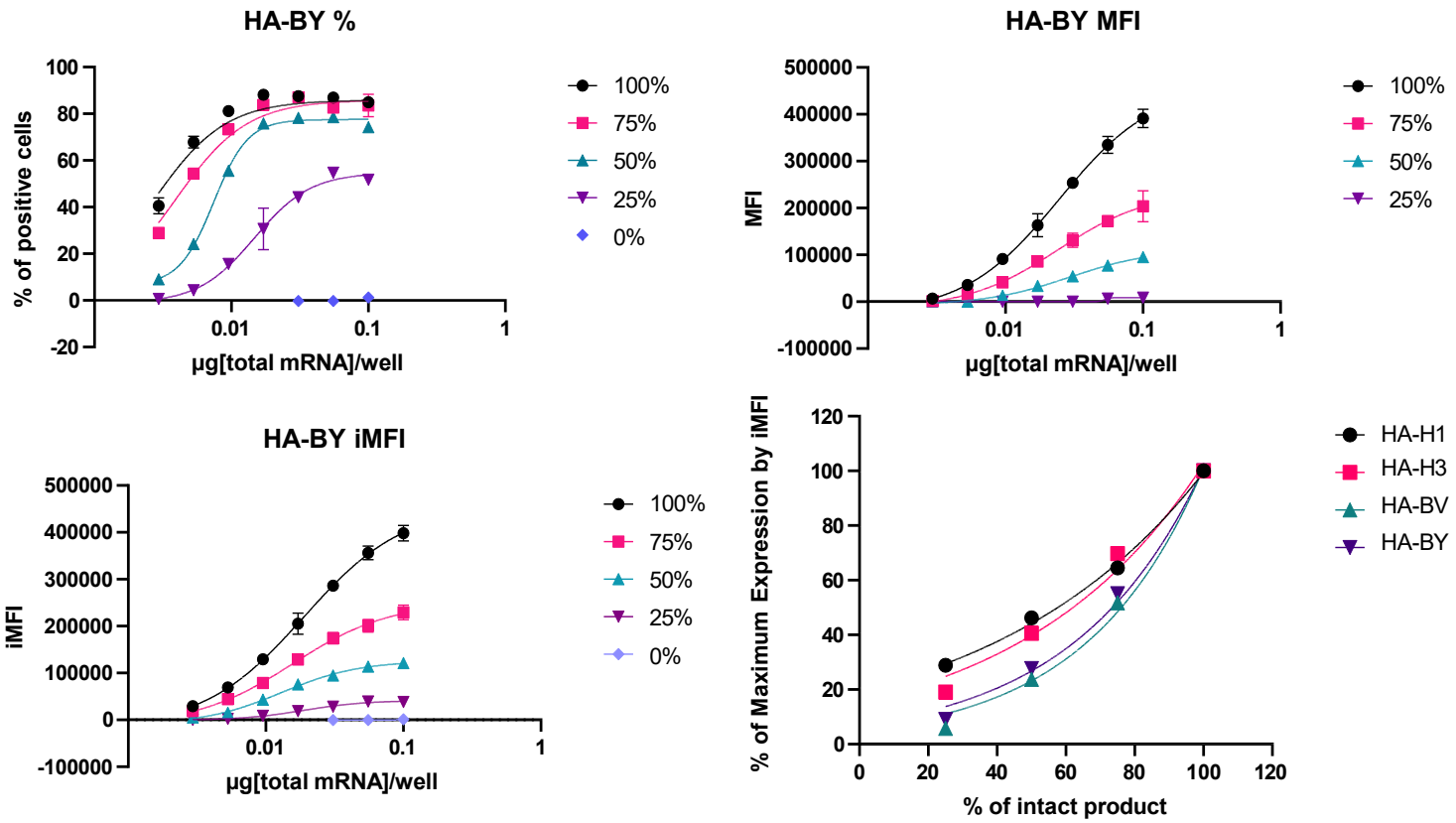
mRNA ratio impact: bivalent VS quadrivalent Drug Proc



Bivalent to *Quadrivalent* :
DP with *four* strains of HA (25% H1) compared with a DP with *two* strains of HA (50% H1)

- Reducing the amount of mRNA by half *did not induce a 2-fold decrease* in relative potency
- MFI shows a significant difference in the *maximum expression* at the higher doses
- *iMFI* shows an important decrease in *maximum expression*

Drug Product *Degradation*: Spiking of a degraded DP

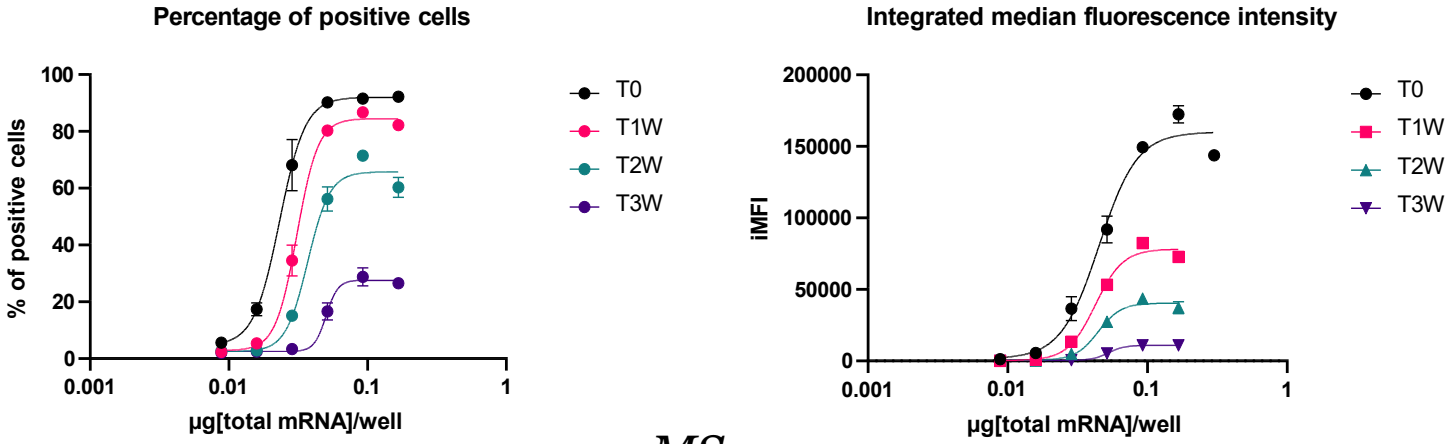


Theoretical levels of *degradation*:
DP with mRNA for four strains of HA *spiked* with the same *degraded* DP

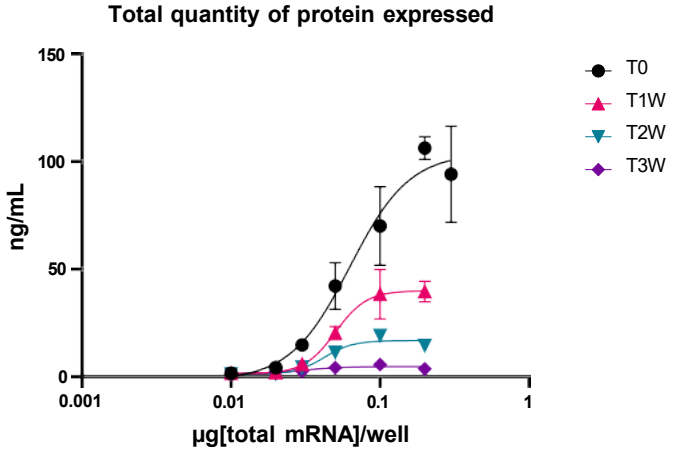
- *Percentage of positive cells*
  Shift of *relative potency* and a drop of the *maximum*
- *MFI/iMFI*
  Decrease in *maximum* expression
- *iMFI*
  Follows an *exponential* relationship with the quantity of *intact drug Product*

Stability studies by *FCM* and *MS*: Dose-responses

FCM



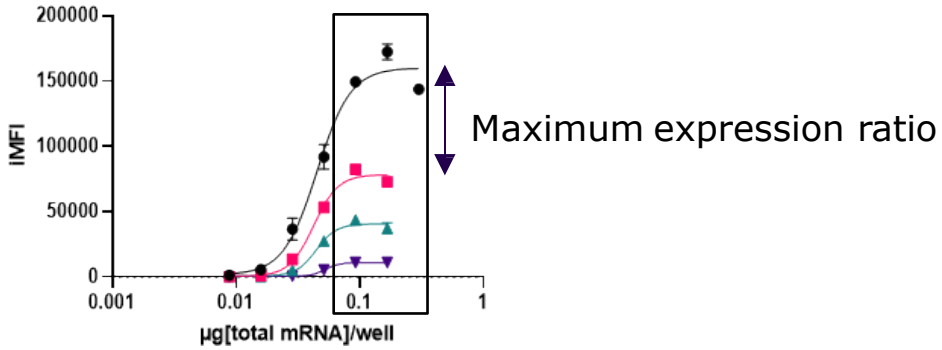
MS



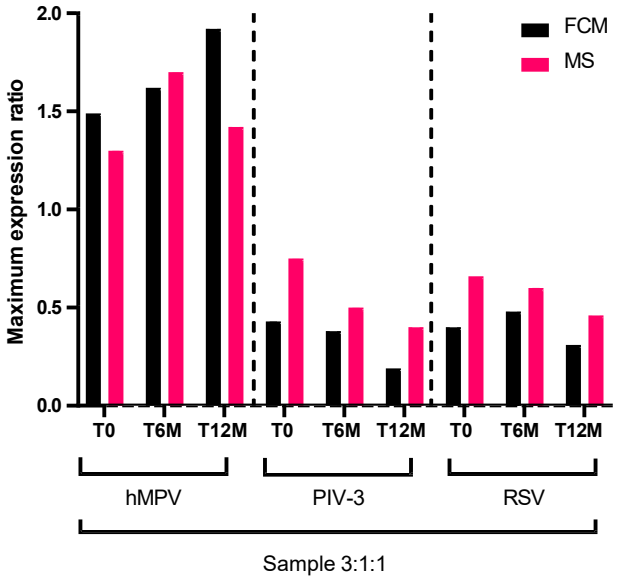
Mass spectrometry evaluation: Comparison with *Flow Cytometry* on a *degraded* DP

- *Percentage of positive cells*
  Shift of *potency* and a drop of the *maximum*
- *iMFI*
  Drop of *maximum* expression
- *Quantity of protein* expressed follows the *same trend as iMFI* but *does not correspond to % of positive cells*

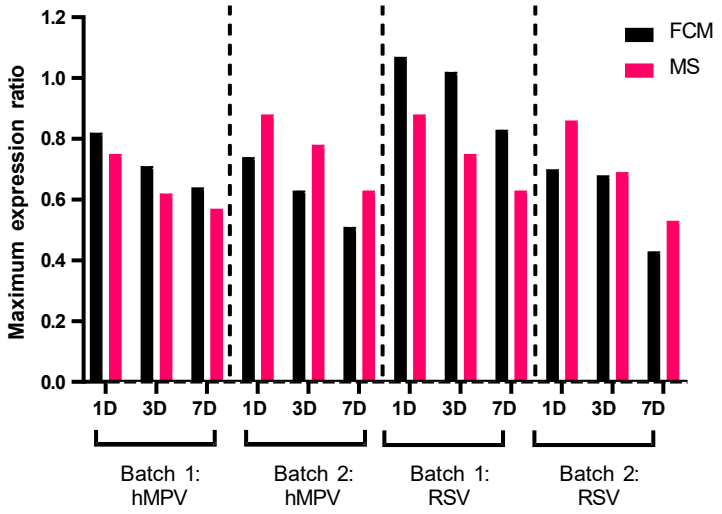
Stability studies by *FCM* and *MS* : Maximum expression



A: -80°C



B: +37°C



Mass spectrometry comparison with *maximum iMFI* on *stability studies* of trivalent DP ratio *1:1:1* and *3:1:1;* (3 highest doses analyzed)

- A: Impact of *mRNA ratio* in the sample containing 3 times the amount of hMPV mRNA observed with *both methods* (1:1:1 reference)
- Stability analysis of samples stored at -80°C, the two methods show the *same trends*
- B: Stability analysis at +37°C show the *same trends*

Conclusions on the cell-based protein *expression t*

Integrated Median fluorescence intensity should be used to have a more comprehensive view of mRNA vaccines *functionality* with antibody-based methods

Mass spectrometry is an antibody-free method to quantify protein expression

However, like flow cytometry, it *does not allow for calculating relative potencies* as described in the pharmacopoeias, due to the loss of parallelism between samples

This could be due to the *complex mechanism of action:* mRNA and LNP may not degrade at the same rate, causing *non homologous samples*

To have a *comprehensive* view of mRNA vaccines *functionality*, the usual relative potency test design *cannot be used*

Perspectives for *mRNA vaccines potency* assays

The antigen is the active component of non mRNA vaccines, so it is necessary to verify its *conformation and quantity*

mRNA-LNP is the active component of mRNA vaccines

Though cell-based protein expression must be characterized, mRNA-LNP *potency* is the result of a *combination* of *biophysical parameters* that can be evaluated separately to be more accurate

Potency could be considered as a combination of :

- **mRNA content Encapsulation rate mRNA integrity**

All those parameters do impact *in-vitro* expression and are *evaluated separately* for release and dose definition The cell-based expression test is *not a potency assay* but allows to verify *functionality* of the Drug Product and that all *CQAs impacting potency are under control.*

sanofi

Thank you

Any questions?