Beyond the Plate Reader: Solving Transduction, Reagent, and Readout Challenges in CGT Potency Assays

Case Studies Across Flow Cytometry and LC-MS

Jessica Bridges Weaver

BioAgilytix



©2026, BioAgilytix, Confidential and Proprietary.

Agenda



- Control Strategy and Potency Assays
- Why Beyond The Plate Reader
- Case Studies
  - Flow Cytometry
  - LCMS
- Conclusions
- Acknowledgments

©2026, BioAgilytix, Confidential and Proprietary.

The Story: Move Bulk Signal to Mechanism Resolved Potency

1. Potency Assurance

Anchor assays to CQAs, mechanism of action, product quality and clinical relevance.

2. Flow Cytometry

Resolve transduction, expression and phenotype at the single-cell level.

3. LC-MS

Measure transgene protein or functional metabolites with molecular specificity and less antibody dependence.

4. Orthogonal Control

Use complementary readouts and phase-appropriate validation to build a defensible CMC story.

©2026, BioAgilytix, Confidential and Proprietary.

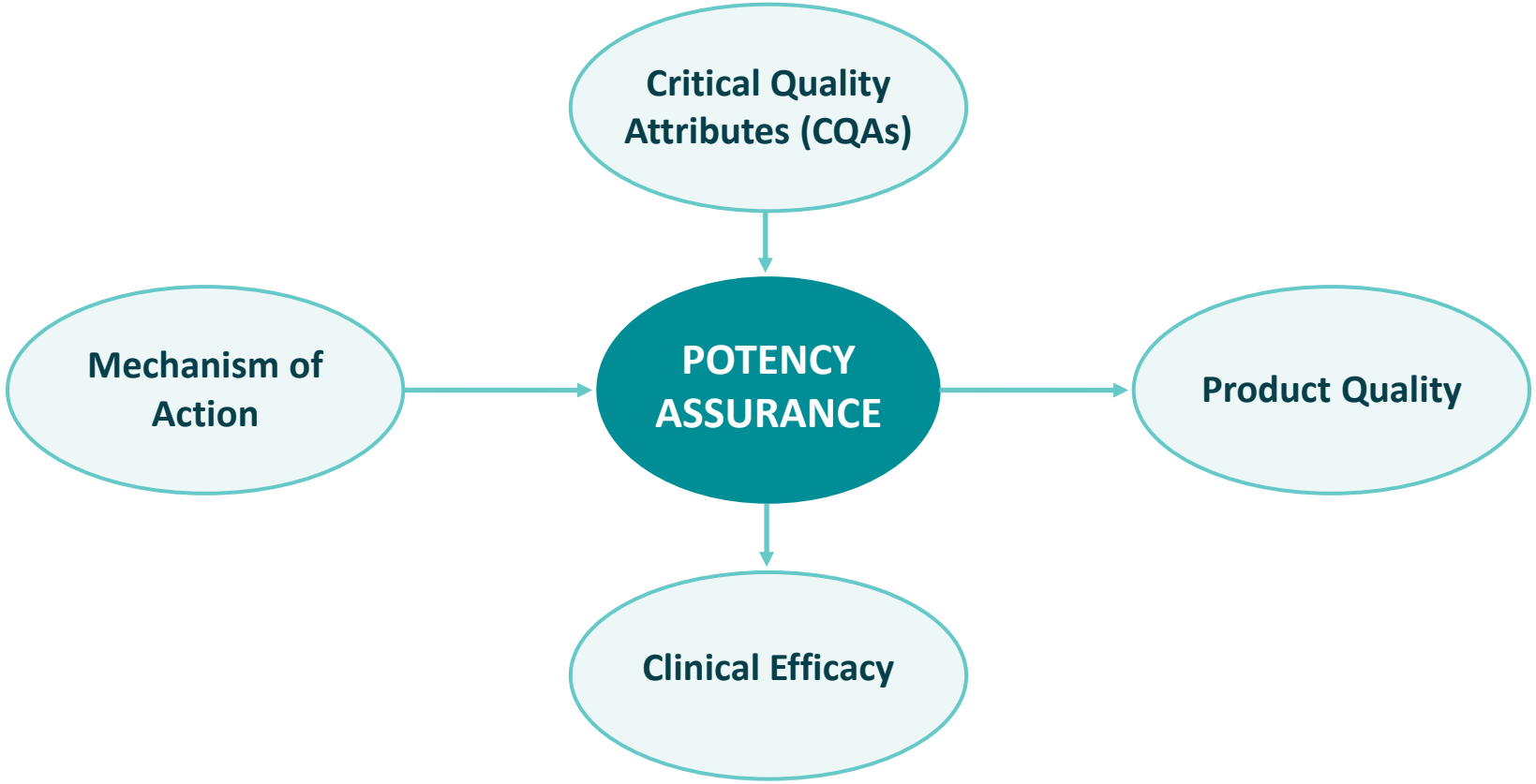
CGT potency sits inside a broader analytical control strategy

SAFETY	IDENTITY	STRENGTH	PURITY	QUALITY
Sterility / Adventitious Agents	Sequence / Capsid / Phenotype	Potency / Expression / Titer	Product- & Process-Related Impurities	Physical Attributes / Appearance

Potency is one critical element of SISPQ — the assay strategy must remain linked to product CQAs and development phase.

©2026, BioAgilytix, Confidential and Proprietary.

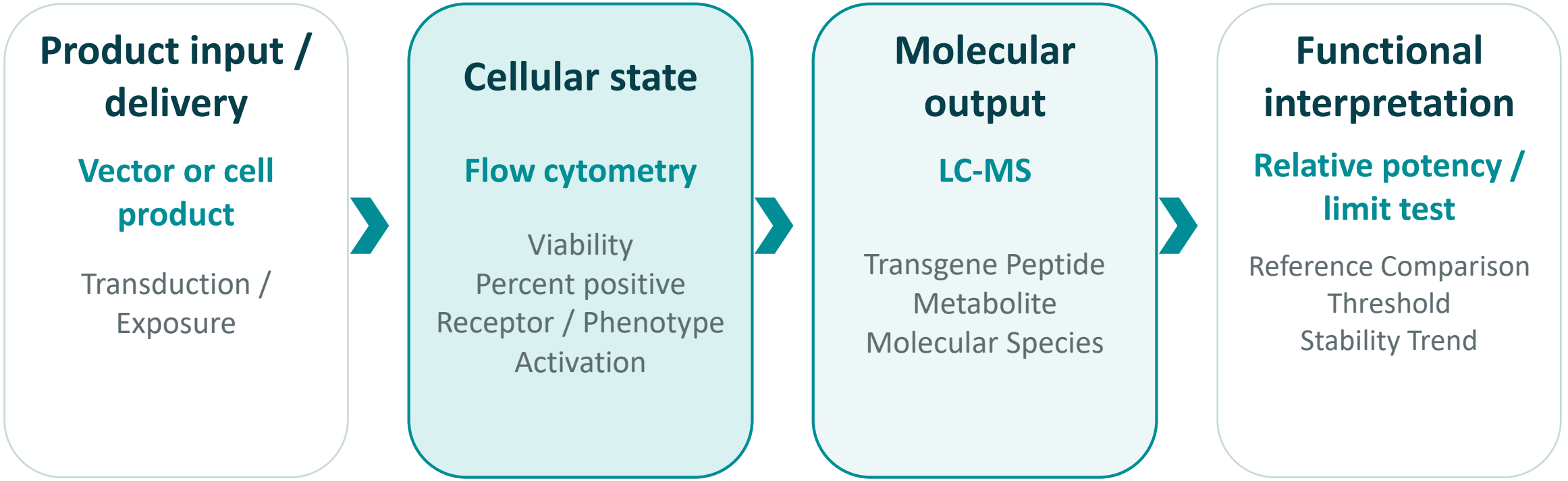
Potency assurance is a matrix, not a single readout



Implication: combine biological and biophysical/physicochemical evidence when a complex MOA cannot be captured robustly by one assay.

©2026, BioAgilytix, Confidential and Proprietary.

An orthogonal potency matrix connects cellular response to molecular mechanism



The matrix is strongest when each assay answers a distinct mechanistic question rather than duplicating the same signal.

©2026, BioAgilytix, Confidential and Proprietary.

Why move beyond the plate reader?

Bulk signal can hide cellular heterogeneity, reagent effects, and molecular ambiguity.

- **Transduction: which cells responded — and how strongly?**
- **Reagents: is the result drifting because the product changed, or because the reagent changed?**
- **Readout: does the signal directly represent the functional molecule or pathway?**

©2026, BioAgilytix, Confidential and Proprietary.

Pros and Cons: What is Gained, What is the Trade-off?



Plate Reader

Bulk Biological Response

- Pros:
  - Simple
  - High Throughput
  - QC Friendly
- Cons:
  - Population Average
  - Reagent Dependent
  - Indirect Readout



Flow Cytometry

Single-Cell Biology

- Pros:
  - Cellular Heterogeneity
  - Percent Transduced
  - Multiparametric Phenotype
- Cons:
  - Gating Complexity
  - Critical Reagents
  - Instrument / Operator Effects



LC-MS/MS

Targeted Molecular Quantitation

- Pros:
  - Direct Molecular Readout
  - High Sensitivity / Selectivity
  - Reduced Antibody Dependence
- Cons:
  - Target is Predefined
  - Matrix/Sample Prep Effects
  - No single cell information



LC-HRMS

High-Resolution Molecular Information

- Pros:
  - Accurate Mass
  - Molecular Identification
  - Variants
- Cons:
  - Data Complexity
  - Greater Analytical Burden
  - Exceeds Routine QC Needs

©2025, BioAgilytix

Platform Selection Requires Scientific and Operational Trade-offs

Plate Reader, Flow Cytometry, LC-MS/MS and/or LC-HRMS

	Plate Reader	Flow Cytometry	LC-MS/MS	LC-HRMS
Scientific Resolution	+	++	++	+++
Lab Footprint / Infrastructure	+	++	+++	++++
GMP Implementation*	+	++	+++	++++
Method / Data Complexity	+	++	+++	++++
Capital / Operating Cost	$	$$	$$$	$$$$

The analytical benefit must justify the additional infrastructure, qualification, validation, and lifecycle burden.

©2026, BioAgilytix, Confidential and Proprietary.

*Relative implementation burden; actual requirements depend on existing infrastructure, intended use, instrument configuration and site quality systems.

Flow and LC-MS solve different parts of the potency problem

Question	Flow cytometry	LC-MS
Unit of information	Single cell / subpopulation	Molecule / peptide / metabolite
Strength	Heterogeneity + multiparameter phenotype	Specificity + direct molecular readout
Typical CGT use	Transduction, expression, phenotype, proliferation	Transgene protein, functional metabolite, molecular characterization
Key dependency	Panel design, controls, gating, cell state	Sample prep, analyte selection, chromatography, instrument response
Best fit in matrix	Cellular consequence of treatment	Molecular evidence of expression/function

©2026, BioAgilytix, Confidential and Proprietary.

Flow cytometry turns transduction into a distribution, not an average

Cells

Start with viable, relevant cells

Transduction

Dose with vector / mRNA / cell product

Panel

Measure expression + phenotype

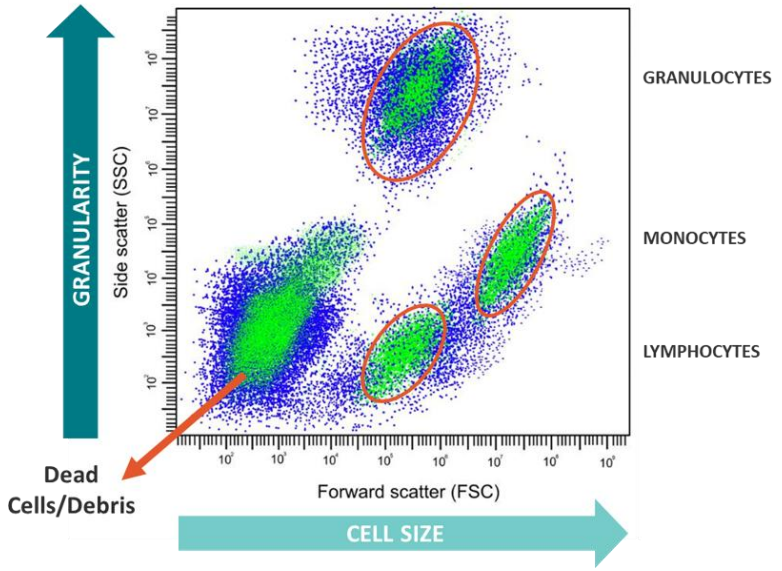
Gating

Resolve viable target population

Potency

Compare % positive, MFI and/or relative response

Design principle: viability and gating decisions are part of the measurement model, not post-processing details.



©2026, BioAgilytix, Confidential and Proprietary.

Flow assay robustness depends on the full measurement system

Biology	Cell health, passage, donor/source, transduction efficiency
Reagents	Antibody lot, fluorophore performance, reference standard, engineered cells
Instrument	Compensation, detector settings, platform transfer
Analysis	Gate hierarchy, viability rule, positivity threshold, MFI statistic
Operations	Operator training, timing, sample handling, data review

Critical point: if the reagent or analysis rule changes, the apparent product potency can shift even when the product has not.

©2026, BioAgilytix, Confidential and Proprietary.

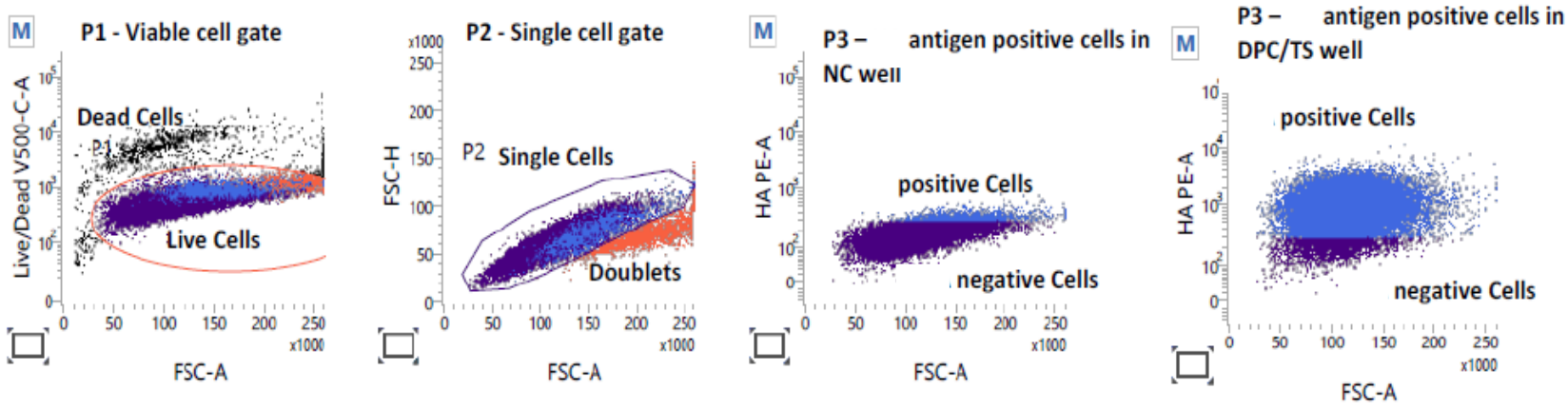
Snapshot for Assay – mRNA-LNP (Intracellular Assay)

BACKGROUND

Non-GMP Transfer and Sample Analysis

- **Modality** - modified mRNA lipid nanoparticle (LNP) vaccine
- **Cells:** HEK293T
- **Readout** - Protein expression is detected by intracellular antibody staining and flow cytometry. Results are reported as the mean percentage of viable single cells positive for expression.

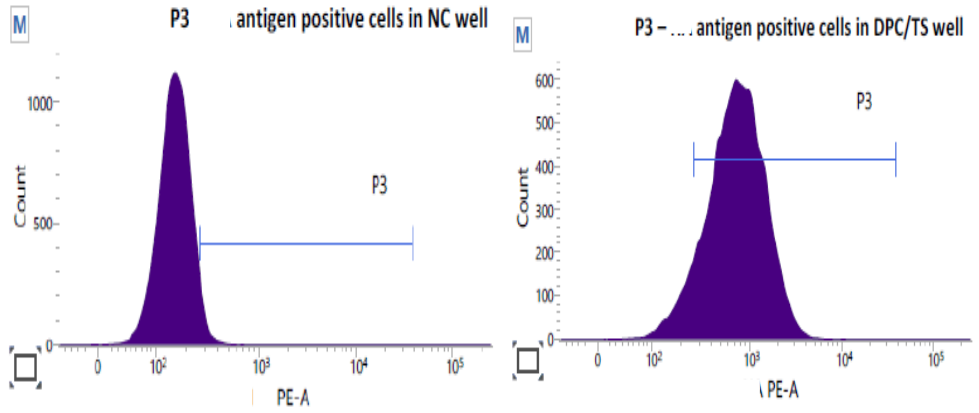
Figure 1: Gating Strategy and representative data



DESCRIPTION OF THE ASSAY

A cell-based flow cytometry assay used to assess the in vitro expression of a protein encoded by the modRNA-LNP vaccine. HEK293T cells are transfected with vaccine samples, stained for intracellular protein using a PE-labeled antibody detection system, and analyzed by flow cytometry. Results are reported as the mean percentage of viable single cells expressing target antigen from two independent assay instances.

Figure 2: Negative and Positive Expression



BioAgilytix
©2026, BioAgilytix, Confidential and Proprietary.

Snapshot for Assay – MPC



BACKGROUND

Development with GMP Phase I Validation

- **Modality** – Mesenchymal Precursor cells (MPC)
- **Cells** - Bone marrow derived MPC
- **Readout** – Flow cytometry to demonstrate positive STRO4 and CD146 expression, with negative CD31, CD45, CD80, CD86, HLA-DR expression.

DESCRIPTION OF ASSAY

This is an immunophenotyping assay for MPC, demonstrating the high expression of positive markers STRO4 and CD146 on MPC cells while control Jurkat cells display high expression of CD31 and CD45 and control Raji cells express CD45, CD80, CD86, and HLA-DR, which are all negative markers for MPC cells.

FIGURE 1: Flow Cytometry Raw Data

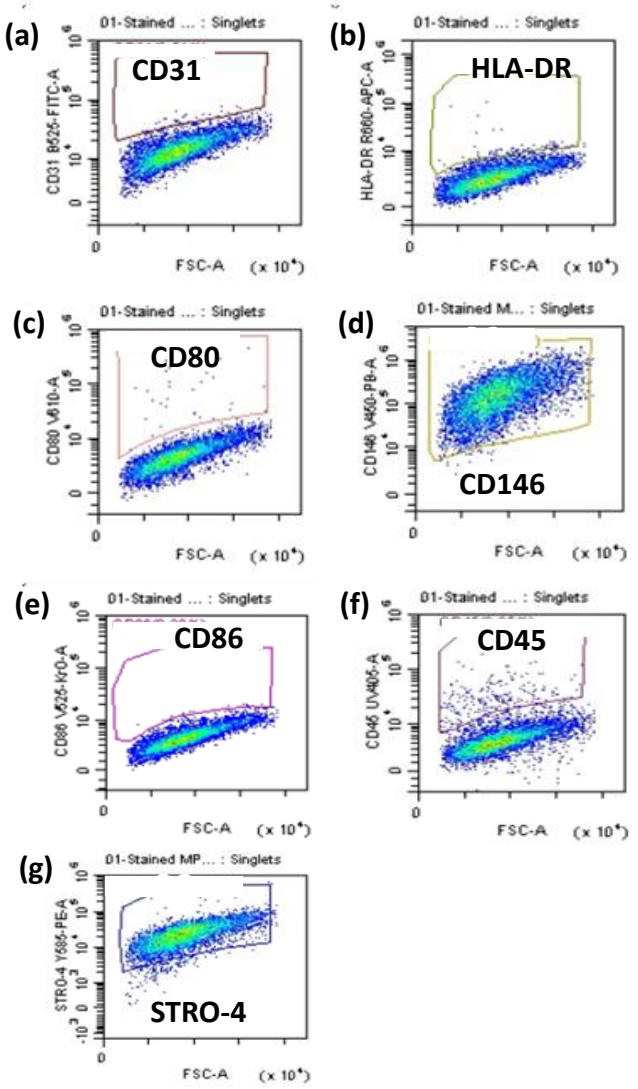


FIGURE 2: CD146 Linearity

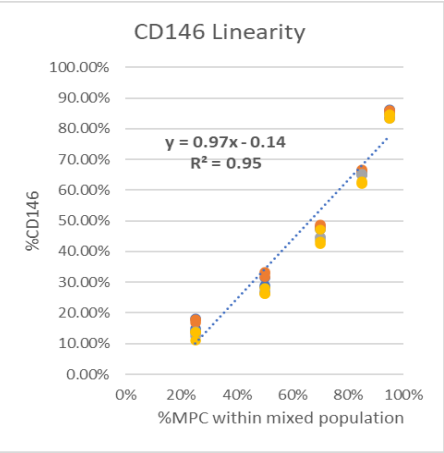
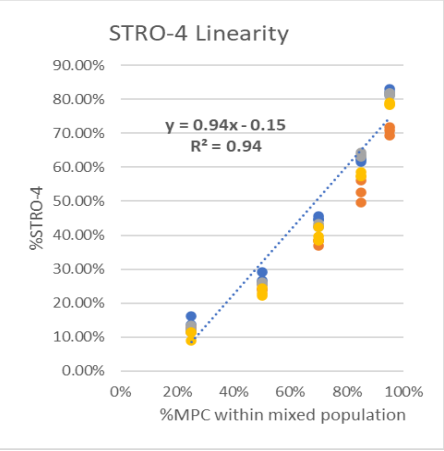
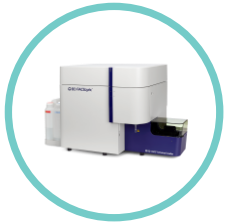


FIGURE 3: STRO-4 Linearity



©2025, BioAgilytix

Snapshot for Assay – mRNA-LNP (Engineered IVE Assay)



BACKGROUND

Non-GMP Transfer and Sample Analysis

- **Modality** - modified mRNA (modRNA) lipid nanoparticle (LNP) vaccine encoding the membrane-bound antigen.
- **Cells** - Expi293F (engineered HEK293)
- **Readout** - Antigen expression is detected by intracellular antibody staining with an AF488-conjugated secondary antibody and flow cytometry. Results are reported as the mean percentage of viable single cells positive for expression.

DESCRIPTION OF THE ASSAY

A cell-based in vitro expression (IVE) assay that evaluates expression of the membrane-bound antigen encoded. Expi293F cells are transfected with modRNA-LNP, stained with two conjugated antibodies, and analyzed by flow cytometry to quantify the percentage of viable single cells expressing target antigen.

Figure 2: DPC Expression example

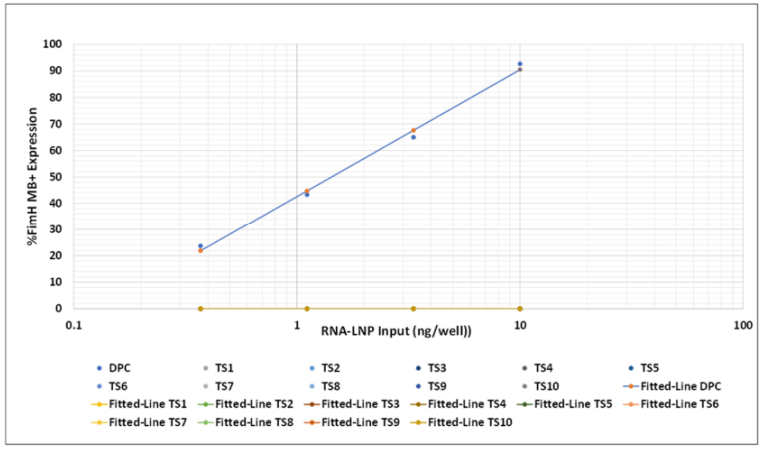
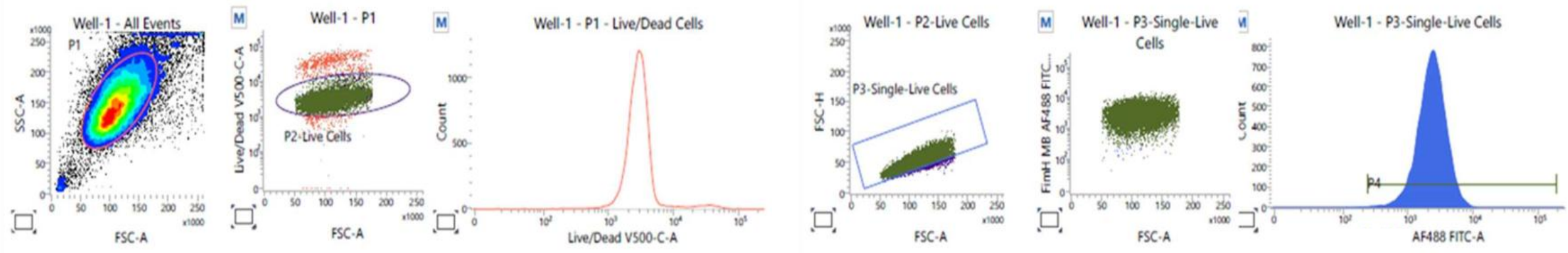
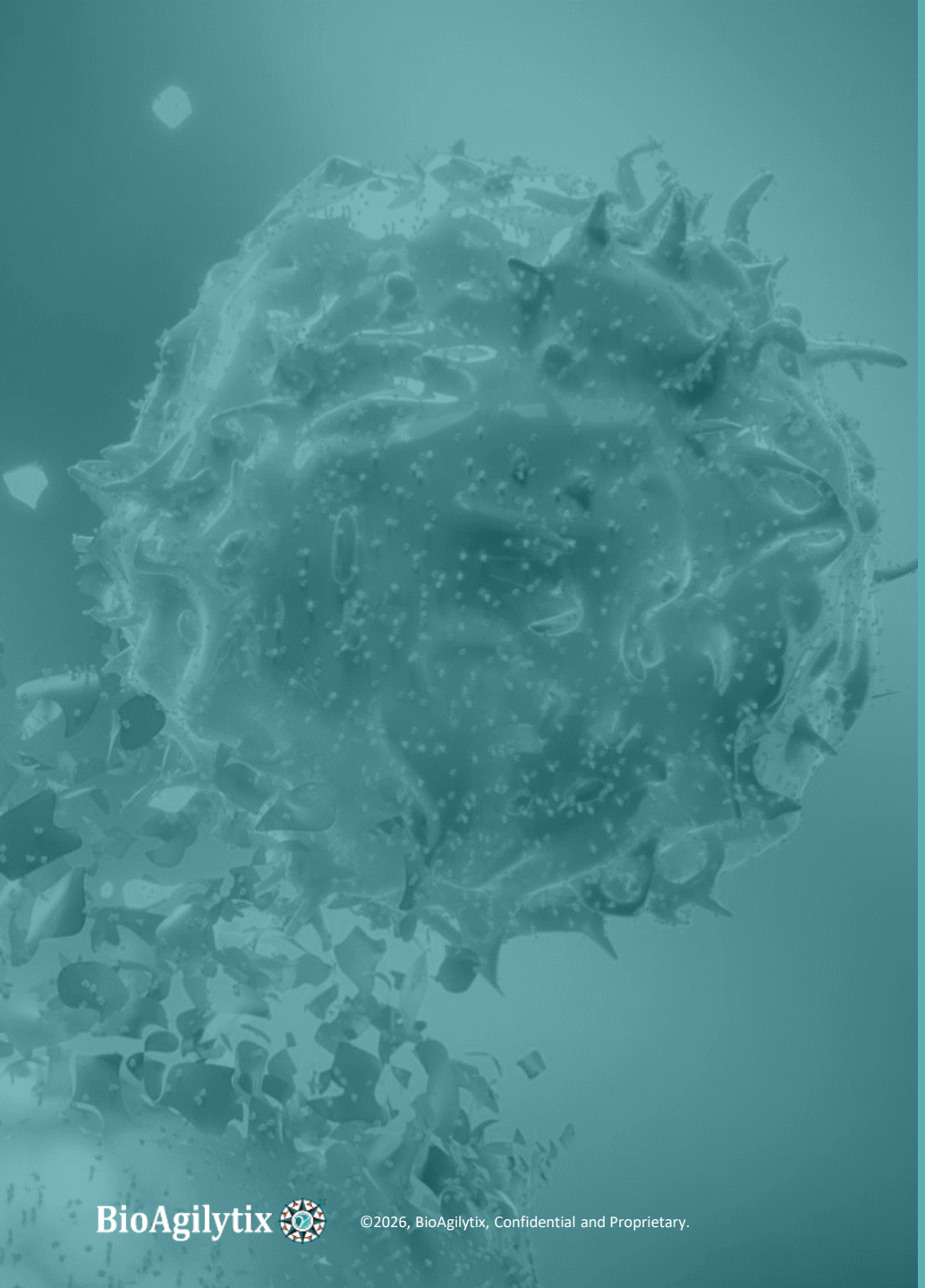


Figure 1: Representative DPC/Test Sample Well Report with Dot Plots and Histograms



©2026, BioAgilytix, Confidential and Proprietary.



LC-MS changes the question from “what signal did the reagent produce?” to “what molecule is present?”

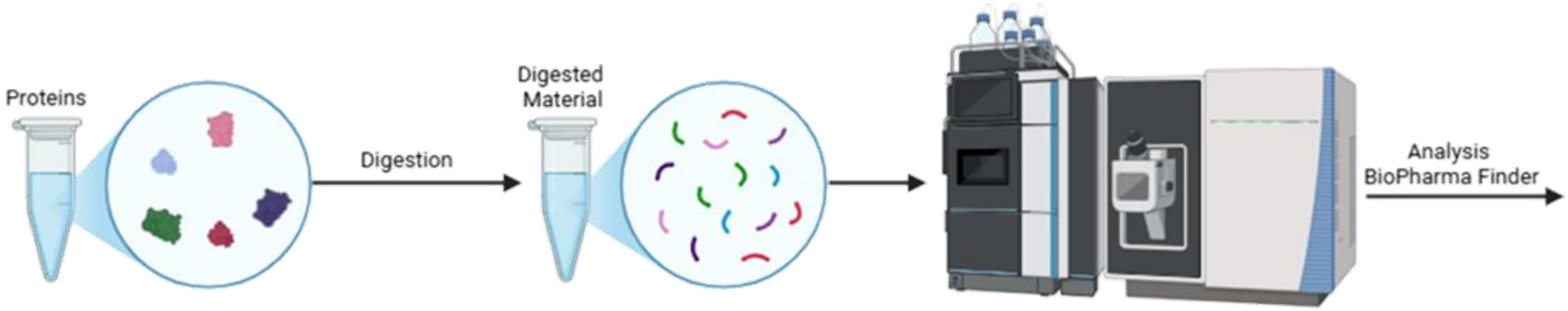
Direct molecular detection can reduce dependence on specialized antibodies and add specificity, multiplexing and mechanistic insight.

- **Reduced Dependence on Affinity Detection Reagents**
- **Molecular specificity**
- **Multiplexing**
- **Isoform / variant information**
- **Adaptable analyte selection**

BioAgilytix ©2026, BioAgilytix, Confidential and Proprietary.

Advantages of LCMS in ATMP Assays

- High Sensitivity and Specificity
- Comprehensive Characterization
- Quantitative Measurements
- Multiplexing Capability
- Reduced Dependence on Specialized Antibodies
- Compatible with GMP Validation



Proteins are detected and quantified in disrupted cells as their surrogate peptides using LC-HRMS/MS

©2026, BioAgilytix, Confidential and Proprietary.

Matrix and instrument effects still matter

LC-MS is not “plug and play”

Sample solvent

High organic content can evaporate rapidly and increase variance; lower organic solvent can improve variance but can affect peak shape.

Injection conditions

Peak shape can be recovered by reducing injection volume while maintaining sufficient signal.

Cell background

Endogenous created significant background signal in the cell system.

Instrument response

The same plate can produce significantly different peak intensity on different systems and should be challenged during assay robustness.

©2026, BioAgilytix, Confidential and Proprietary.

Snapshot for Assay – AAV

(Functional Protein Expression)

BACKGROUND

- Development with GMP Phase 2 Validation
- **Modality** – Adeno-Associated Virus (AAV)
- **Cells** – HEK293
- **Readout** – Shimadzu Nexera Series UPLC System on SCIEX Triple Quad™ 7500 MS

DESCRIPTION OF ASSAY

Following cell transduction, secreted protein with the appropriate functional binding characteristics is selectively enriched, digested, and analyzed by LC-MS using protein-specific surrogate peptides.

Development focused on identifying a robust molecular readout by screening candidate peptides for specificity, sensitivity, background interference, and concentration response. The best-performing peptides were advanced into the final workflow. The resulting phase-appropriate method was designed as a limit test, using non-transduced controls to establish assay background and an LOQ-based threshold for positive functional protein expression.



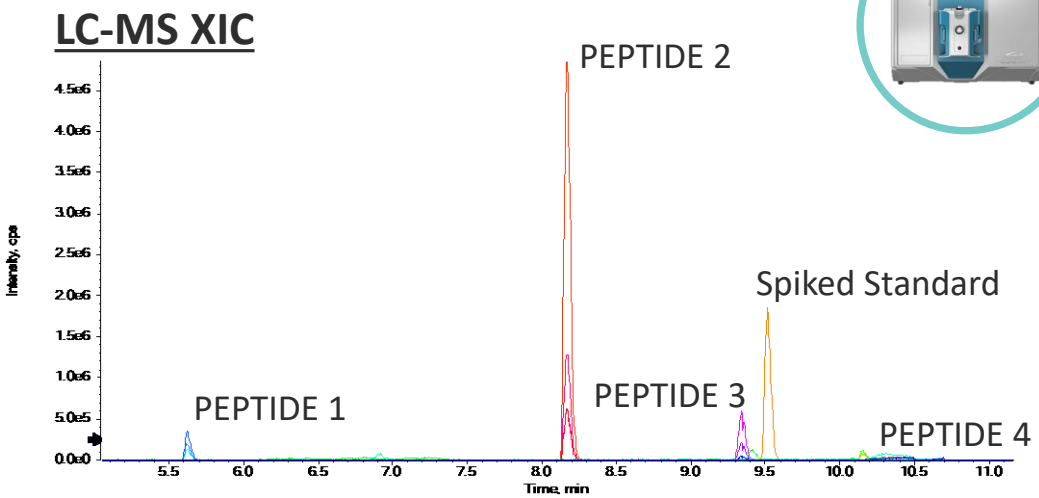


Figure 1: Trypsin digest and LC-MS analysis of recombinant human RS1 protein to test detection of candidate peptides

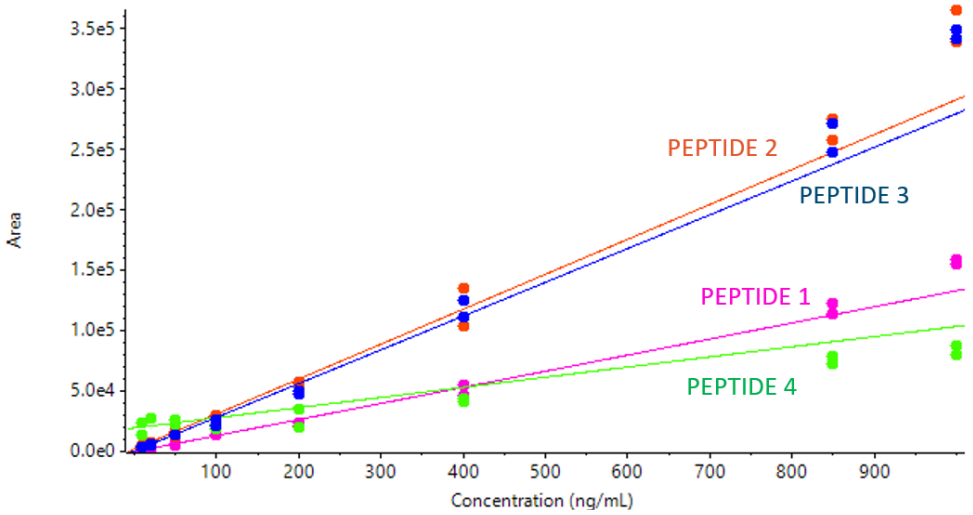


Figure 2: LC-MS Analysis – Concentration Response

©2026, BioAgilytix, Confidential and Proprietary.

Snapshot for Assay – AAV

(Functional Metabolite)

BACKGROUND

- Ongoing Development for Phase 2 Validation
- **Modality** – Adeno-Associated Virus (AAV)
- **Cells** – Engineered HRPE
- **Readout** – Shimadzu Nexera Series UPLC System on SCIEX Triple Quad™ 7500 MS

Figure 1: Reconstitution Solution

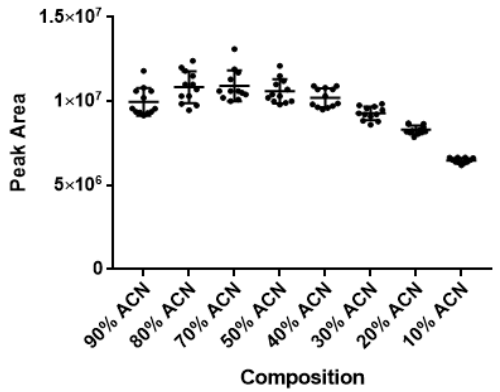
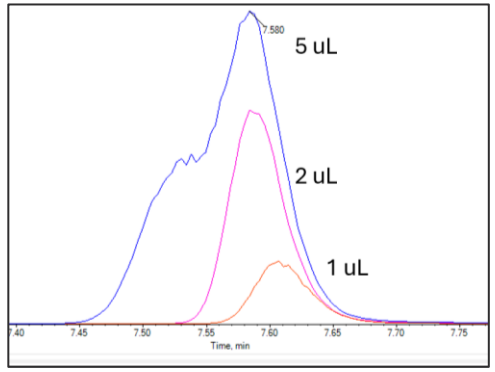


Figure 2: Test Injection Volumes





DESCRIPTION OF ASSAY

Following cell transduction, cells are lysed and the intracellular metabolite is extracted, separated, and quantified by LC-MS. Development addressed both analytical and biological sources of variability. Sample preparation and injection conditions were optimized to improve chromatographic reproducibility, while system exploration identified endogenous metabolite background from the cell line as an important assay consideration.

A dose response was established, and the method was configured for relative potency by comparing reference and test samples using a parallel-line approach. Instrument-to-instrument differences in raw response were mitigated through internal-standard normalization, supporting method robustness across systems. Relative potency development remains ongoing, with efforts focused on reducing variability and defining the assay range.

Figure 3: Product Dose Response

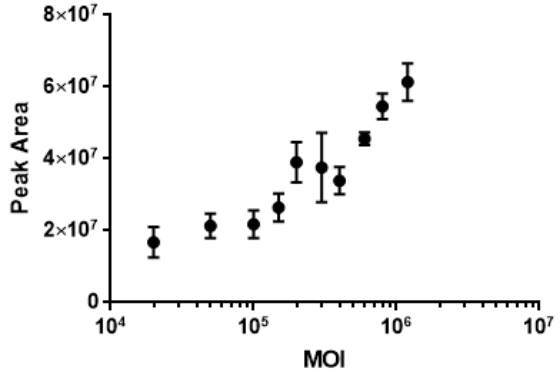
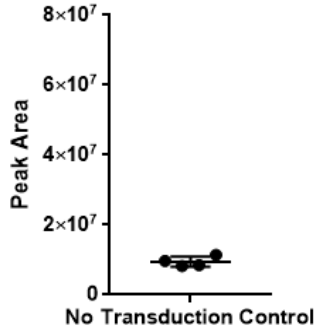


Figure 4: Background Signal



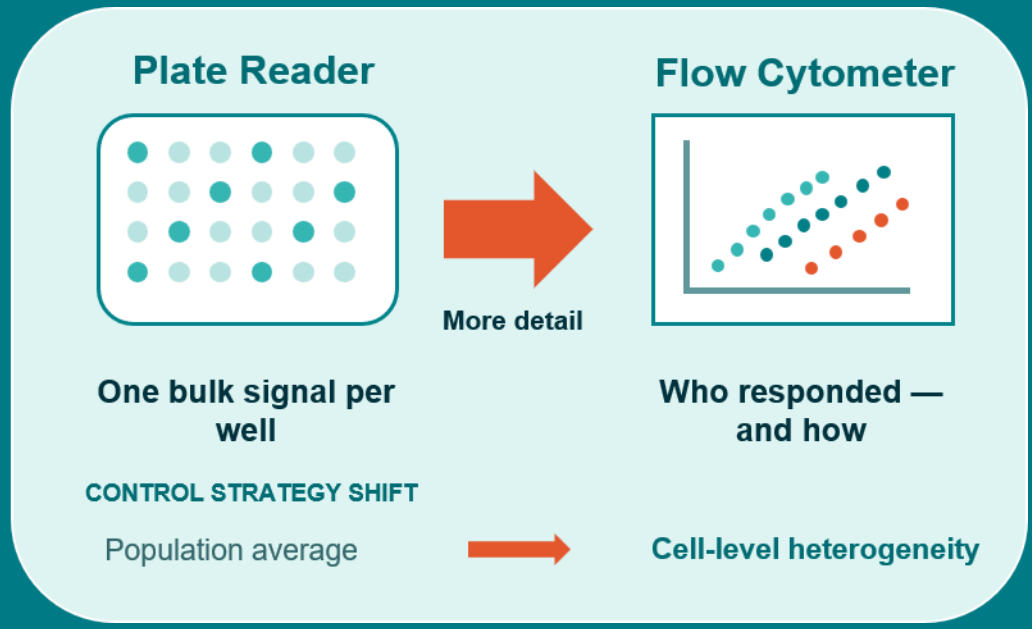
©2026, BioAgilytix, Confidential and Proprietary.

Beyond the Plate Reader

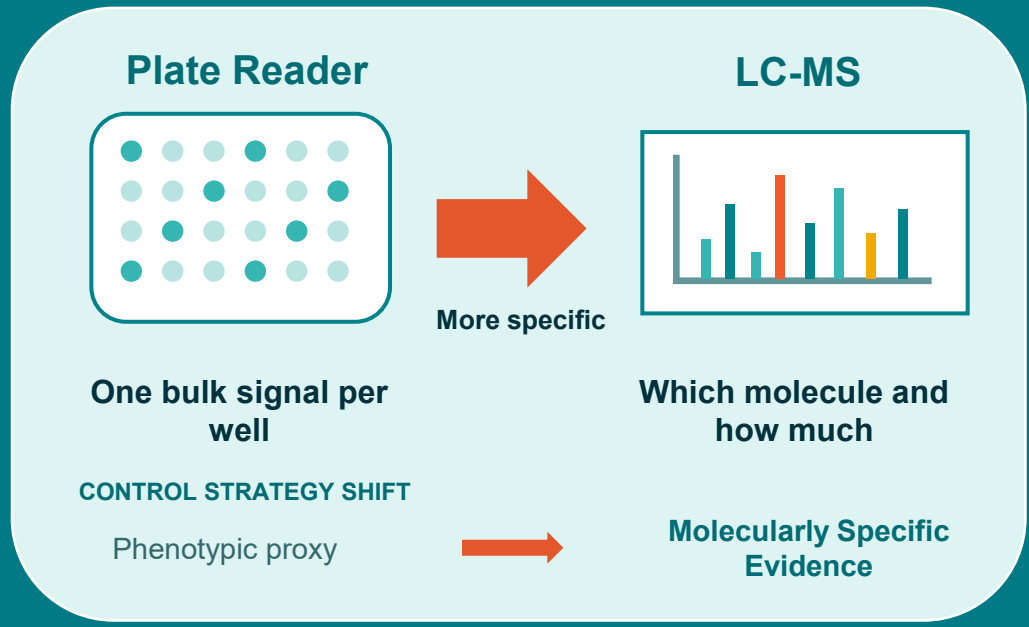
Control Strategy Decision – NOT a Technology Decision

- Flow cytometry resolves who responded and how cellular heterogeneity shapes potency.
- LC-MS provides molecularly specific evidence of transgene expression or downstream function.
- Orthogonal, phase-appropriate methods reduce dependence on any single reagent, signal or platform.

FROM AGGREGATE SIGNAL TO SINGLE-CELL RESOLUTION



FROM AGGREGATE SIGNAL TO MOLECULAR SPECIFICITY



BioAgilytix
©2026, BioAgilytix, Confidential and Proprietary.

Practical design checklist for non-traditional CGT potency assays

- ✓ Does the readout map to a defined MoA step or potency related CQA?
- ✓ Can the method distinguish product change from reagent, cell or instrument change?
- ✓ Are viability, gating, background and matrix effects explicitly controlled?
- ✓ Is the reference standard / control strategy sustainable across the lifecycle?
- ✓ Can the assay be transferred and operated under the intended GMP environment?
- ✓ What orthogonal evidence would make the potency conclusion more defensible?

©2026, BioAgilytix, Confidential and Proprietary.



Thank You!

ACKNOWLEDGEMENTS

BioAgilytix, Hamburg Germany
Julia Hecker, PhD
GMP Development & QC Team

BioAgilytix, RTP, North Carolina
Reema Davis, PhD
Bhoomi Jani Patel, MS
Bryan Thacker, PhD
Manoj Baskaran, MS
Likun Duan, PhD

CMC Development Team
GMP QC Team

Sponsors

©2026, BioAgilytix, Confidential and Proprietary.