Potency Assays for ATMPs – Selection of Assay Platforms and Data Processing Methods are Key Aspects for Successful Control Strategies

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BioAgilytix

Part 1:

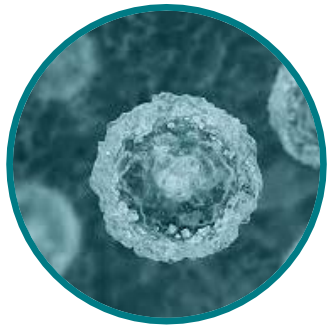
PCR, immunoassays, and flow cytometry as key components to the control strategies of ATMPs

Learning Objectives

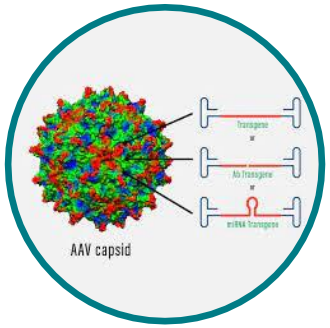
Summary

- Understand the analytical control strategy for ATMPs
- Discuss techniques applied to the control of ATMPs and how they are utilized; flow cytometry, ddPCR, qPCR, and immunoassays (including ELISA and MSD)
- Phase appropriate considerations for method utilization and qualification
- Understand the significance of potency assays in control strategies and the regulatory expectations for gene therapies
- Discuss example case studies to demonstrate the analytical technologies and approaches applied to potency assay development for gene therapies

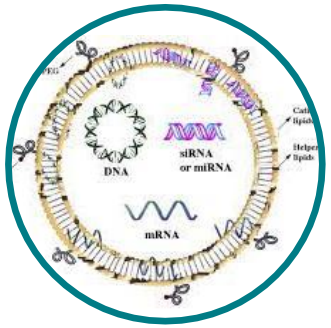
Advanced Therapy Medicinal Products (ATMPs): *Gene and Cell Therapies (GCT)*



Stem Cells

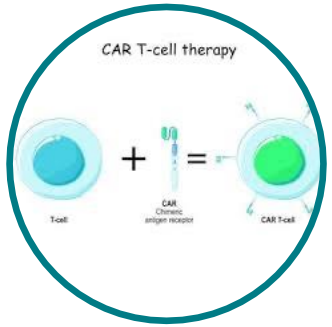


AAV

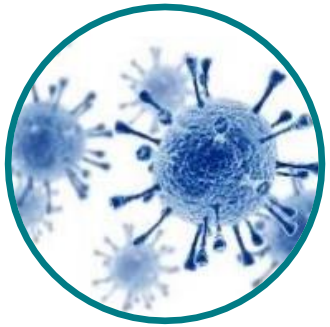


Lipid Nanoparticles

Tissue Products

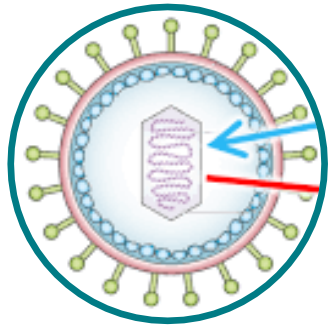


CAR T



Lentivirus

Naked or Paired Nucleic Acid



Engineered Oncolytic Virus

What is a CMC Analytical Control Strategy?

CMC – Chemistry, Manufacturing and Controls

- Section of regulatory filing: analytical testing is the controls aspects
- Characterization, tests and specifications designed to assure SISPQ (Safety, Identity, Strength, Purity and Quality) for a product or substance
- Addresses and monitors CQAs (critical quality attributes)
- Contains “control strategy” which is typically 10’s of tests driven by: required safety, manufacturing process, formulation and matrix, drug properties, and development data
  - A combination of GMP and characterization assays, combination of PAT and release tests

What Drives a Control Strategy?

Development Phase, CQAs, and SISPQ

Clinical Phase of Development

The phase of development will affect the rigor and demands of the assays and the dimensions of the control strategy as the processes, methods, and product evolve

SISPQ: Safety, Identity, Strength, Purity and Quality

The guiding principles and foundation of the cGMP control and monitoring of a drug substance/API or drug product

CQA: Critical Quality Attribute

"Physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality”

- ICH Guideline Pharmaceutical Development Q8(R2)

Challenges to CMC from ATMPs

Complex and Multiple MOAs

- Matrix approach (multiple assays)
- Functional assay (close as possible to the MOAs)
- Required to be quantitative and stability indicating
- MOA characterization (does not need to be GMP as not part of the control system)

Complex Assay Procedure

- Additional procedural steps and additional critical reagents
- Larger specifications on target titer/co

Reference Standard

- Well-characterized, qualified and stored appropriately

High Degree of Variability Between Batches

- Changes in process, scale-up, raw materials, plasmid, promoter, vector, etc.

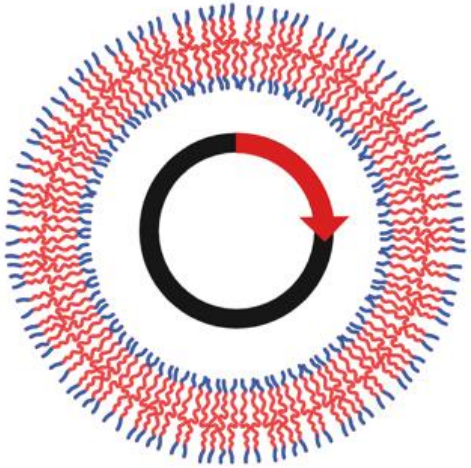
Limited Lot Size/Limited Material for Testing – Small Batches

- Variability in starting batches
- Limited lot size/lack of appropriate reference materials
- Increase in comparability studies

Control Strategy for a pDNA or mRNA (LNP) Product

PhysicoChemical
- Physical properties
- Quantity/titer
- Compendial tests

Characterization/ID
- DNA/plasmid ID
- Stability



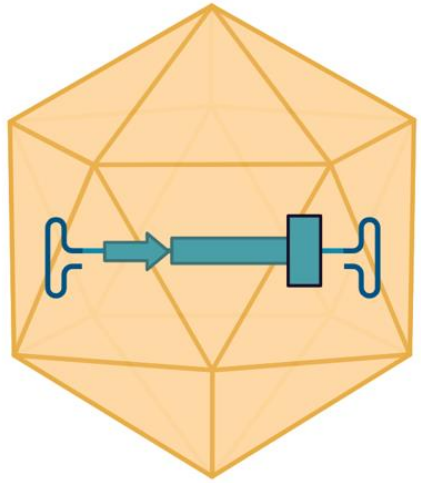
Biological Activity
- Infectivity
- Target gene expression
- MOA relative potency assays

Purity/Impurity
- Plasmid purity
- HCPs
- HC DNA
- Related impurities and residuals

Safety
- Sterility
- Compendial tests

Control Strategy for an AAV

PhysicoChemical
- Physical properties
- Quantity/titer
- Compendial tests
- Capsid quantitation
- Compendial tests
- Empty/full capsid



Biological Activity
- Infectivity
- Target gene expression
- MOA relative potency assays

Characterization/ID
- DNA/Plasmid ID
- Stability
- Capsid protein PTM

Purity/Impurity
- Capsid purity
- Plasmid purity
- HCPs
- HC DNA
- Related impurities and residuals

Safety
- Sterility
- Compendial tests

What Might be in a Control Strategy for an AAV ("SISPQ")

	Test			Test
Strength	**Vector Titer**		Potency	**Infectious Titer**
Identity	**GOI Sequence**		Potency	**Particle to infectivity ratio**
Strength	**Gene expression**		Potency	**Transgene Expression**
Identity	**Pro-viral Sequencing** (DNA sequencing)		Potency	**Enzymatic Activity; Relative Potency**
Strength	**Virus/Capsid titer**		Potency	**Stability (long term; short term)**
Physical	**Capsid Occupancy or Empty/Full Evaluation**		Potency	**Relative Potency (cell culture/vector transduction / ELISA)**
Physical	**Osmolality**		Potency	**Relative %CAR Expression** (cell culture/vector transduction/flow cytometry)
Physical	**pH**		Potency	**Infectious Titer** (transduction/cell culture/qPCR)
Physical	**Aggregates**			
Physical	**Appearance**			
			Safety	**Replication-Competent Virus**
Purity	**Process-related impurities: Benzonase, Resins, etc.**		Safety	**Adventious Viruses**
Purity	**Plasmid Purity**		Safety	**Endotoxins**
Purity	**Expressed Purity**		Safety	**Mycoplasma**
Purity	**Residual Plasmid DNA**		Safety	**Adventitious Viruses: Adeno-Associated Virus** (qPCR)
Purity	**Residual HC-DNA (SV40 T-Ag, E1A)**		Safety	**Replication Competent Lentivirus**
Purity	**Residual HCP**		Safety	**RCL (EOP) End of Product**
Purity	**Other Residual Proteins/DNA**		Safety	**Adventitious Viruses: In Vitro Adventitious Agents** (cell culture)
Purity	**Residual Genomic DNA** (qPCR)		Safety	**Viral Safety Testing (Cell therapies)**
Purity	**p24 Concentration** (ELISA)		Safety	**Bovine Polyoma virus**
Purity	**Pluronic, Tween, Triton**		Safety	**Bovine viral contaminants**
Purity	**Residual Benzonase**			

Common Techniques for CMC Testing of AAV (GCT/ATMP)

Protein Simple Maurice

- Empty/Full Capsid
- Glycoform Analysis
- Protein Purity
- Reagent Characterization

Bio-Rad QX200 and QXOne Droplet Digital PCR Systems

- Vector Copy Number
- Relative Potency
- Gene Expression
- Residual Plasmid
- Host Cell DNA
- MOA Identification

Flow Cytometry

- Cell attributes
- Gene Expression
- Cell activity
- Receptor biding/expression
- Multi-parameter analysis
- Reagent Characterization

Thermo Scientific Orbitrap Exploris

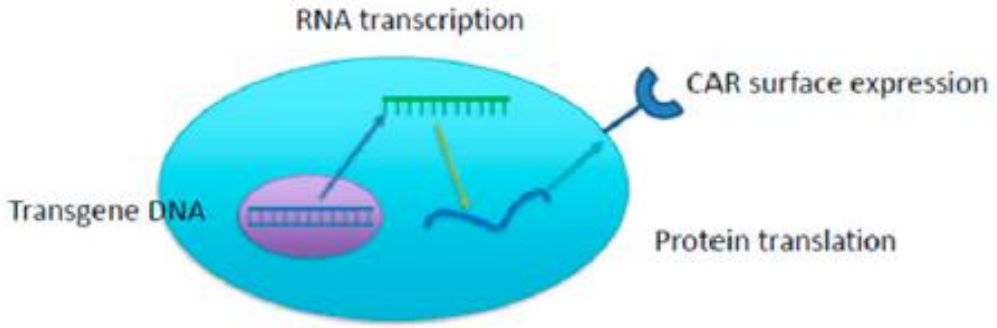
- LCMS Peptide Map
- Glycoform and PTM Characterization
- Impurity ID and Identity
- Capsid Characterization
- Reagent Characterization
- Potency Readout

Quantitative Strategy for Cell Therapies

Cellular Kinetics, Persistence

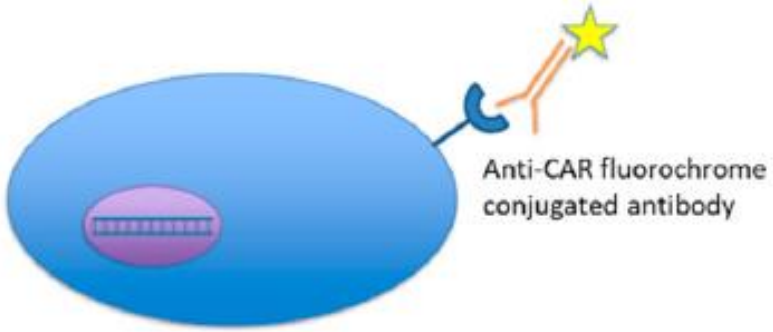
Quantitative PCR

CAR-T concentration is inferred from the level of transgene DNA



Flow Cytometry

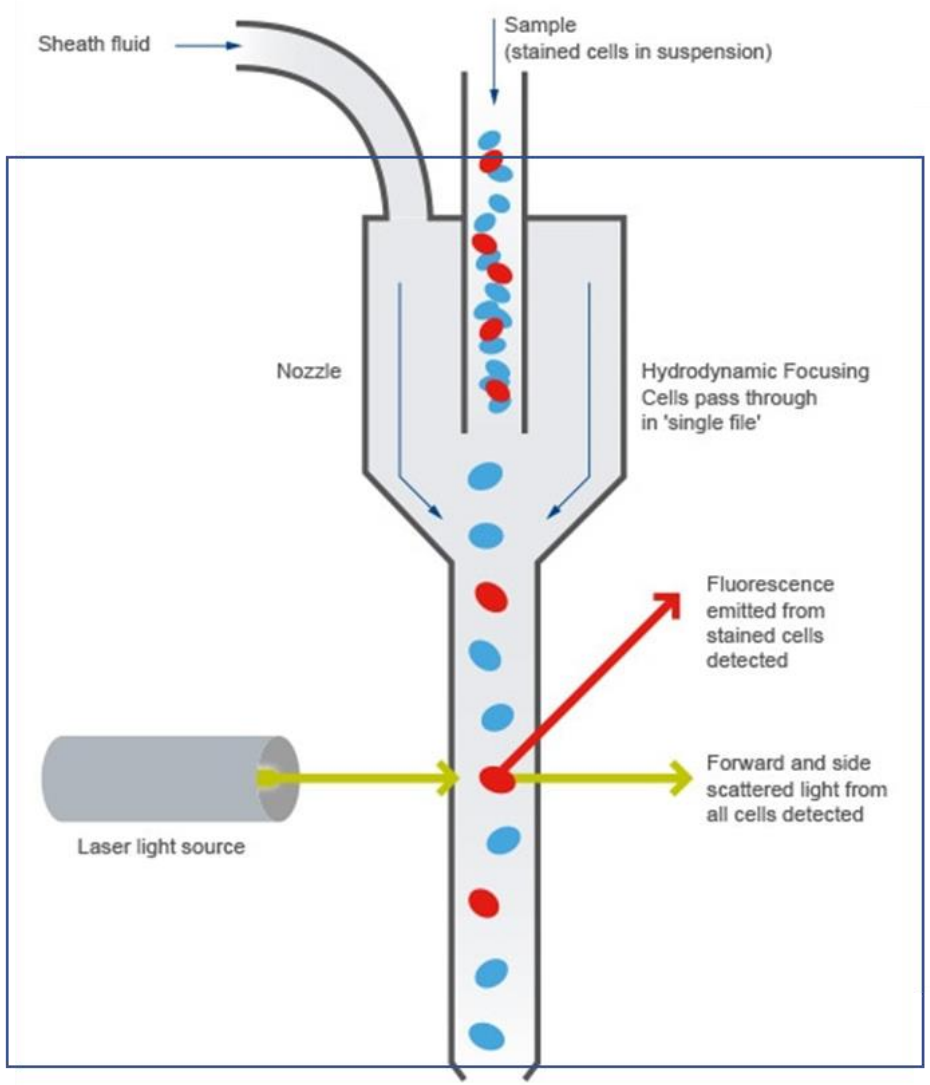
direct measure of the CAR-T therapeutic agent

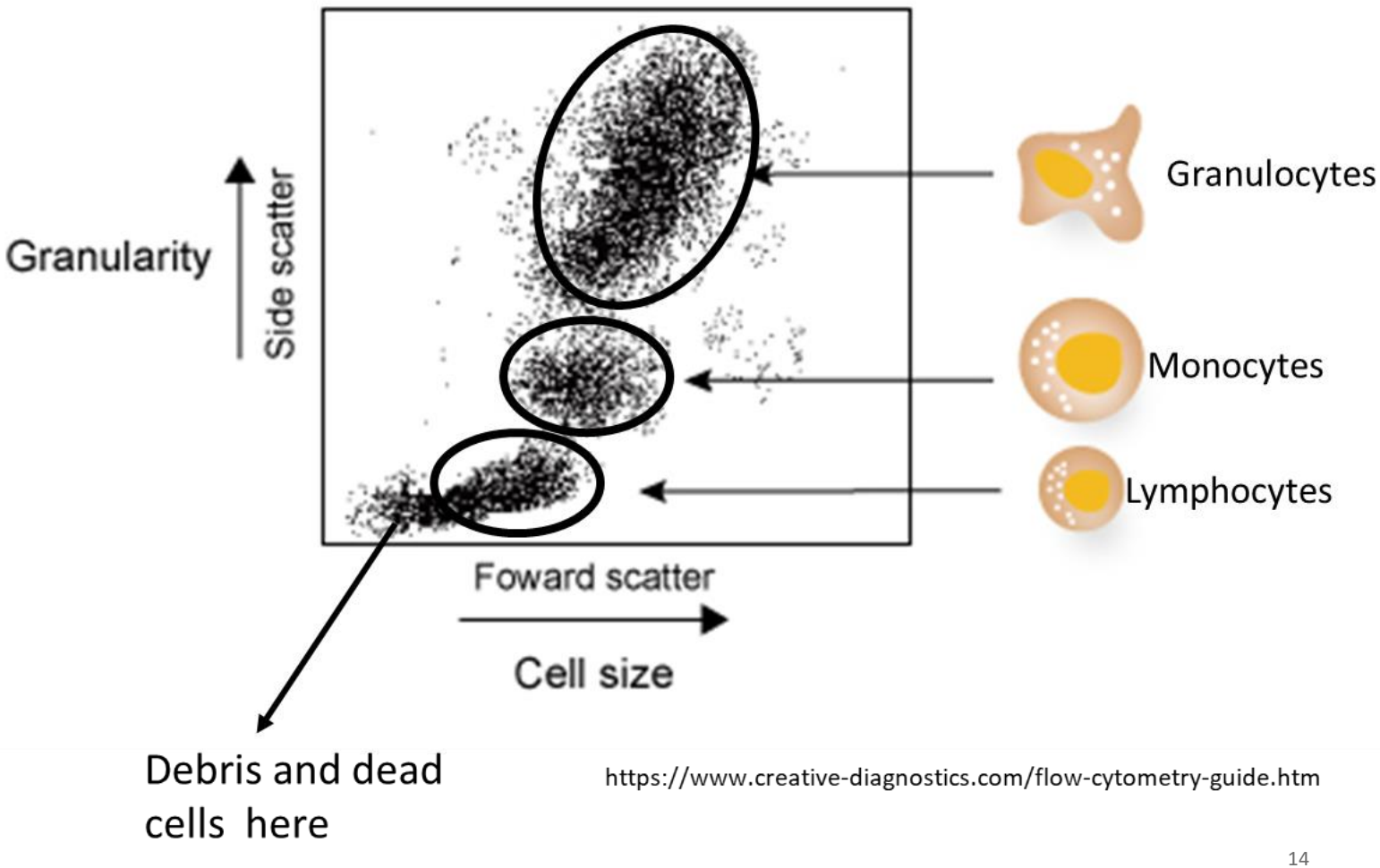


Hays et al. Int. J. Mol. Sci. 2023; 24, 695.

Flow Cytometry

Flow Cytometry – Platform Basics





https://www.creative-diagnostics.com/flow-cytometry-guide.htm

Advantages of Flow Cytometry Over Cell-based Assays

1. High-Throughput: Flow cytometry allows for the rapid analysis of a large number of cells

2. Multiparametric Analysis: Flow cytometry allows for the simultaneous measurement of multiple parameters in a single experiment and provides a more comprehensive understanding of cellular characteristics compared to some cell-based assays

3. Quantitative Measurements: Flow cytometry provides quantitative data for multiple attributes.

4. Cell Sorting Capability: Flow cytometers equipped with cell sorting capabilities can isolate specific cell populations based on their characteristics.

5. Single-Cell Analysis: Flow cytometry can analyze and sort individual cells, enabling the study of cellular heterogeneity within a population

6. Real-Time Analysis: Flow cytometry provides real-time data acquisition allowing analysis of fast kinetics, transient changes, or dynamic interactions within a cell population.

7. Automated Data Analysis: Flow cytometry data analysis can be automated, facilitating the handling of large datasets using a range of software tools.

8. Transferability: The movement or transfer of experiments between instruments or even between platforms is relatively robust.

9. Flexibility: All the above attributes makes flow a highly flexible and adaptable technique amenable to cell-based determination.

Adeno-Associated Virus (AAV)

MOA: AAV3 Capsid that contains a single-stranded DNA genome carrying a gene

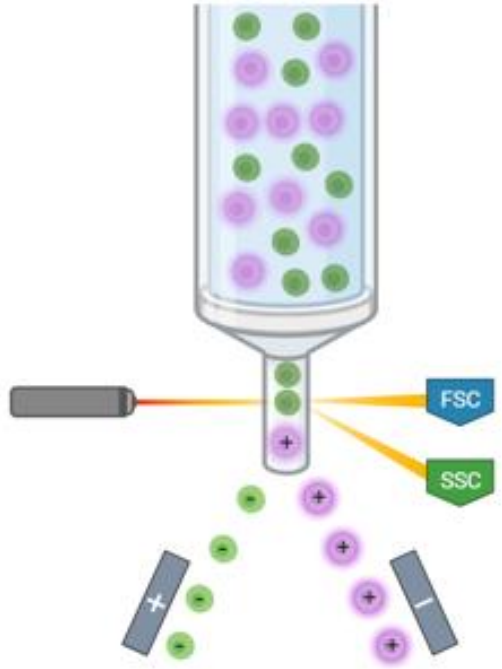
- Protein replacement therapy

Functional Flow Cytometry Assay:

- Flow cytometry provides quantitative data, allowing for accurate measurements of cellular features, such as protein expression levels, cell cycle distribution, and apoptosis. This is particularly useful when precise quantification is required for experimental analysis.
- Assay development/Optimization challenges:
  - Sourcing critical reagents
  - KO vs WT cells
  - Custom antibodies

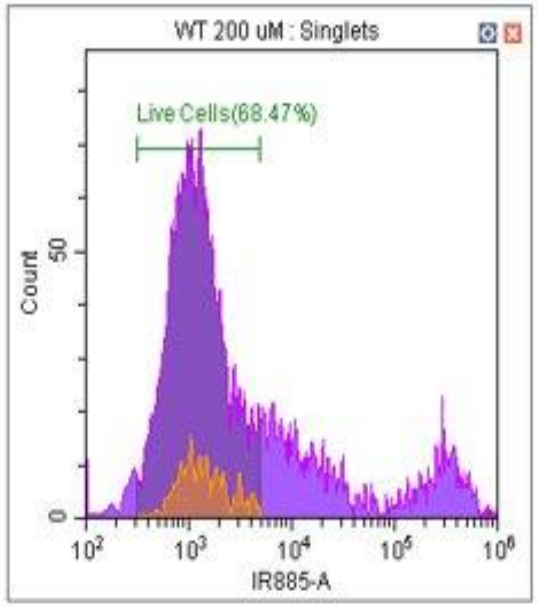
Assay Lifecycle Challenges:

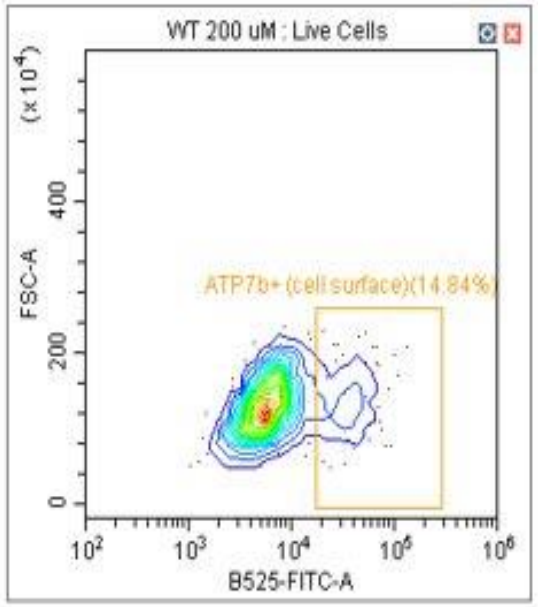
- Long-term stability of custom critical reagents
- Bridging of new lots of critical reagents



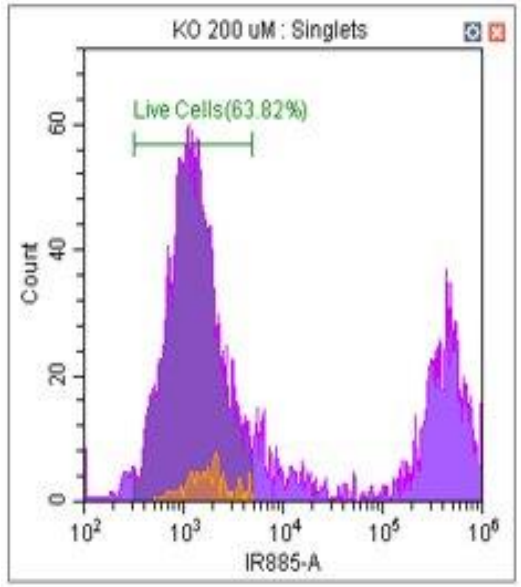
Representative Data

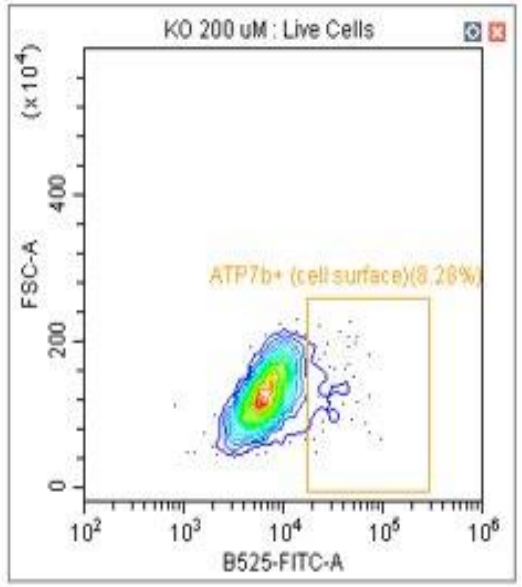
WT Cells, 200 uM Stimulant X





KO Cells, 200 uM Stimulant X





Note – Begin with 8-point curve for practical reasons… not optimal… end with 12

Snapshot for Assay (Phase I) - CAR-NK

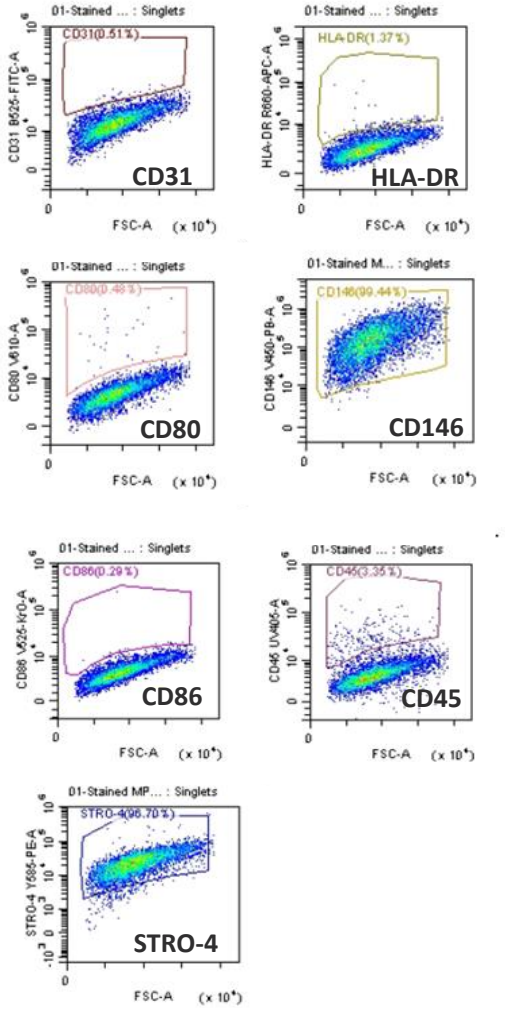
Background:
Modality – Irradiated uAPC cells (CAR-NK therapy)

MOA – Ki67 is absent in resting cells (G0) but present in all active phases of the cell cycle.

Cells – irradiated uAPC and non-irradiated K562

Readout – Flow cytometry to demonstrate the lack of proliferation in irradiated uAPC cells over time

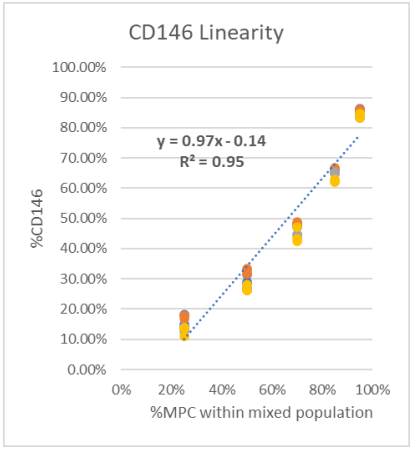
Description of Assay:
This procedure demonstrates the lack of Ki67 staining in the nucleus of irradiated uAPC cells compared to the control non-irradiated K562 cells cultured simultaneously over a period of 21 days.

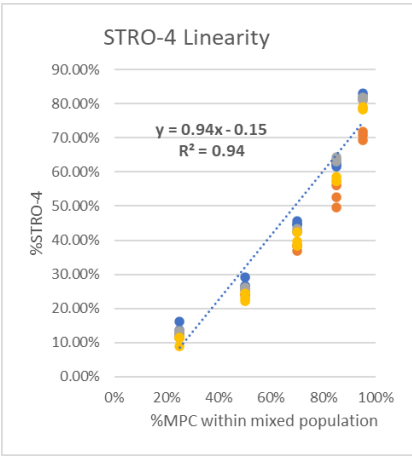


Representative data from assay

GMP Phase I Qualification:

Assay Performance Characteristics:



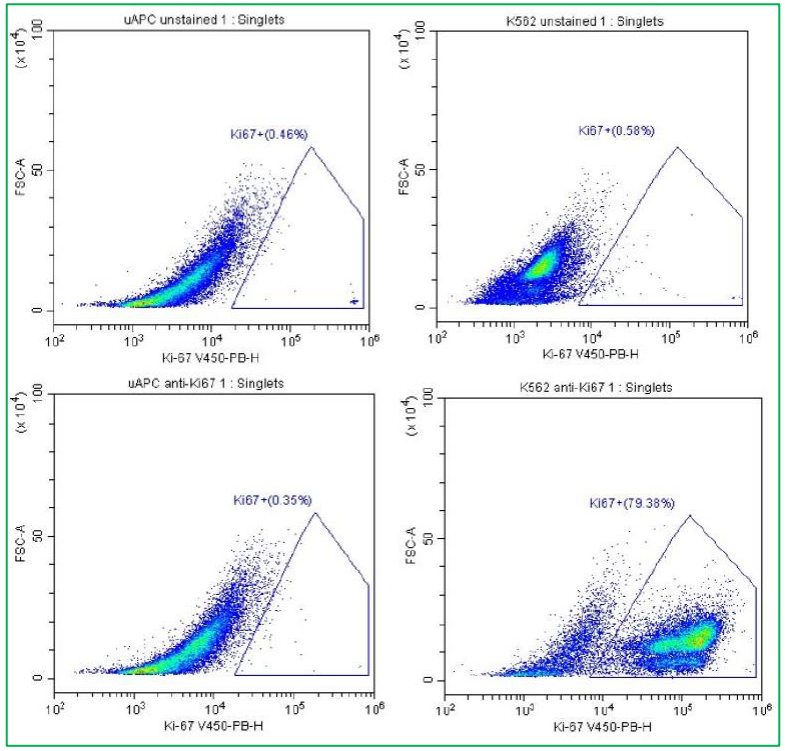


Specificity: STRO-4 and CD146 isotype antibodies generated a response similar to unstained cells demonstrating specificity

Snapshot for Assay (Phase I) - MPC

Background:
Modality – Mesenchymal Precursor cells (MPC)
Cells – Bone marrow derived MPC
Readout – **Flow cytometry** to demonstrate positive STRO4 and CD146 expression, with low CD31, CD45, CD80, CD85, HLA-DR expression.

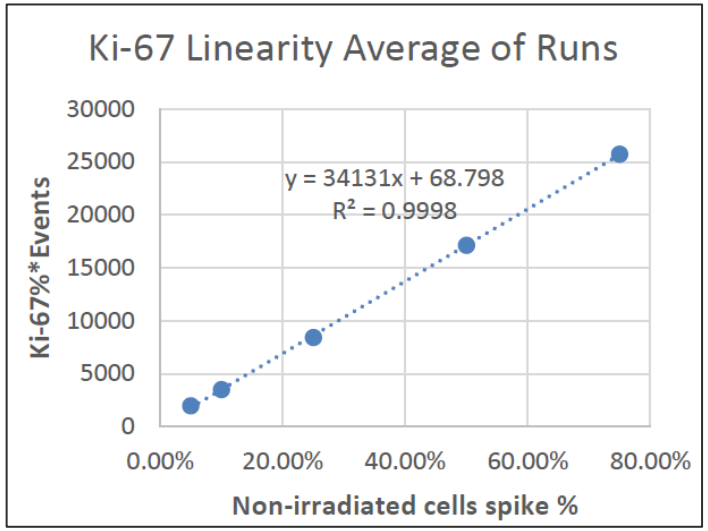
Description of Assay:
This procedure demonstrates the high expression of positive markers STRO4 and CD146 on MPC cells while control Jurkat cells display high expression of CD31 and CD45 and control Raji cells express CD45, CD80, CD86, and HLA-DR, which are all negative markers for MPC cells.



Representative data from assay

GMP Phase I Qualification:

Assay Performance Characteristics:

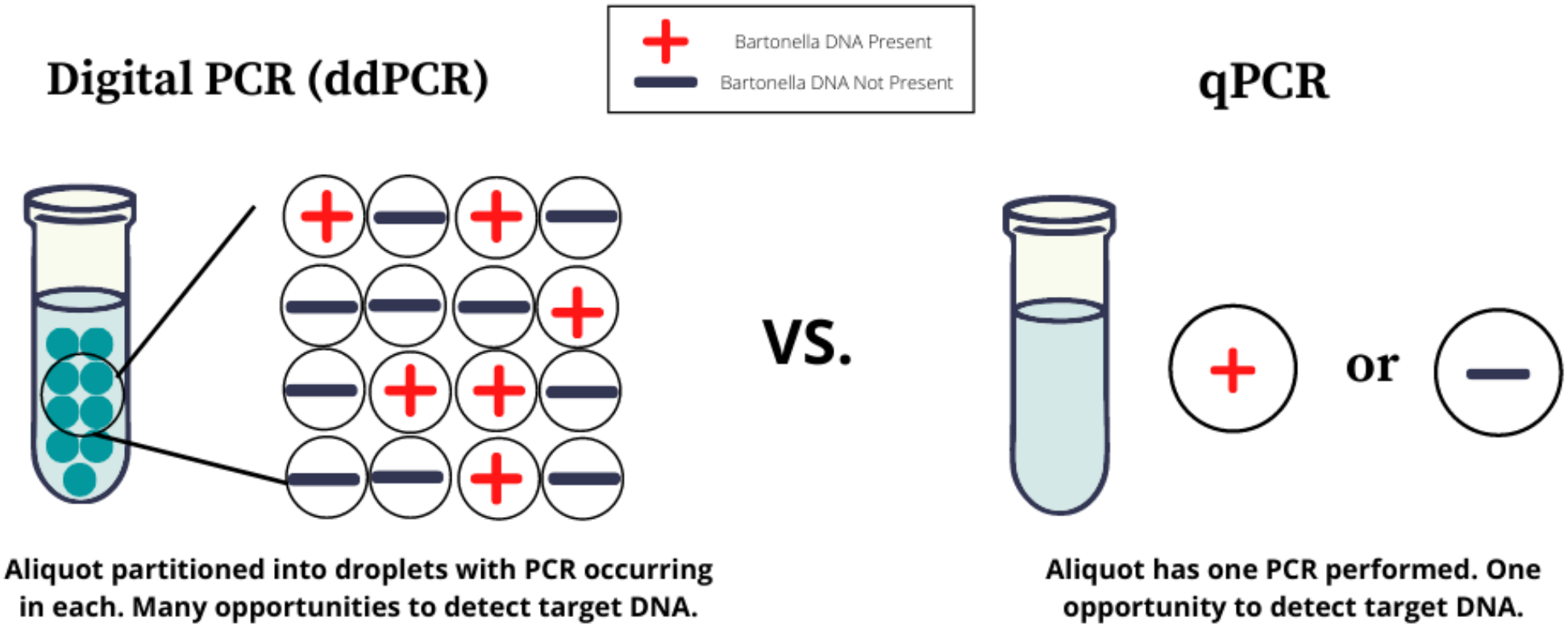


ddPCR and qPCR

The Technology Platforms

	dPCR	qPCR
Quantitative (with standard curve)	+	+
Absolute quantitation	+	-
Susceptible to interferents/inhibitors	low	variable/high
Multiplex capable	+/++	++
Sensitivity	++	++
Precision for rare events	++	+
Reverse transcriptase-incorporated workflow	+	+
Cost of instrumentation	$$$	$/$$
Cost of reagents/consumables	$$$	$$
Average run throughput	5h	1.5h
Reactions per plate	96	96/384

ddPCR vs qPCR



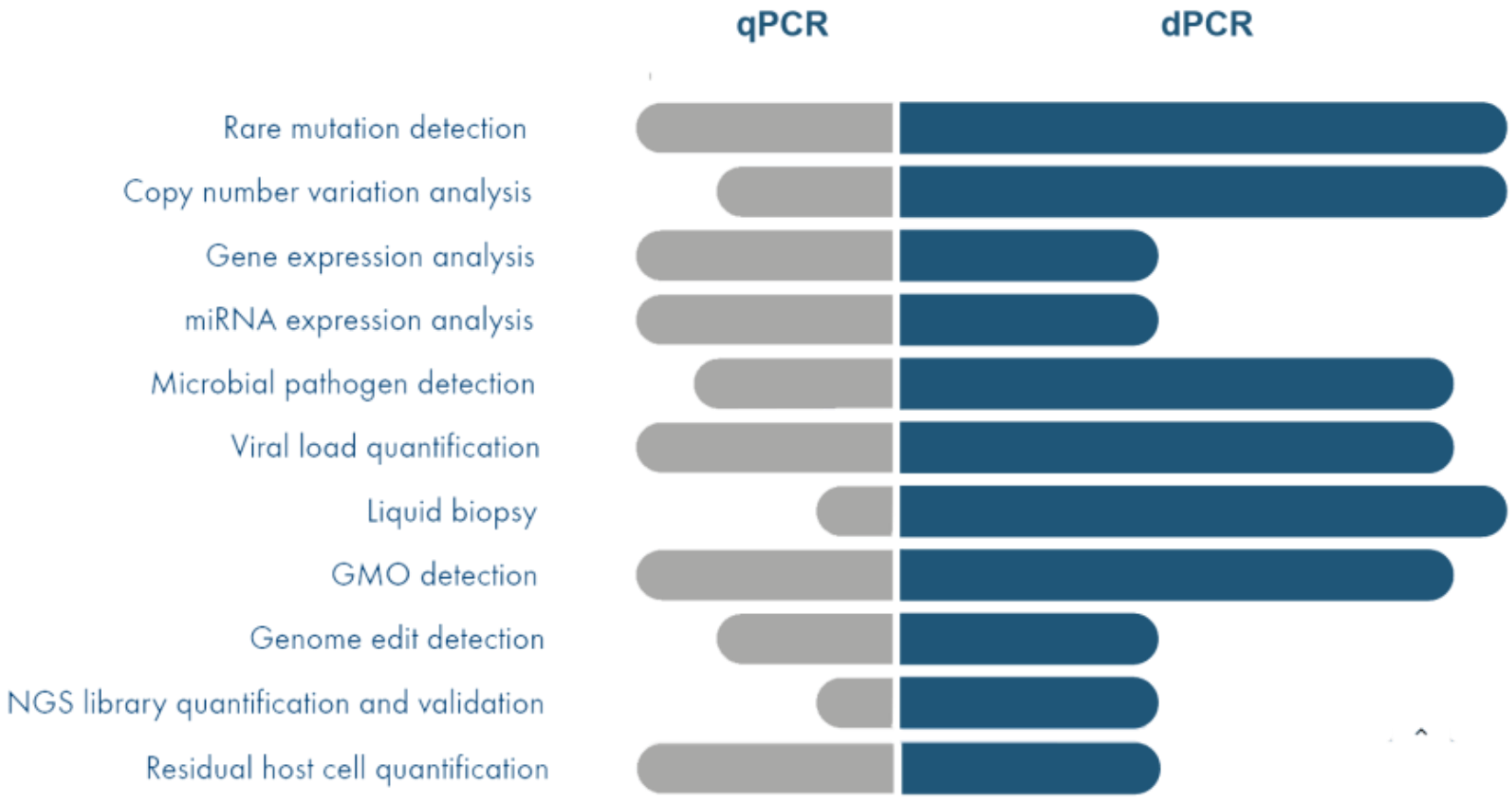
Molecular Applications in Cell Therapy Drug Development

Chemistry, Manufacturing, and Controls (CMC)

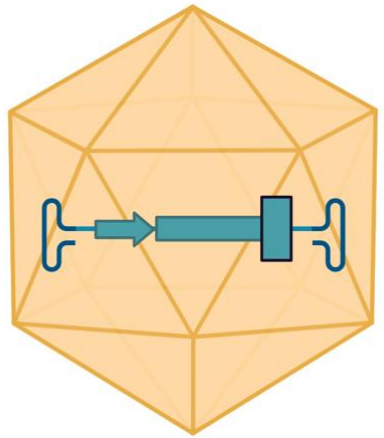
- Vector Copy Number (VCN)
- Replication Competent Lenti/Retrovirus (RCL/RCR)
- Adventitious agents
- Residual plasmid
- Host cell DNA
- Potency

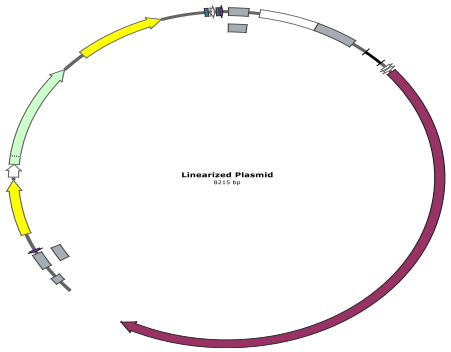
Currently >30 potency or other assays at various stages of development

Applications of ddPCR vs qPCR



Vector Copy Number Determination



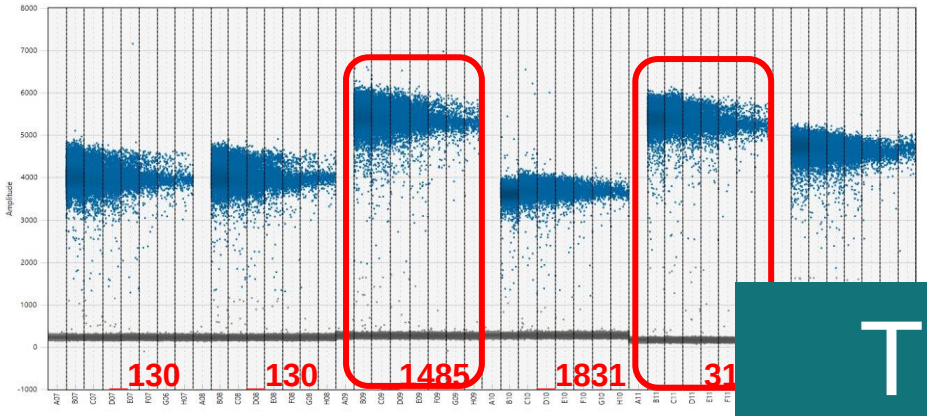


Linearized Plasmid xxx

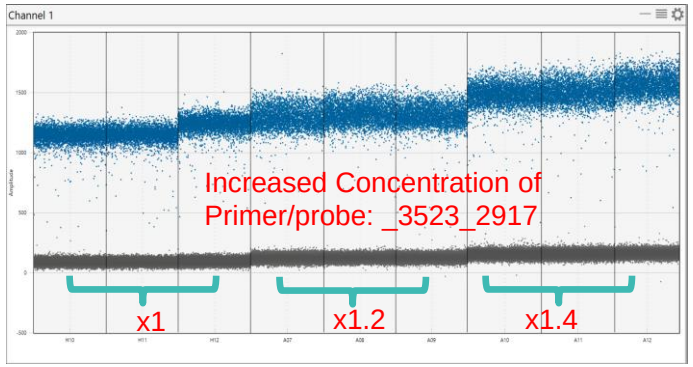
- How many copies of plasmid in the viral vector?
- Are all active genes balanced?
- How is this determined?
- Design and optimize primer/probe sets
- Test with naked plasmid and then AAV viral vector

Vector Copy Number Determination

Create primer/probe sets and screen

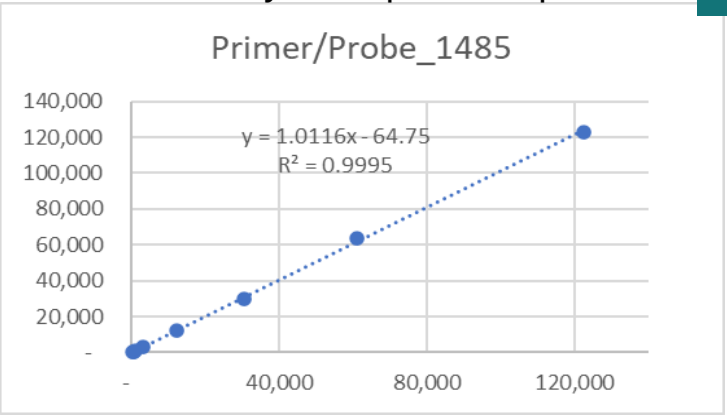


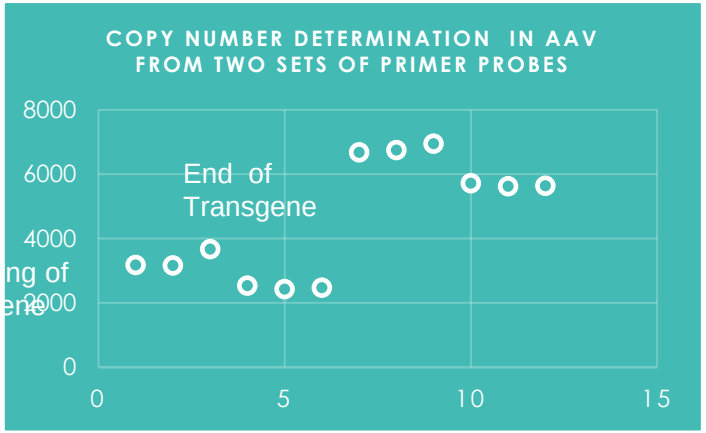
Optimize the concentration of primer/probe



The Story Continues

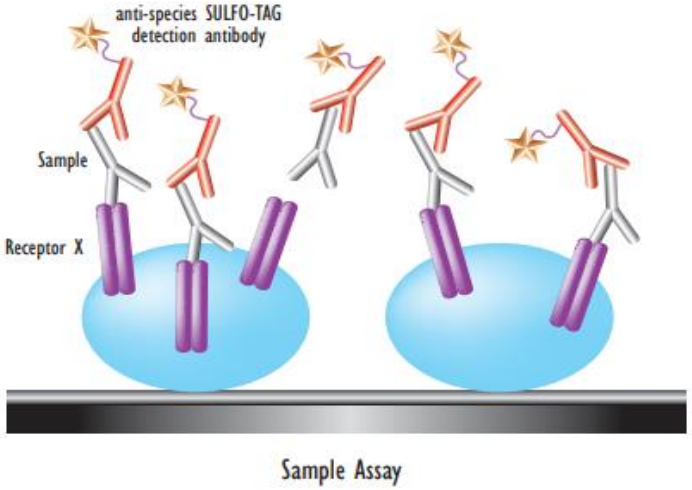
Evaluate linearity of response to plasmid





Trace Analysis and Immunoassays

MSD (Meso Scale Discovery)



MSD-ECL Imager 600

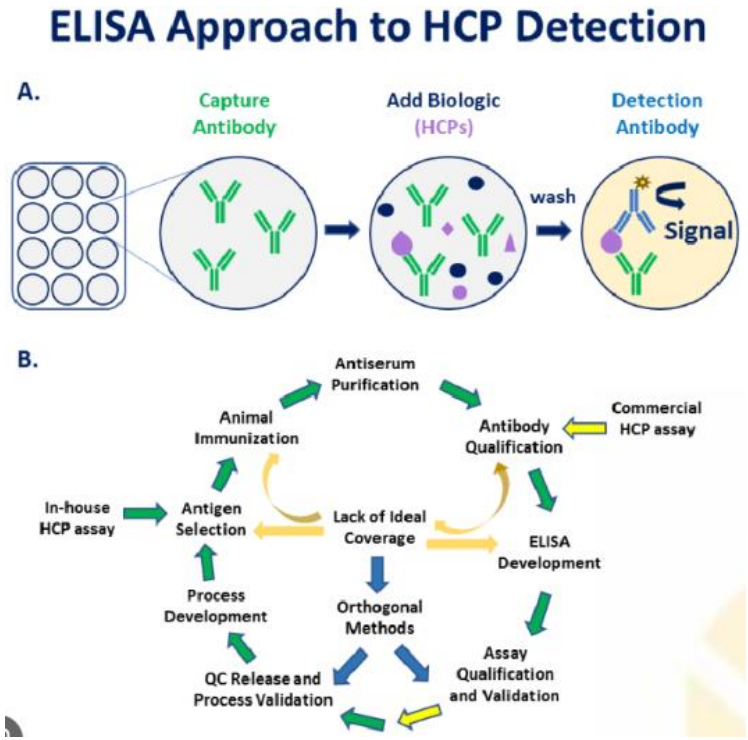
MSD SQ120 Quickplex

How do we leverage MSD capabilities?

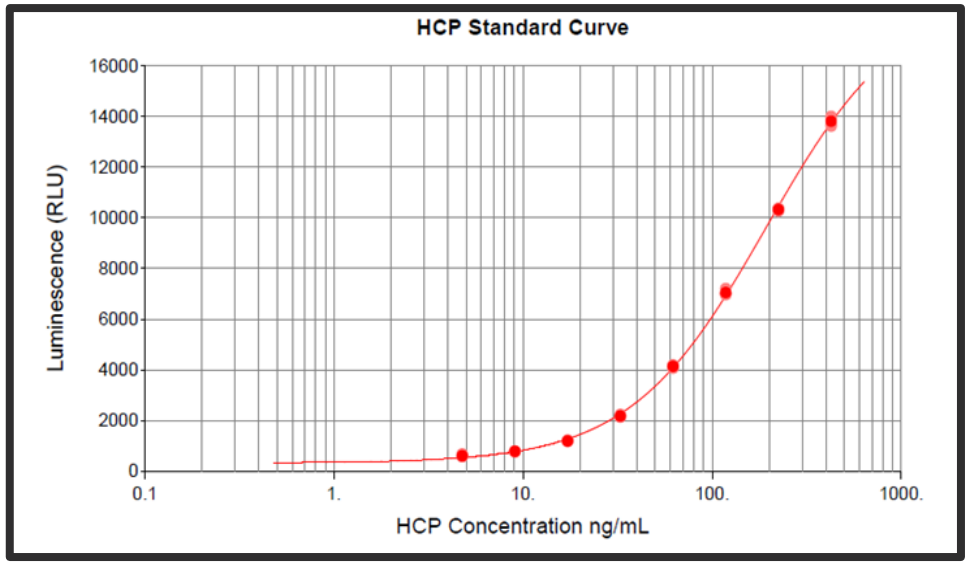
- Sensitivity
- Dynamic range
- Custom compatibilities
- Multiplex capabilities
- Off-the-shelf “Plex” panels (developed for biomarkers but useful to CMC applications)

MSD Utilization in HCP Analysis

Modality – fusion protein
MOA (drug) – toxin fragments are delivered to cells to inhibit protein synthesis
Readout – MSD ELISA measuring the amount of process related impurities (Host Cell Proteins, HCP) present in samples, measured by luminescence



Representative Data



Advantages of MSD approach:
- Broad dynamic range (perform fewer dilutions)
- High sensitivity to preserve material (low volumes available here)
- Robust method (Phase III validated)

Limitations:
- Uniquely MSD

Summary

Reviewed tools used in the control of ATMPs:

- Flow Cytometry
- PCR – ddPCR and qPCR
- Immunoassays by MSD

Some are familiar tools from protein biopharmaceuticals applied to the control of ATMPs

Tools from other analytical disciplines (molecular) are becoming common and powerful in CMC control strategies

They have their strengths and opportunities

Part 2:

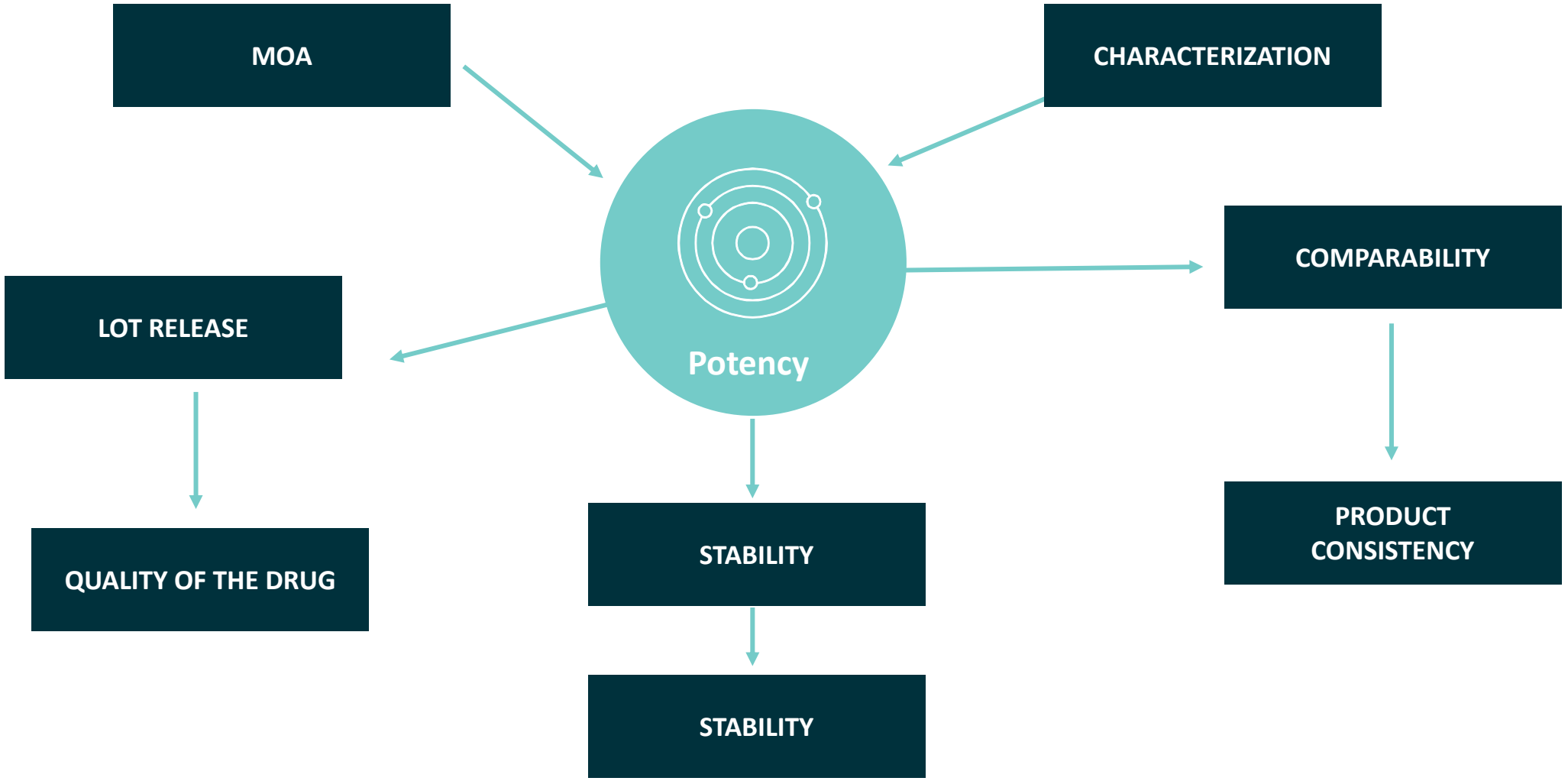
Potency assays for ATMPs – effectiveness as part of your control strategy

Learning Objectives

Summary

- Understand how potency assays fit in the control strategy and regulatory expectations for gene therapies
- Review the different analytical technologies needed to assess the potency of gene therapies
- Discuss example case studies of potency assay development for gene therapies

Potency Assays for Gene Therapy



Potency Assay Expectations

Key takeaways from regulatory guidance

- Reflective of MOAs, activity, and/or intended biological effect
- Quantitative, and feasible for a QC laboratory
- Meet pre-defined acceptance and/or rejection criteria
- Demonstrate lot to lot consistency and stability
- Include appropriate reference material, standards/controls
- Validated for linearity/range, accuracy, precision, specificity, sensitivity, and robustness

ATMP “Potency” Assays: Matrix Approach

A single assay is not achievable:

- There are multiple therapeutic genes involved (delivered in one or more vectors).
- There are multiple pathways affected by a therapeutic gene and each pathway is UNIQUELY required by the MOA.

Monitoring multiple attributes is needed:

- For a single therapeutic gene, if a single assay could not meet all expectations for potency test, multiple attributes of this single therapeutic gene need to be monitored (esp in early phases).

Often phase-selective

Often directed by discussion with the Agency

Always dictated by Safety and Science

Potency – Matrix Approach

ASSAY	DEMONSTRATES	EXAMPLE ASSAY FORMATS
Infectivity	Demonstrate that there is successful delivery of the transgene to the target cells	Viral Titer, Infectious Titer, qPCR/ddPCR, Flow Cytometry
Expression	Demonstrate the presence of expressed genes and proteins	qPCR, ddPCR, Western Blot, ELISA, MSD, Flow Cytometry, LCMS
Activity/Functional	Demonstrate that the genes and/or proteins expression are functional and generate the intended response	Highly dependent on the functionality of transgene product and final readout. Enzymatic, Cytotoxicity Assay, Proliferation Assay, ELISA, Flow Cytometry

Case Study #1
Oncolytic Virus - Early Phase

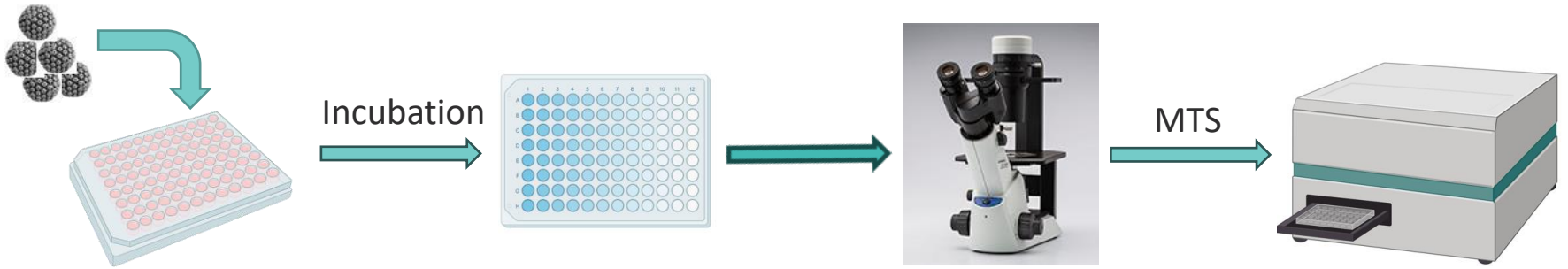
CASE STUDY 1

Background Information

MOA: Oncolytic Virus, lysis of cancer cells and upregulation of immune response

Infectivity and Potency (EC50) Assay:

- Infectious Titer, TCID50 – cytopathic effects
- EC50 by cell viability - absorbance readout
- Assay development/optimization challenges:
  - Subjective
  - No quantitative control
  - High variability



CASE STUDY 1

Product Change

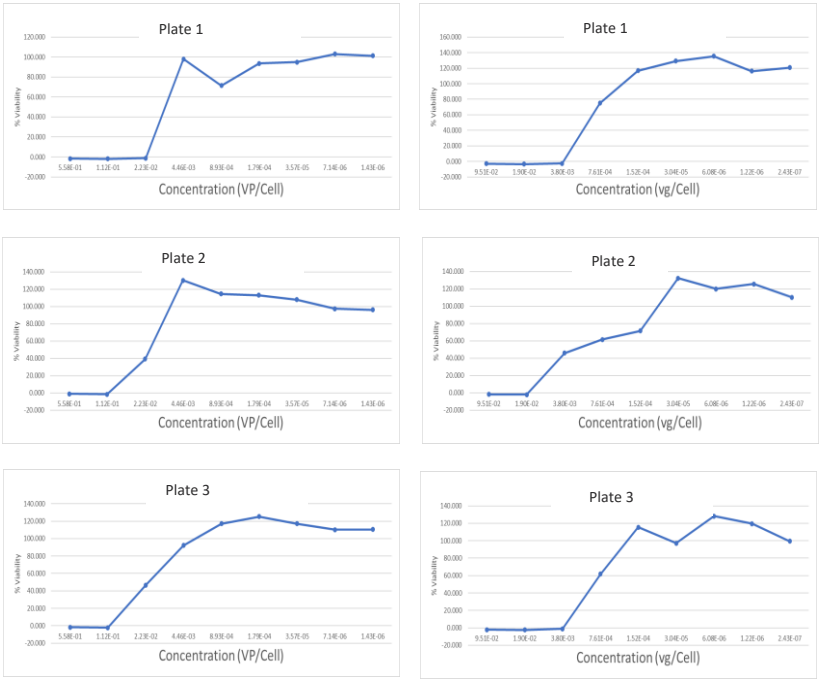
The drug product (still in preclinical/Phase I) was modified after initial method development to be at 1/30 of the effective concentration.

Resulted in:

- Minor changes in TCID50/EC50 assay (sample prep; see results right)
- Changes to sample prep for identity/strength assay
- In addition to changes to assays there were also adjustments to acceptance criteria and preliminary specifications to be considered.

Summary:

- Simple assays for very early (IND) phase
- High variability for most
- Adapted for formulation/product change



Before change

After change

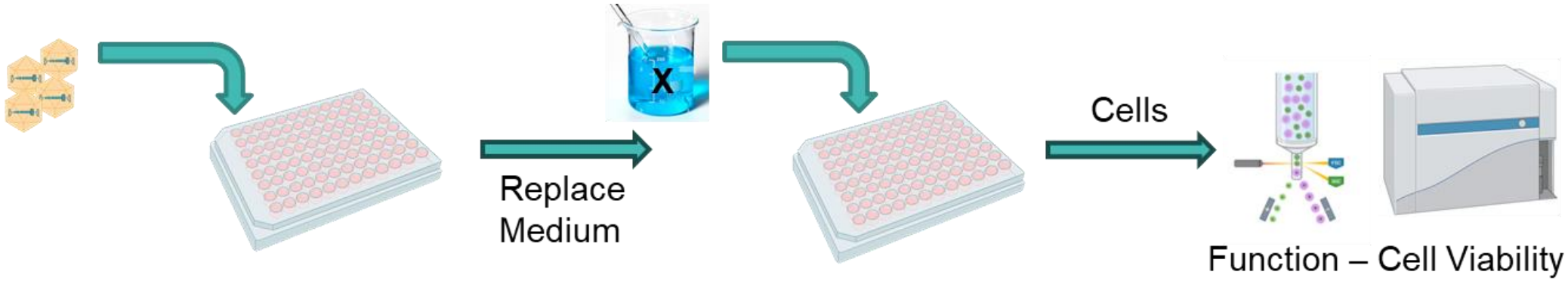
39

Case Study #2
AAV - Progression into Phase II

CASE STUDY 2

Functional Assay Using Flow Cytometry

MOA: AAV carrying one transgene. Protein Replacement Therapy.

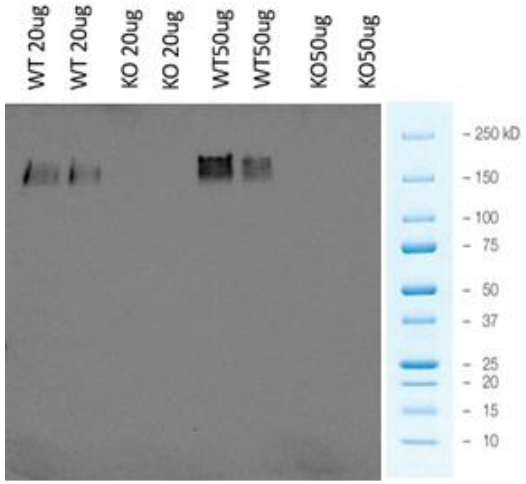


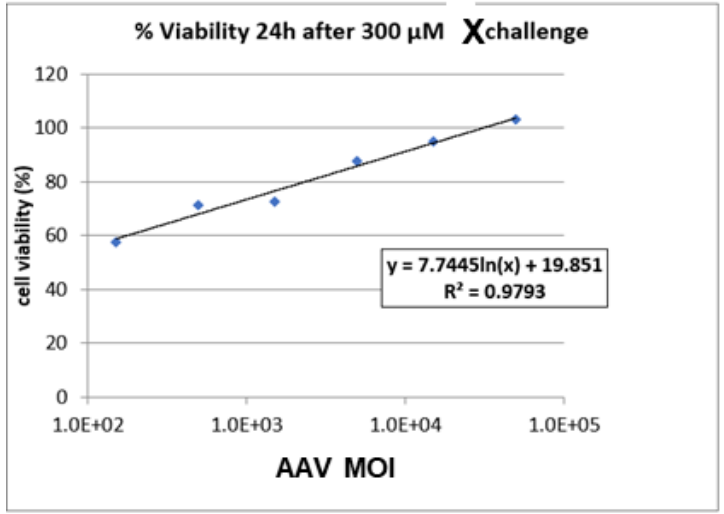
Potency Assay Matrix:

- Expression at mRNA level (qPCR)
- Expression at protein Level (Western Blot)
- Functional Assay (Flow Cytometry)

Assay Challenges:

- KO vs WT cells
- Critical reagents

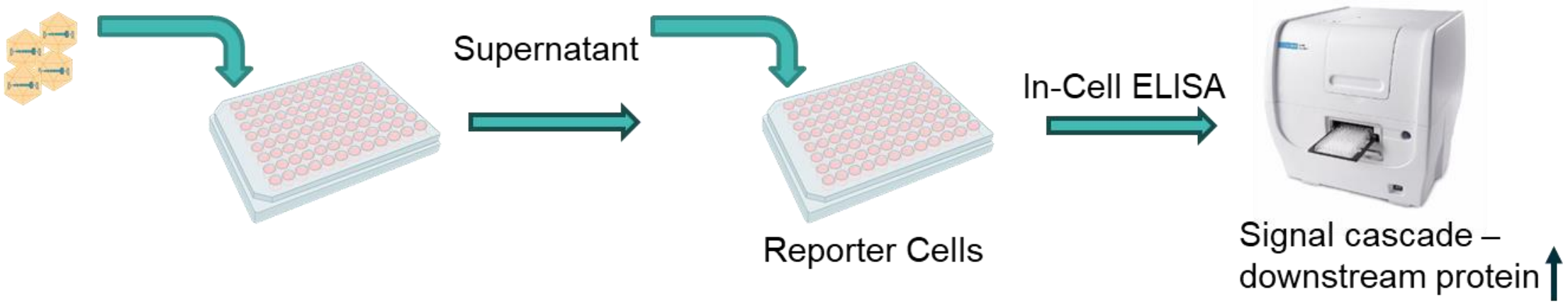


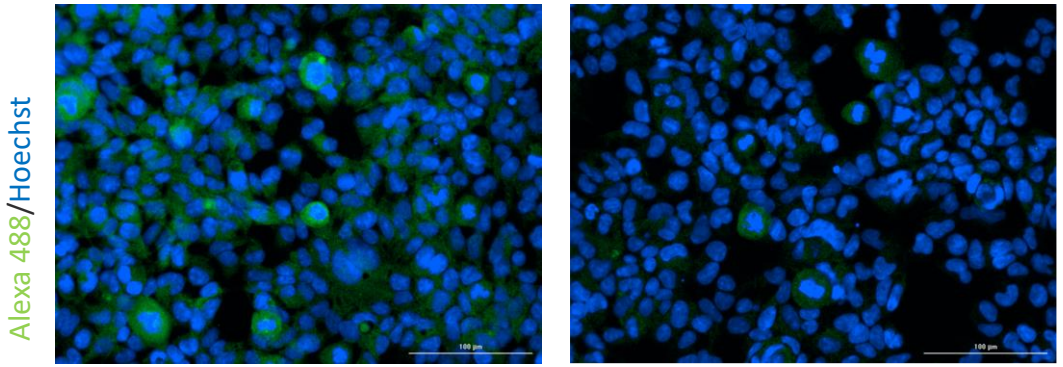


CASE STUDY 2

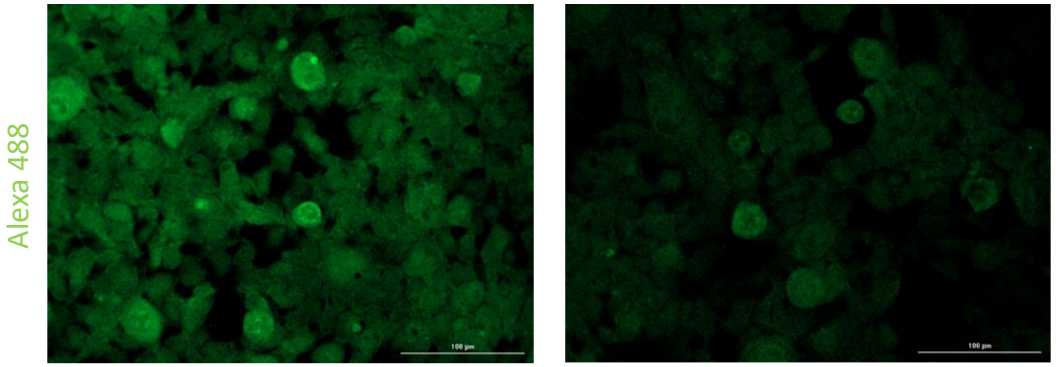
Functional Assay Using In-Cell ELISA

MOA: AAV carrying one transgene. Protein Replacement Therapy.





Target Protein and Cells



Target Protein

Case Study #3
Nanoparticle Containing Plasmid DNA - Moving to Phase III

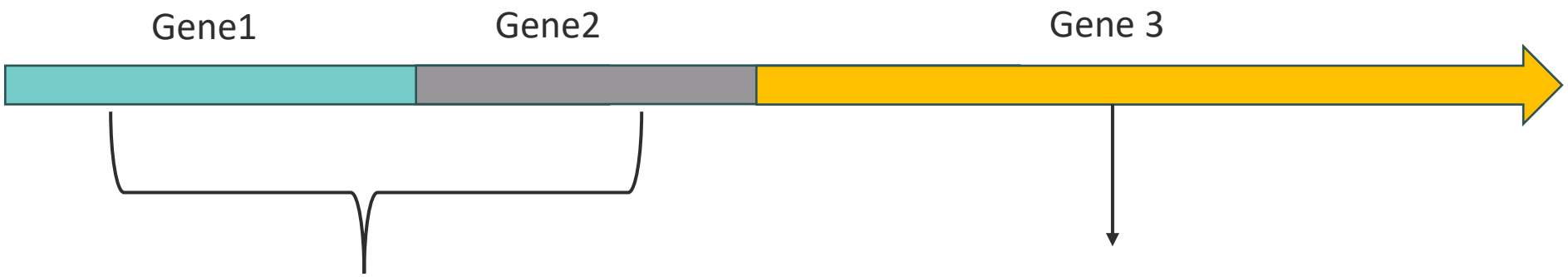
CASE STUDY 3

Background Information

MOA:

DS: plasmid DNA carrying three genes

DP: nanoparticles of a polymer excipient and DNA plasmid API, and further coated with a copolymer

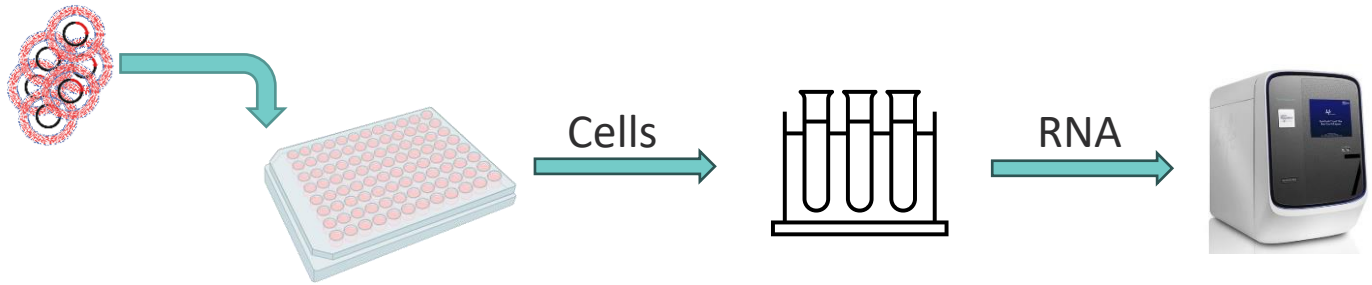


RNA products of these two genes stimulate a pathway, resulting protein X expression.

This gene encode protein Y.

CASE STUDY 3

Potency Matrix_Phase 1

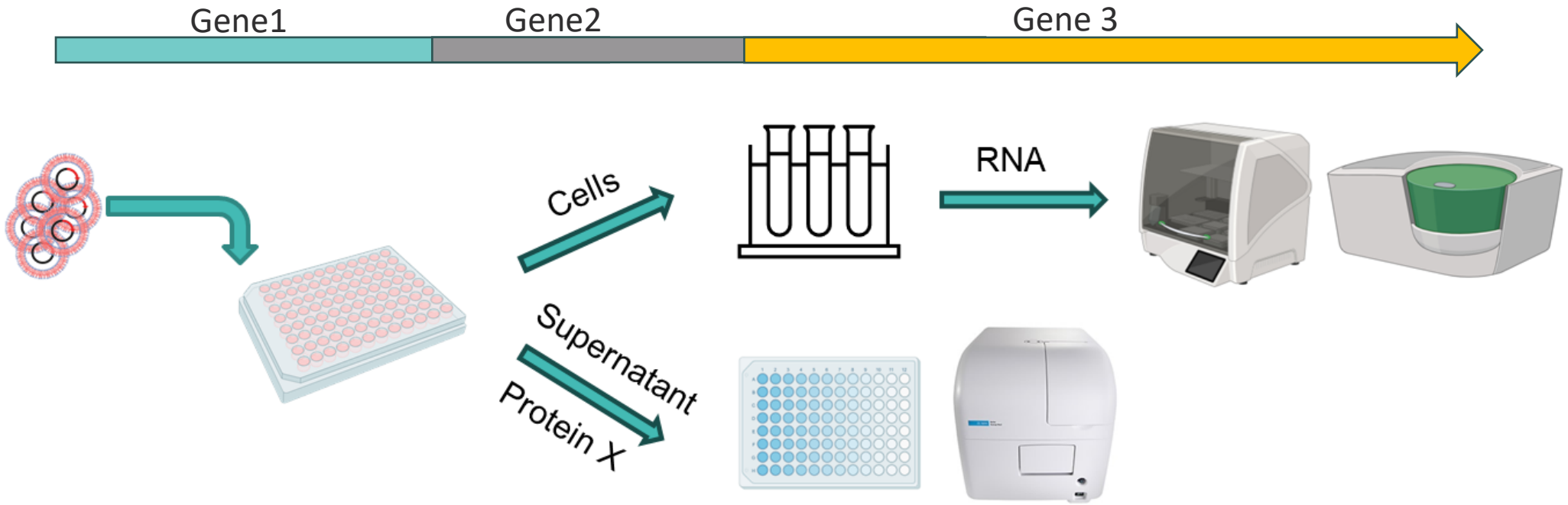


Gene 1 and 2: Expression (RT-qPCR, 2 methods)

Gene 3: Expression (MSD)

CASE STUDY 3

Potency Matrix_Phase 2

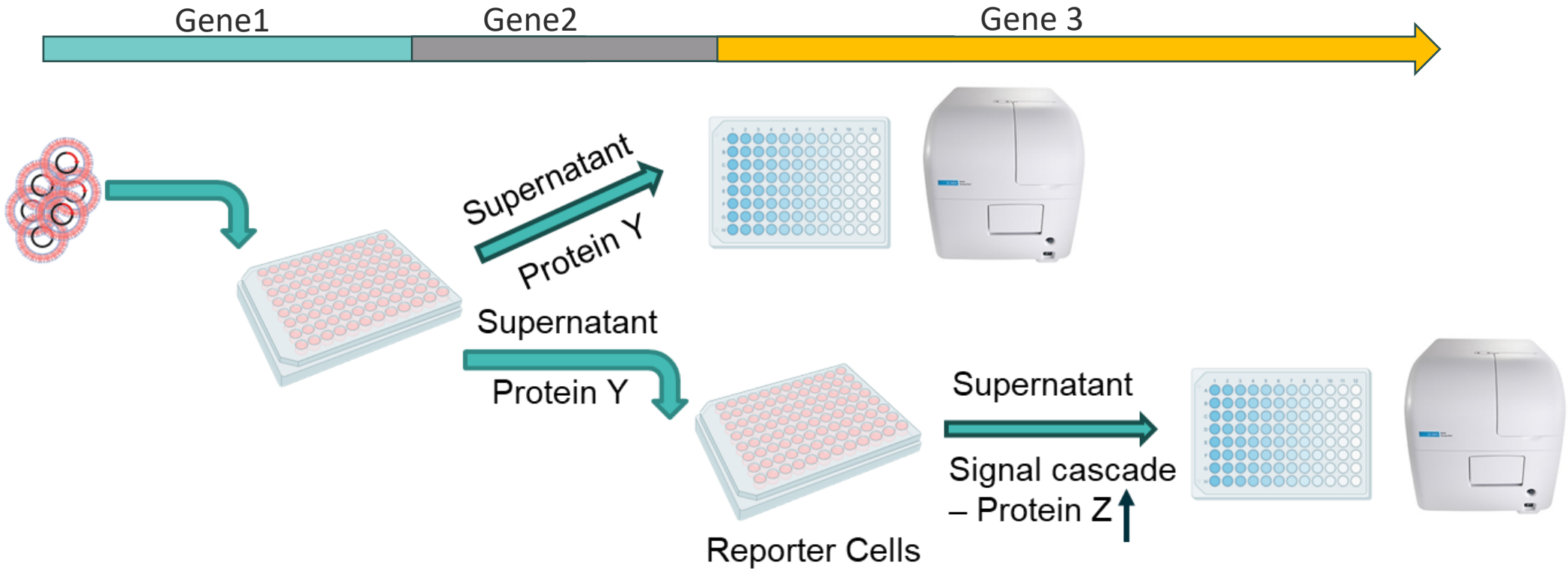


For Gene 1 and 2:

- Expression (RT-ddPCR, duplex method, 2 reportable results)
- Functional (ELISA, for Protein X)

CASE STUDY 3

Potency Matrix_Phase 2



For Gene 3:

- Expression (ELISA, for Protein Y)
- Functional (Colorimetric enzyme assay, for protein Z)

CASE STUDY 3

Potency Matrix_Phase 2

SUMMARY:

- 3 Transgenes
- 2 Expression Assays (3 results)
- 2 Functional Assays
- 3 Cell Lines
- DS transductions are different procedurally than DP transduction.

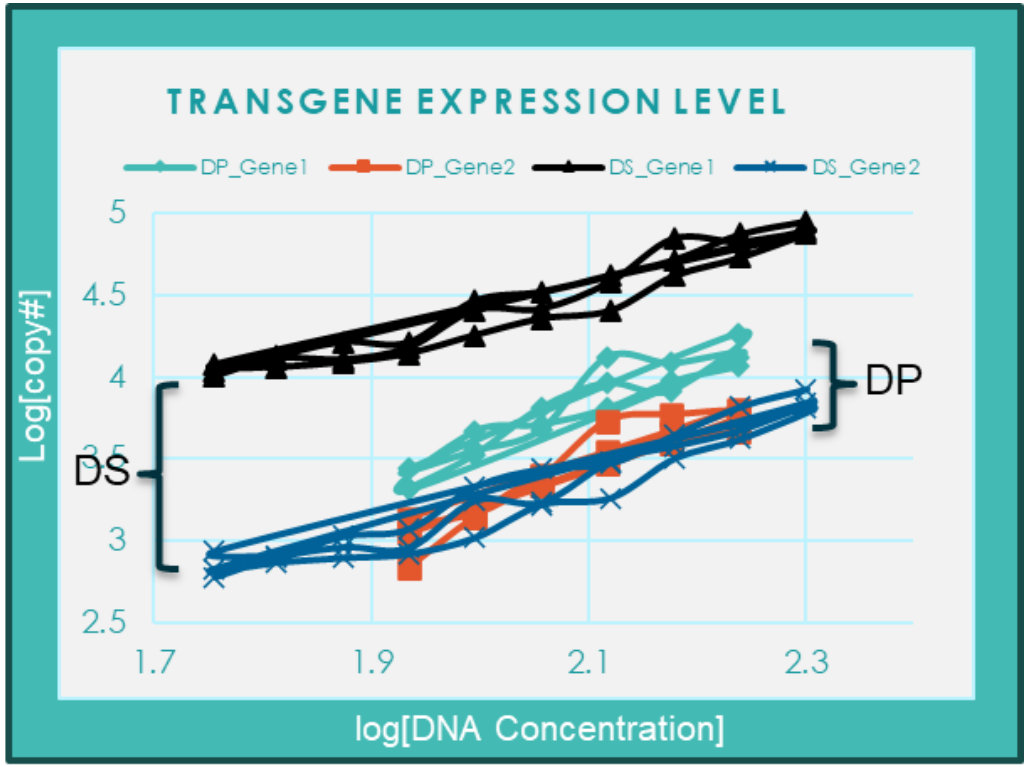
8 Assays, 10 reportable results

Assay Challenges:

- Expression level of Gene 1 and 2 are different
- DS and DP have different behaviors (transduction)
- Variance is HIGHER with DP than DS in some assays
- Assays still require evolution including adaptation to final product and to reduce variance

CASE STUDY 3

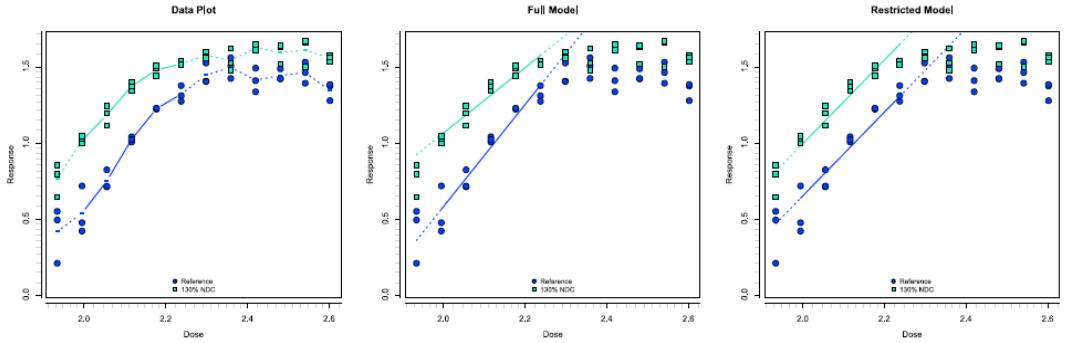
Potency Matrix_Phase 2



Potency estimation

Relative potency	130% NDC	Reference
Potency ratio	1.33220	
95% Confidence interval	1.27144 - 1.40414	
Relative confidence interval	95.44% - 105.40% (9.96%)	
EC 50		
Estimated EC50 value	115.03345	129.51310

Graphics

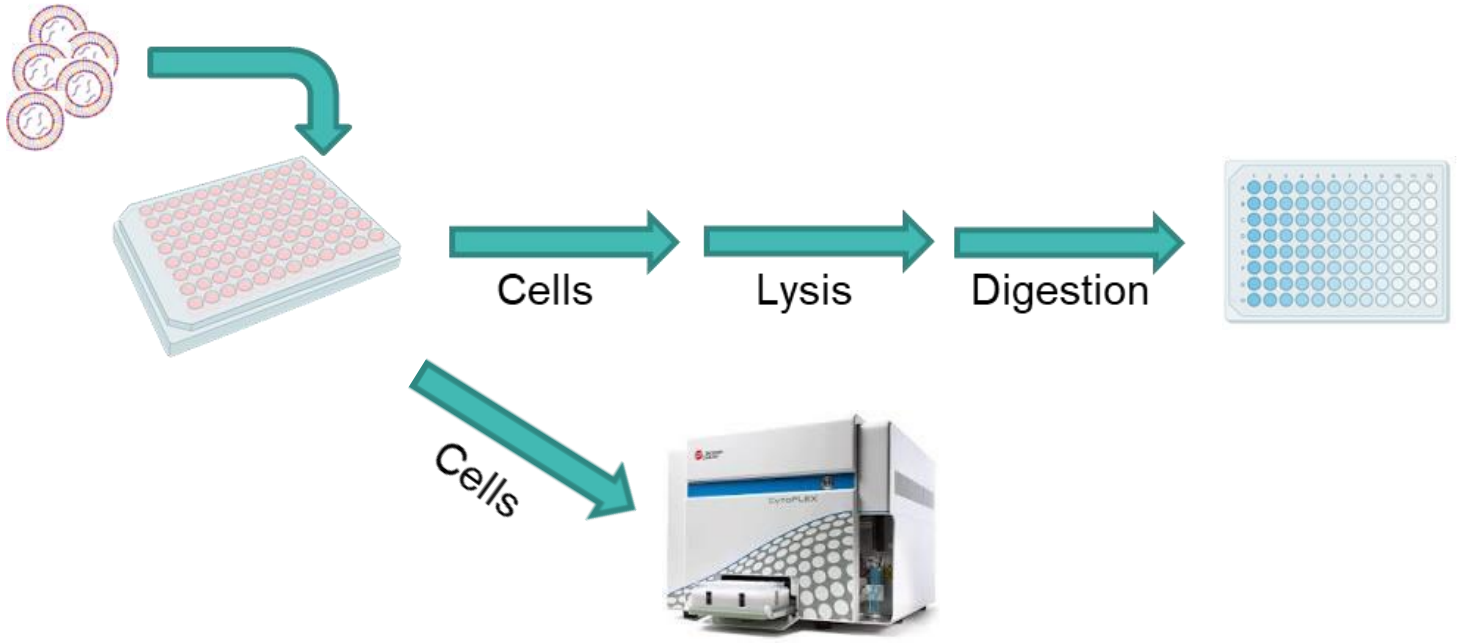


Case Study #4
mRNA in LNP_Early Phase Screening

CASE STUDY 4

Vaccine Development

MOA: mRNA in Lipid Nanoparticles



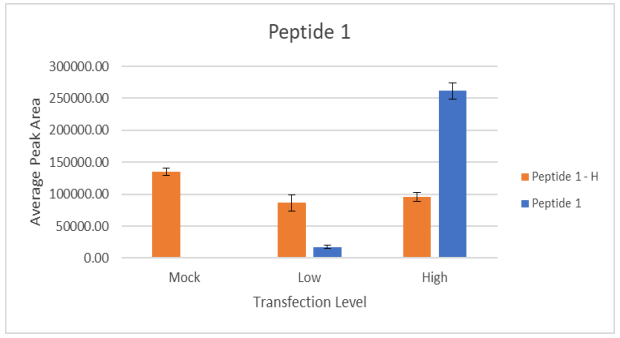
Flow Cytometry

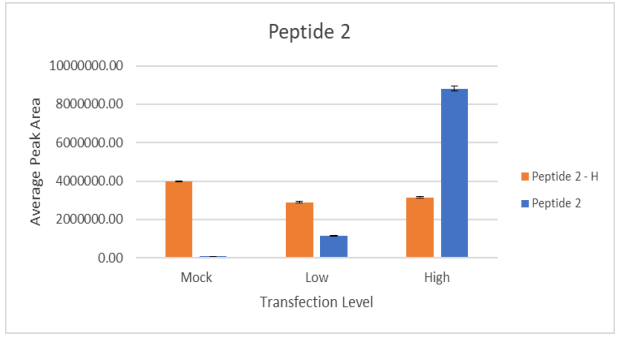
Detect expressed protein(s) by targeted LC-HRMS as their peptide surrogates

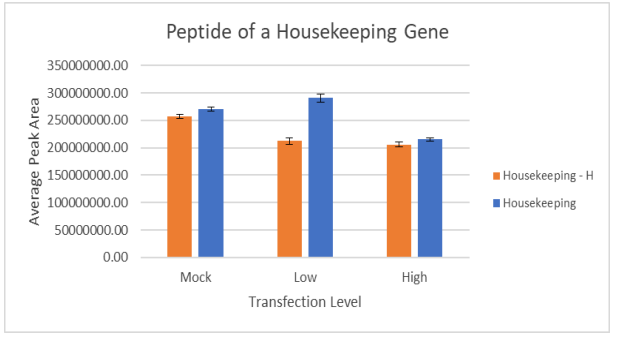
CASE STUDY 4

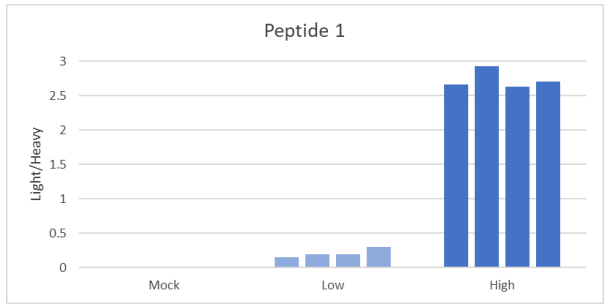
Pilot Study Results

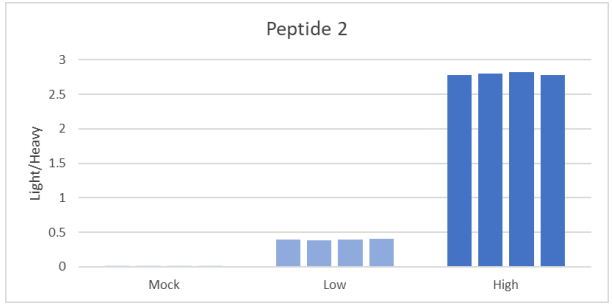
MOA: mRNA in Lipid Nanoparticles

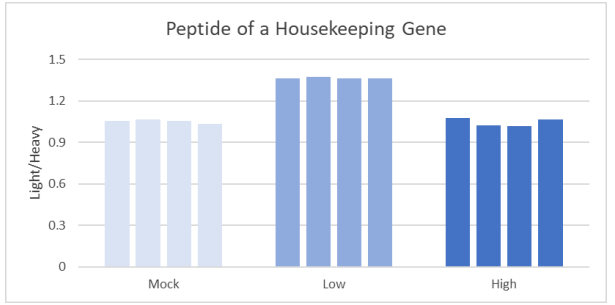












Conclusions

Highlights and recommendations for gene therapy potency assay

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Questions?

Thank You!

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