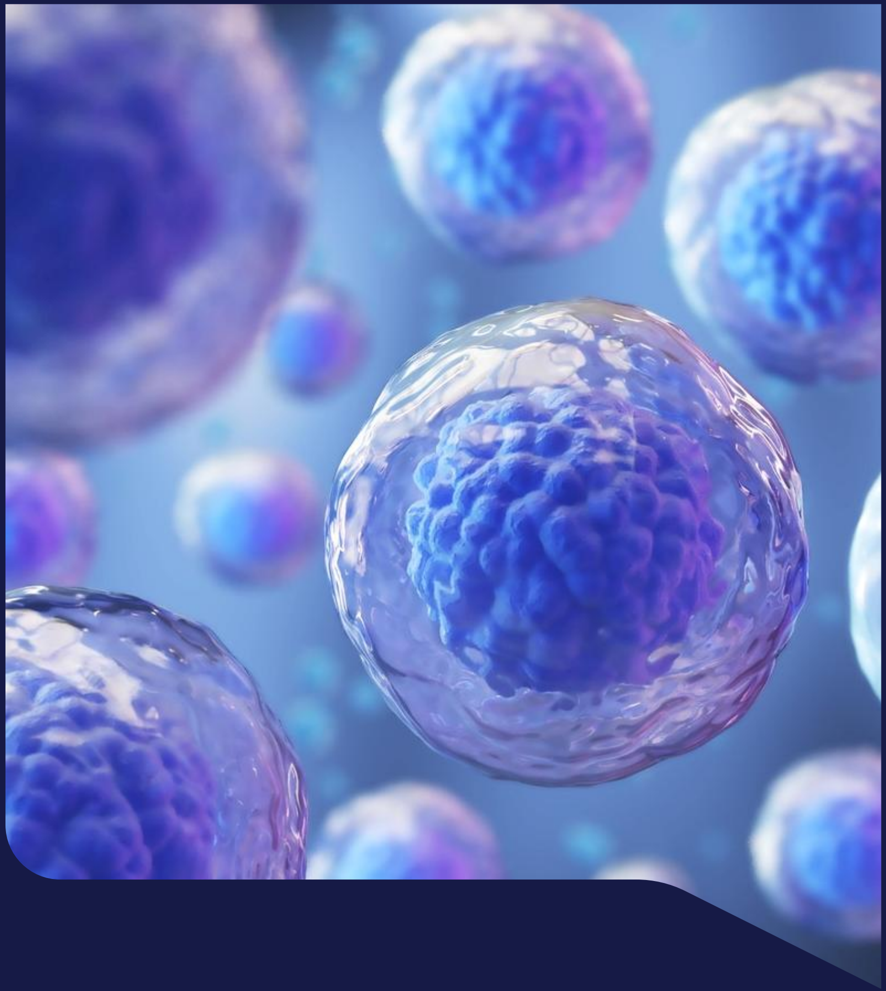
Designing Robust Cell-Based Potency Assays:

Strategic Frameworks and Emerging Biological Readouts



Pedro Morales

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Catalent™ Pharma Services

Agenda

01 Introduction & Considerations

02 Readouts

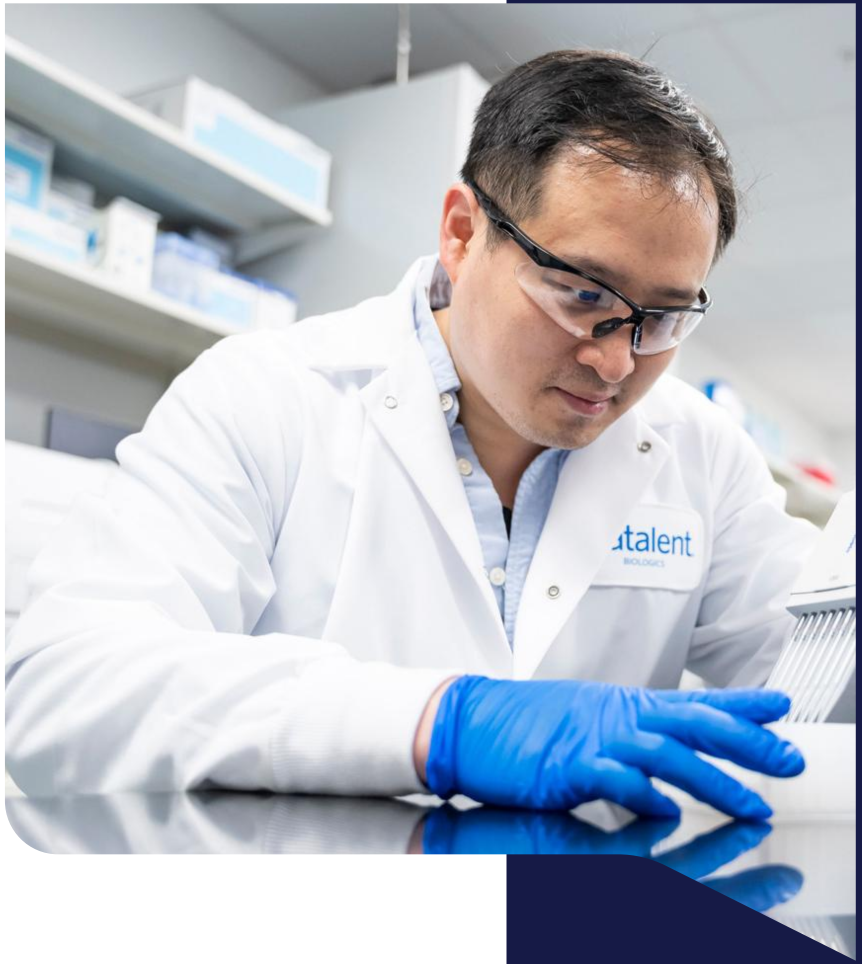
03 Strategy

04 Roadmap Visualization

05 Examples: Dose Response Curves

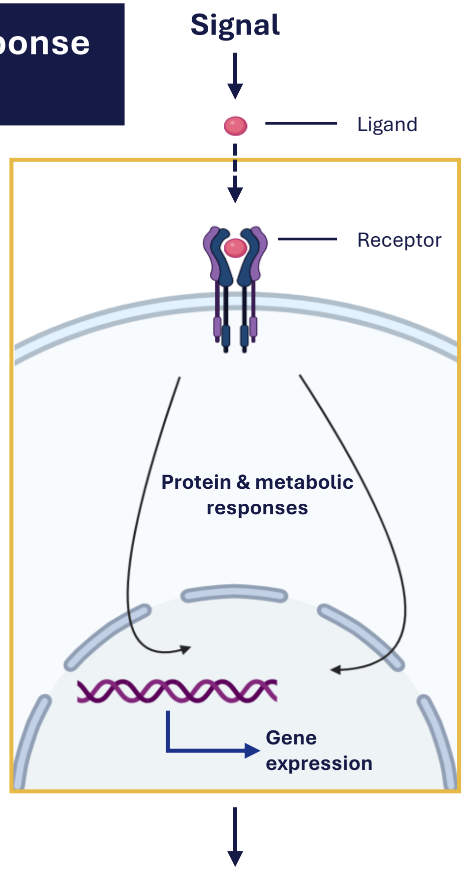
06 mRNA Transcription Assay

07 Q&A



Increased Regulatory Expectation for Robust Cell-Based Potency Assays in Biologics & CGT

Harnessing Cell Response for Use in Bioassay



Cellular responses
- Morphological Change
- Metabolic Change
- Proliferation, Survival

ICH Q6B -> validated cell-based potency assays

For commercial release & stability of large molecules for licensing in multiple countries

Must reflect the **MoA** of large molecules

Be **stability indicating** -> key for ensuring **Safety, Efficacy, Purity, Identity** and **Potency** of medicinal products

May be also utilized for **manufacture comparability assessments, extended characterization** and **formulation development**

ATMPs like CGT require a customized **"matrix"** panel of cell-based potency assays

For manufacturing, purification and release decisions

Cell-Based Potency Method Development

Aspects to Consider

Cell Stimulation

- Direct analyte interaction
- Ligand interaction
- Serum/growth factor starvation
- Co-culture required
- ECM interaction necessary
- Others, such a biomechanical, hypoxia, etc.

Biological Response

- The biological response should reflect the mechanism of action & be measurable
- Understand procedure to control for biological response drifts
- Start assay trending early to include relevant variables

Curve Shapes

- Most biological responses can be analyzed with 4-PF curves, but straight-line analysis is also an option
- Ensure curve represents optimized method before validation

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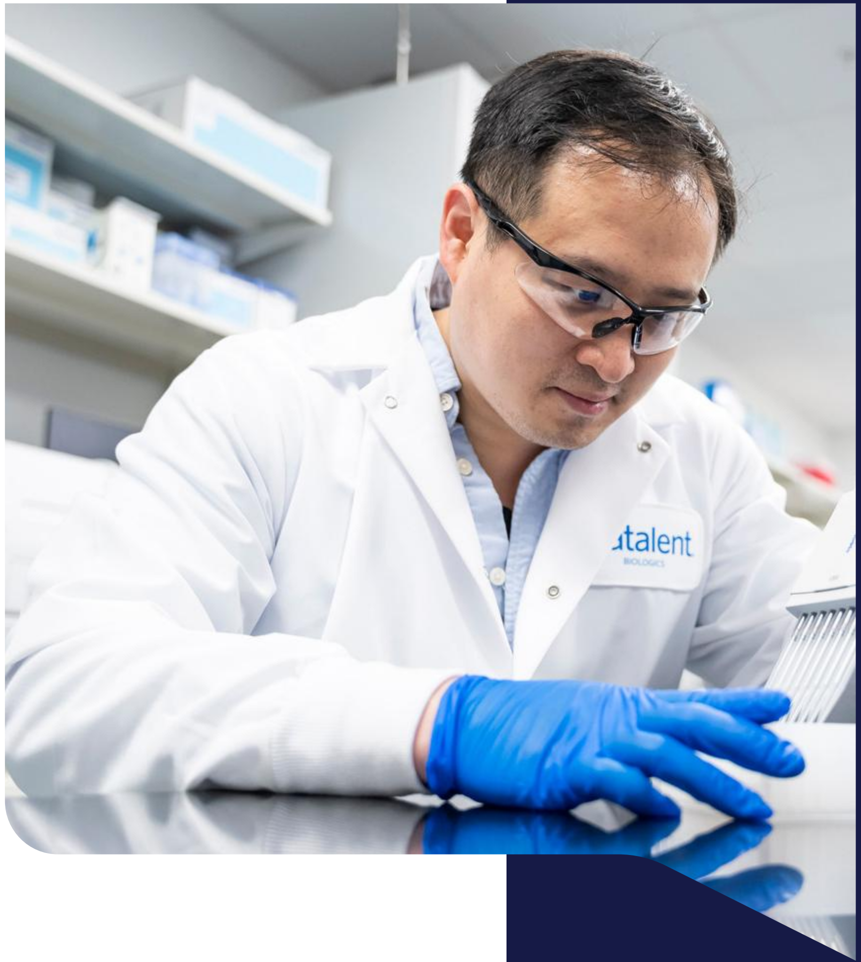

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Bioassay Readouts: Where Biology Meets Chemistry

Each of these assays use luminescent, colorimetric or fluorescent readouts to detect & quantify specific biochemical or cellular processes

Cytotoxicity 01

- CytoTox-Glo™ assay measures a distinct protease activity associated with cytotoxicity
- alamarBlue™

Enzymatic 02

- Caspase-Glo® assay measures caspase-3/7, enzymes that play a role in apoptosis

Reporter 03

- cAMP-Glo™ assay measures cAMP, a second messenger important in many biological processes, by converting it to a luminescent signal

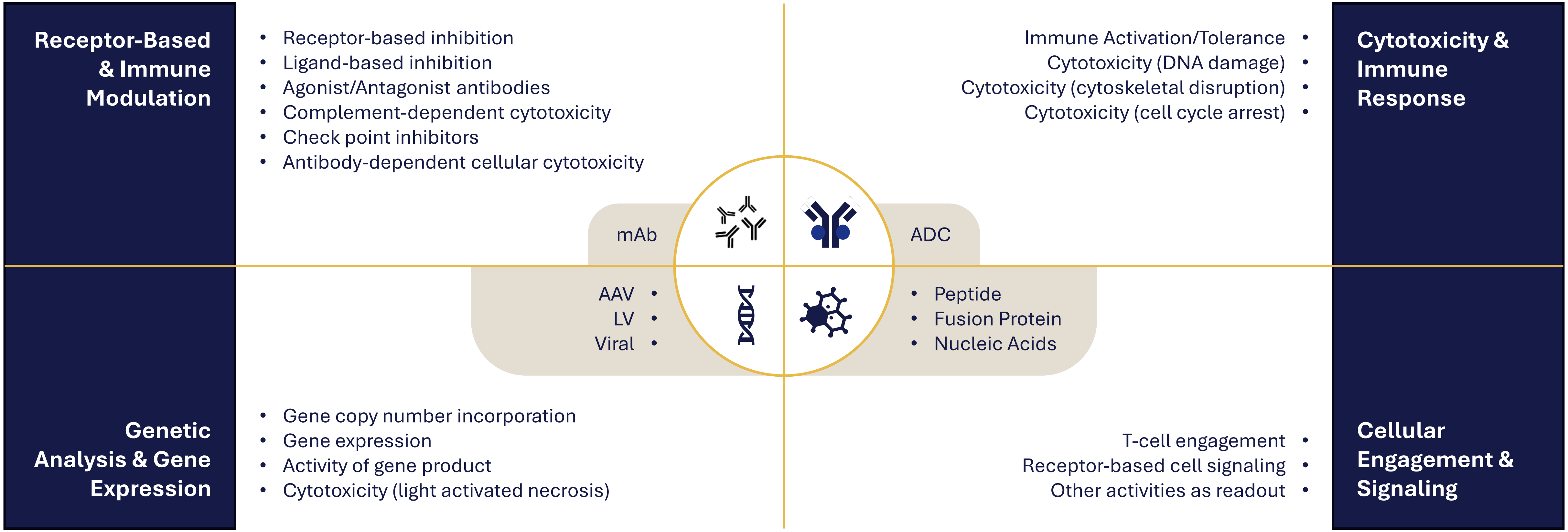
Metabolic 04

- CellTiter-Glo® assay measures ATP as an indicator of metabolically active cells
- alamarBlue™

Nucleic Acid 05

- Quantitative PCR (qPCR) and droplet digital PCR (ddPCR) which are used to quantify DNA or RNA from cells

Bioassay Selection Framework for Different Biotherapeutic Formats



Receptor-Based & Immune Modulation

- Receptor-based inhibition
- Ligand-based inhibition
- Agonist/Antagonist antibodies
- Complement-dependent cytotoxicity
- Check point inhibitors
- Antibody-dependent cellular cytotoxicity

Cytotoxicity & Immune Response

- Immune Activation/Tolerance
- Cytotoxicity (DNA damage)
- Cytotoxicity (cytoskeletal disruption)
- Cytotoxicity (cell cycle arrest)

mAb

ADC

- AAV
- LV
- Viral

- Peptide
- Fusion Protein
- Nucleic Acids

Genetic Analysis & Gene Expression

- Gene copy number incorporation
- Gene expression
- Activity of gene product
- Cytotoxicity (light activated necrosis)

Cellular Engagement & Signaling

- T-cell engagement
- Receptor-based cell signaling
- Other activities as readout

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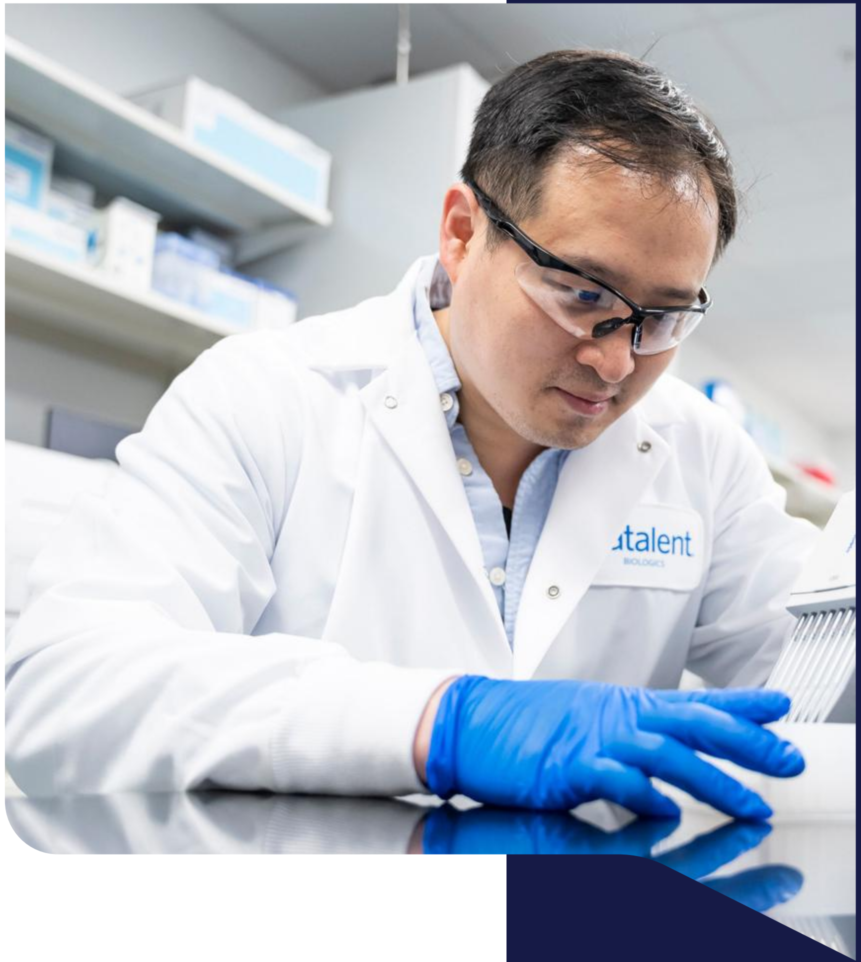

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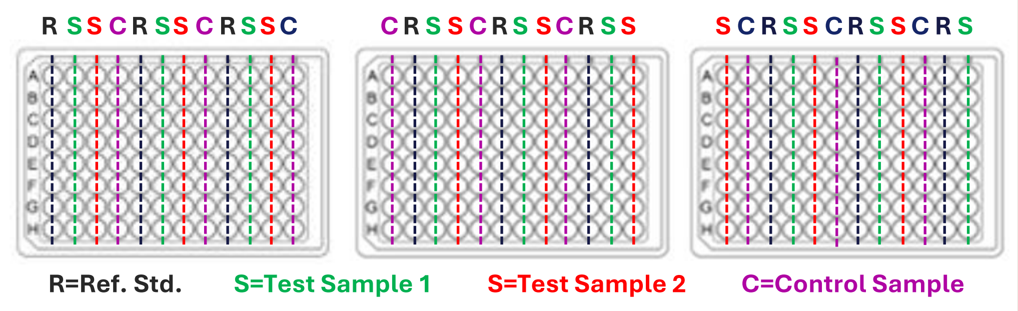
Cell-Based Assay Strategy

Part 1 Cell Culturing Optimization

- Utilization of well characterized cells
- Evaluate continuous culture conversion to “thaw & use”
- Monitor critical parameters:
  - Reagents, cell viability, passage number, culture conditions & population doubling
- GMP banks produced based on mock banks:
  - Master & working banks recommended
  - Generated & tested under protocol (QA & client approved)
  - Tested for mycoplasma, bioburden & functionality

Part 2 Assay

- Plate layout/uniformity (temperature/evaporation)
- Convert continuous culture to “thaw and use”
- Randomize distribution of replicates across plate
- Maximize throughput (horizontal vs vertical)
- Inclusion and trending assay control sample



Pre-Validation Activities

- Performed under an agreed plan
- Involve multiple analysts/plate readers

Phase Specific Method Validation

- Under protocol with criteria per pre-validation
- Minimum of two analysts
- Based on client needs and minimum requirements of Catalent SOPs

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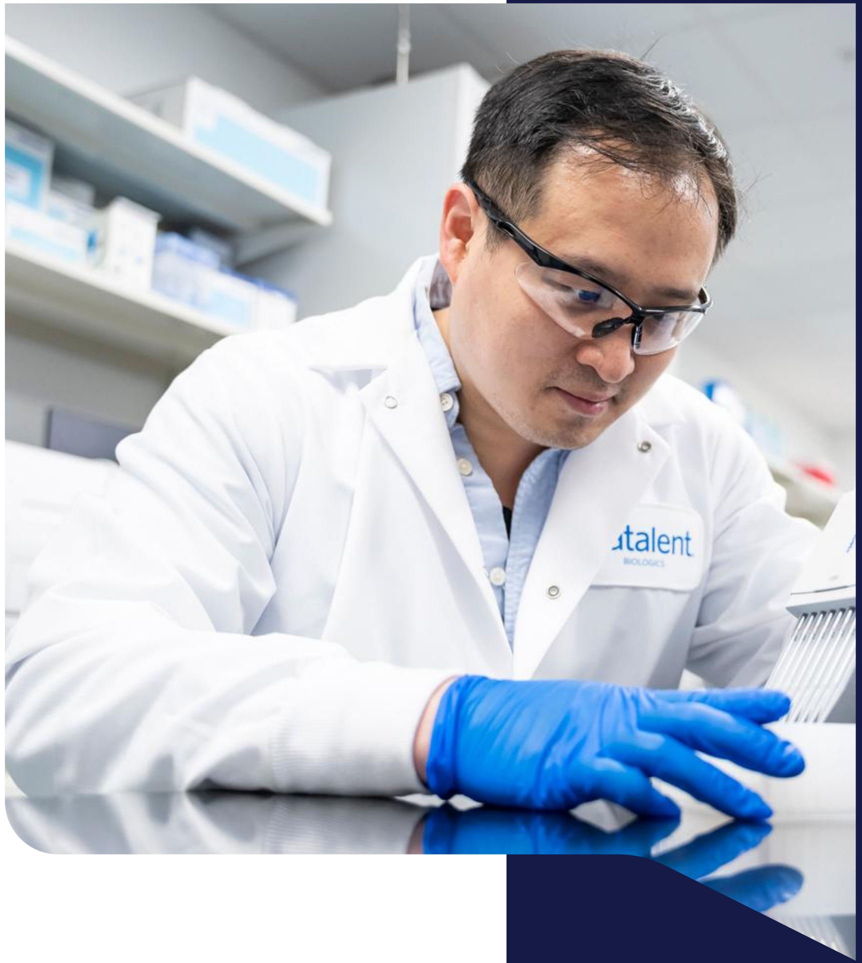
04	**Roadmap Visualization**
05	Examples: Dose Response Curves
06	mRNA Transcription Assay
07	Q&A



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Roadmap to Validated Cell-Based Assay

Aspects to Consider

Sample & Reagent Management

- Sample handling
- Critical reagents
- Drug Substance/ Drug Product scope

01

Assay Performance

- Incubation times
- Number of plates
- Assay parameters
- System suitability
- Robustness

02

Process Control & Validation

- Locking steps
- Locking parameters
- Assay acceptance criteria
- Specifications
- Critical steps

03

Data Integrity & Trending

- Data trending
- True ACS lot
- Specificity

04

Operator Considerations

- Training

05

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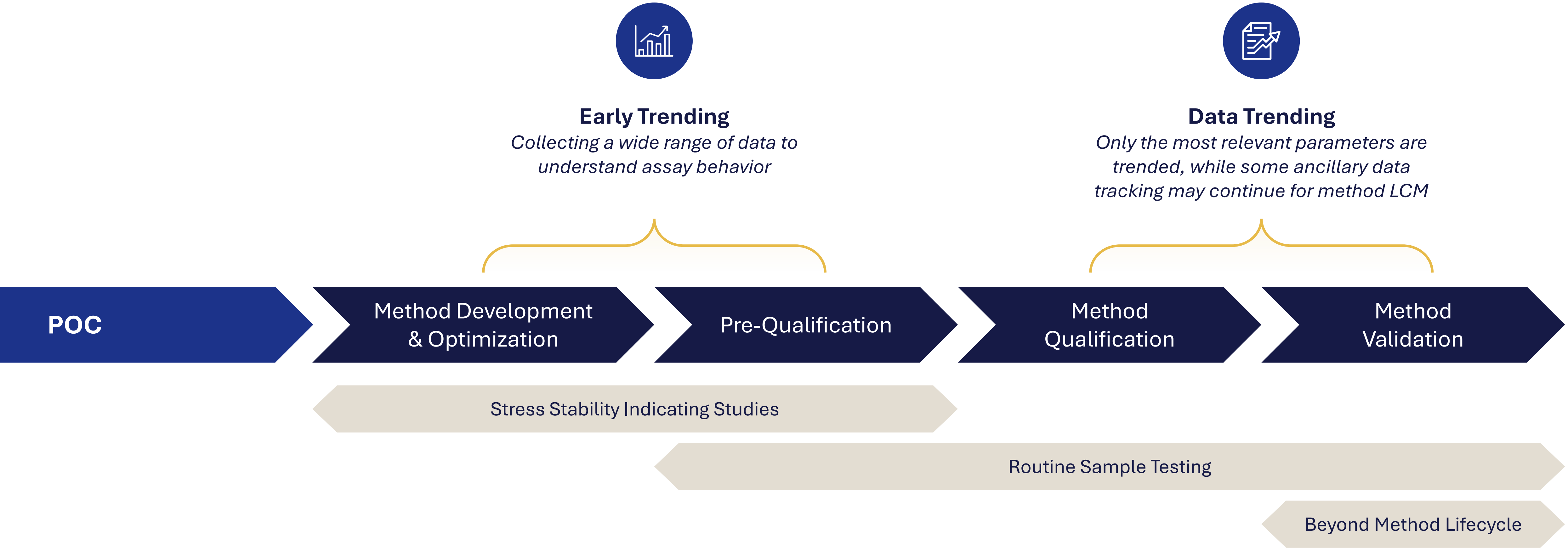
Roadmap to Validated Cell-Based Assay

From Initial POC Through Full Method Validation & Lifecycle Management (LCM)



Early Trending
Collecting a wide range of data to understand assay behavior

Data Trending
Only the most relevant parameters are trended, while some ancillary data tracking may continue for method LCM

POC

Method Development & Optimization

Pre-Qualification

Method Qualification

Method Validation

Stress Stability Indicating Studies

Routine Sample Testing

Beyond Method Lifecycle

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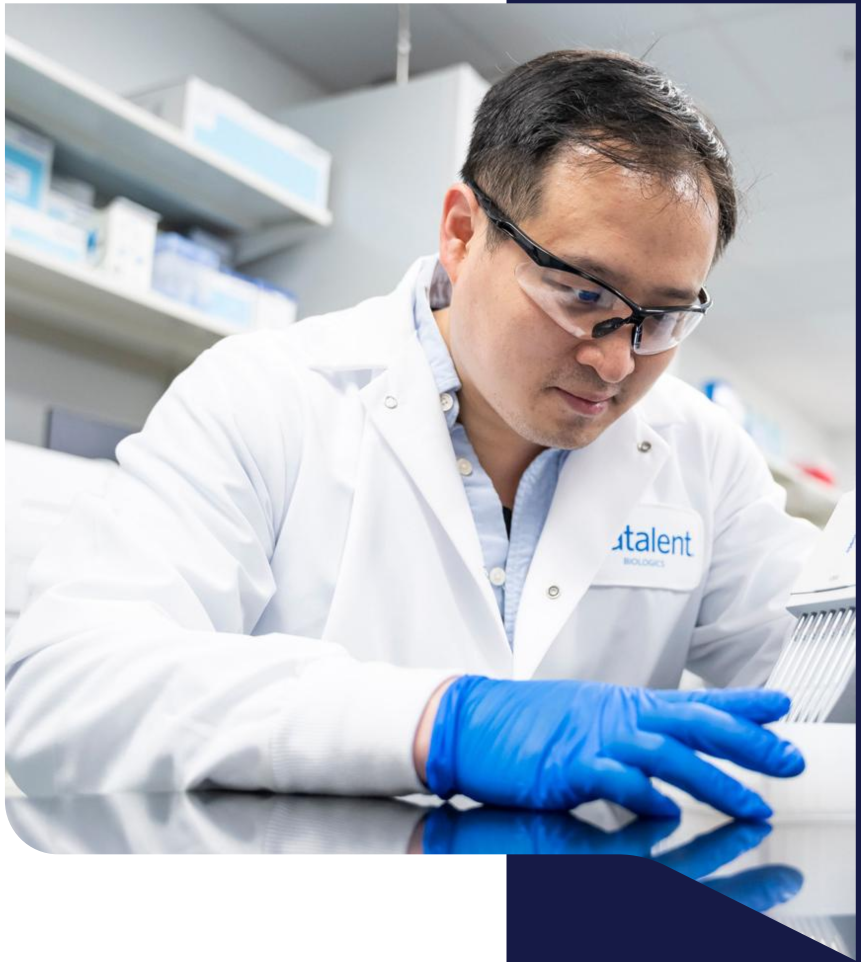

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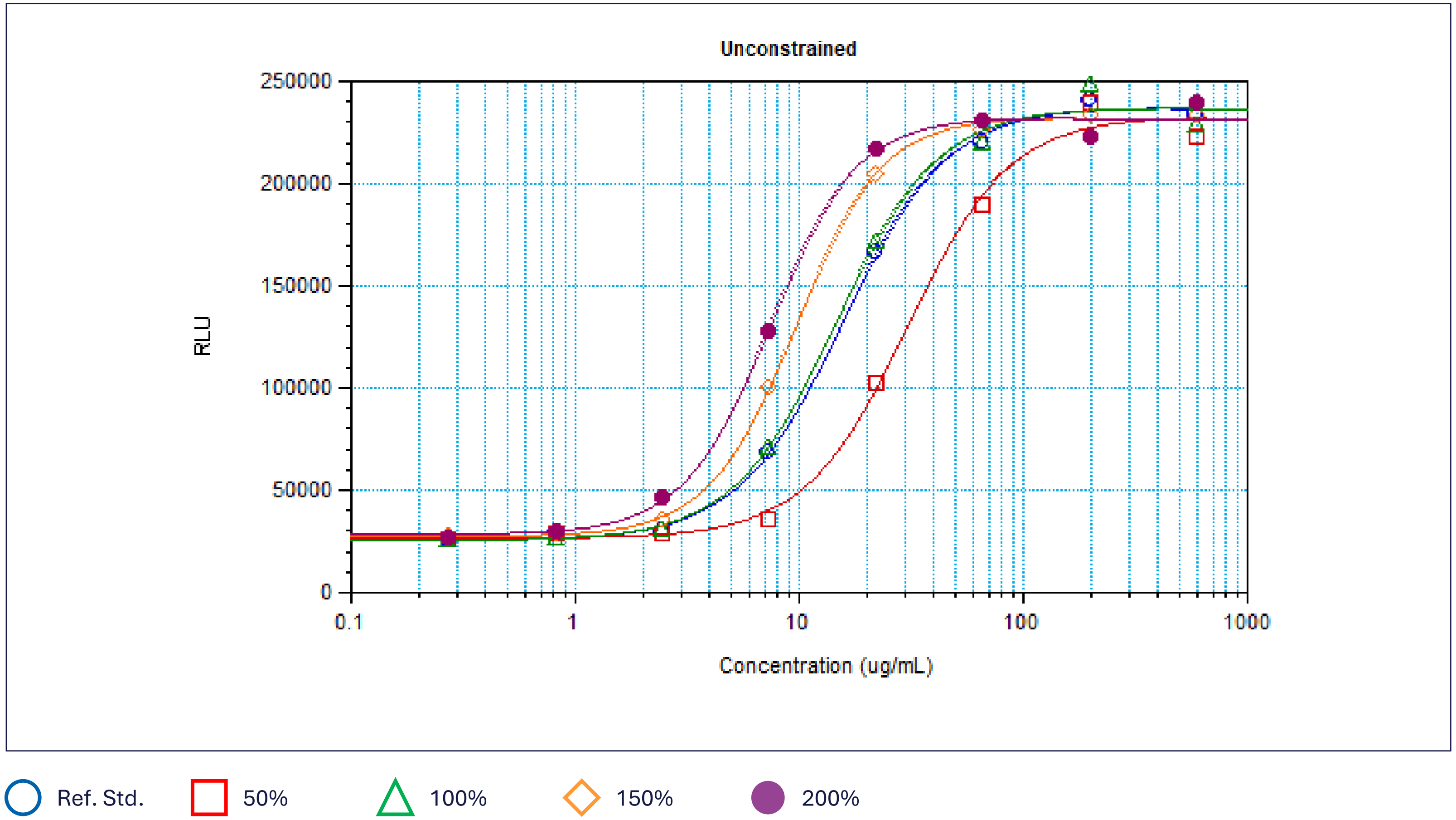
Optimal Bioassay Dose Response Curves

Example

Drug dilution series generate biological responses

Accuracy/Precision across range of method

Example of well-balanced curves, with defined upper & lower asymptotes, more than one point at the linear range, good parallelism & responses across the assay range



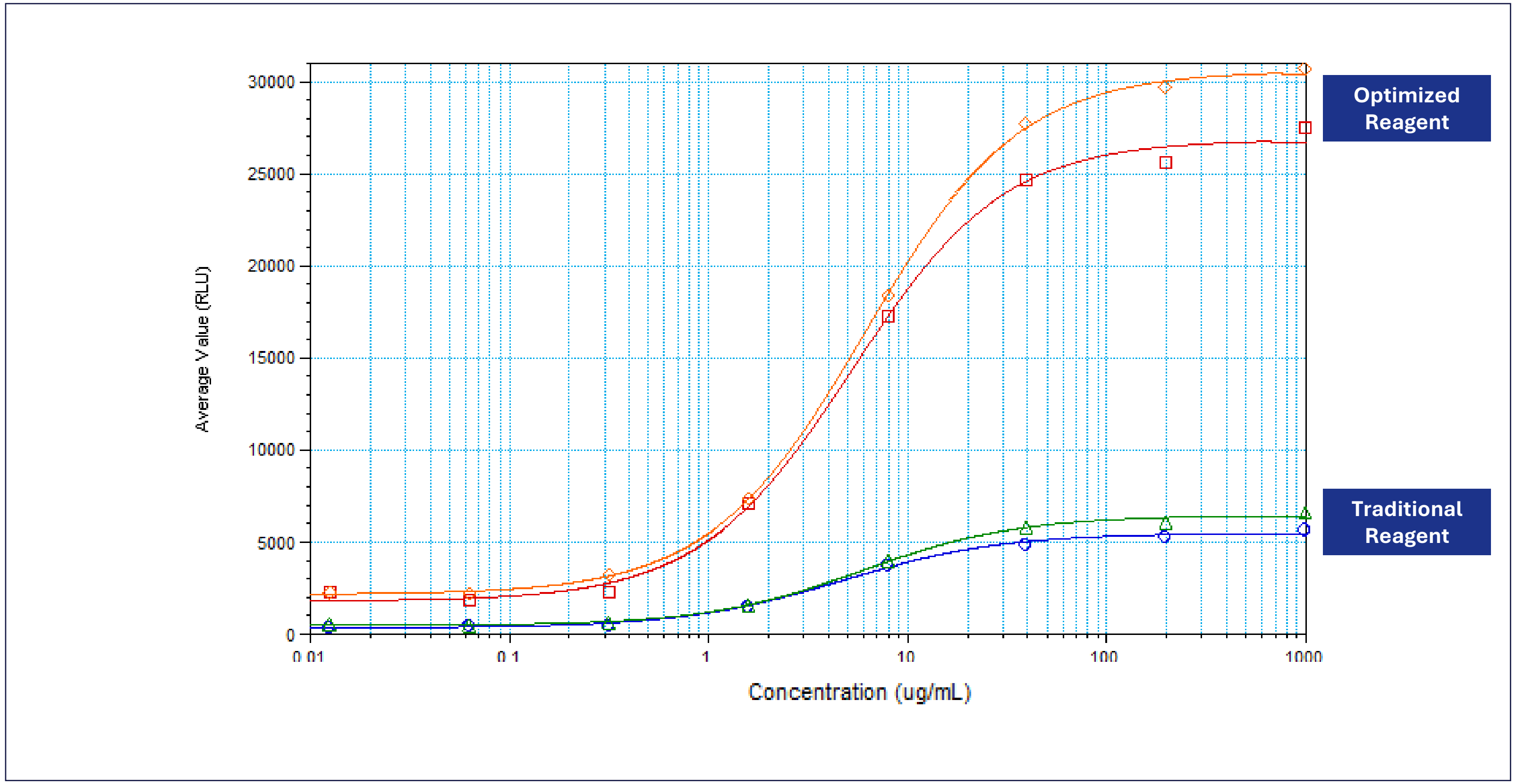
Alternative Optimized Reagents

Example

Optimization of System Parameters

- Detection reagent choice: traditional versus optimized reagent

Trade-off between light intensity and duration of the signal using an optimized reagent



Optimized Reagent

Traditional Reagent

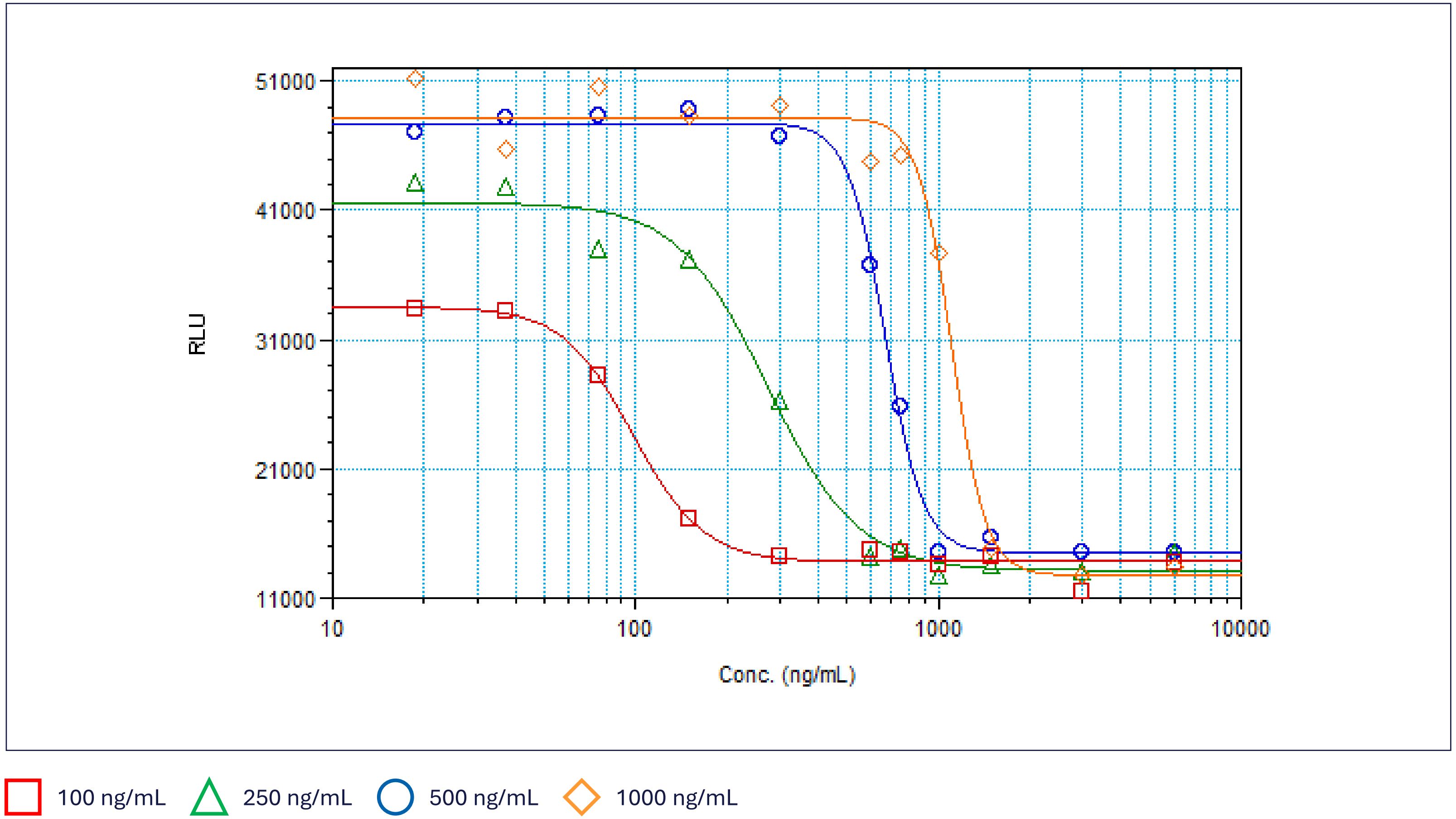
Ligand Activity Optimization

Example

Optimization of System Parameters

- mAb binds to ligand and neutralizes activity (apoptosis in this example)
- Choice of ligand concentration critical for optimal dose response

Ligand-induced cell stimulation must be evaluated for activity and not just rely on vendor's label claim concentrations



Incubation Conditions During Reactions

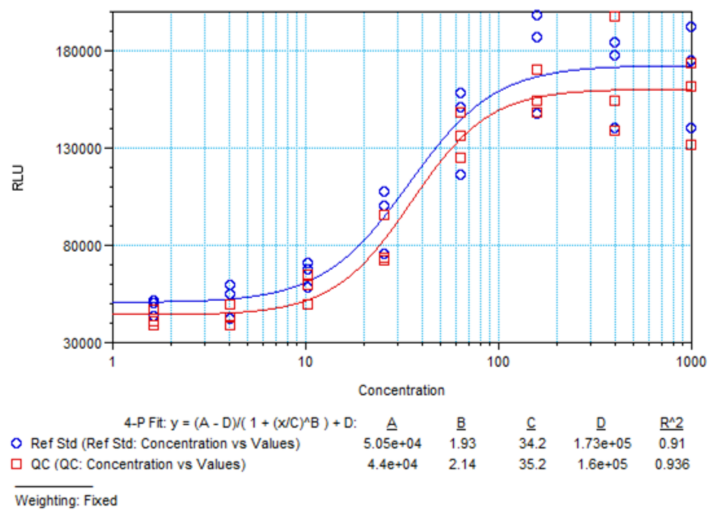
Example

Optimization of incubation with Luciferase substrate

- Identical dilutions used on 2 plates, one not cooled prior to substrate addition and the other cooled to RT for 15 min.
- Cooling may increase ambient O_2 solubility which is required during end point of reaction (Luciferase substrate requires ATP, oxygen and metallic cation).

Understanding factors potentially interfering with full reaction is important to avoid variability

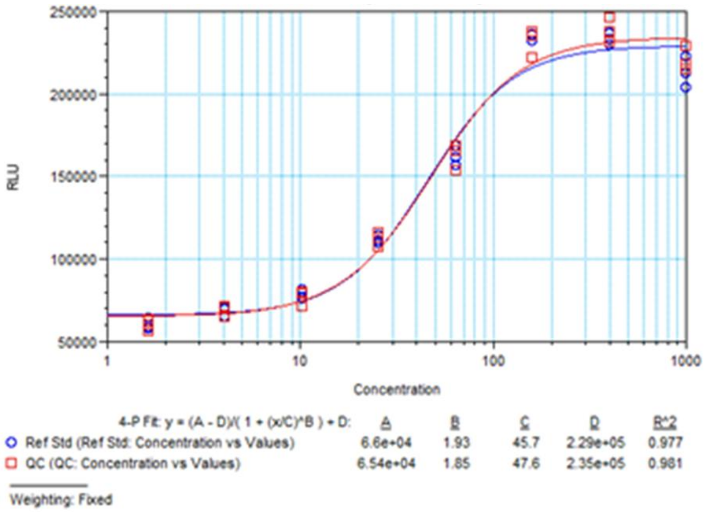
Not Cooling



4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
○ Ref Std (Ref Std: Concentration vs Values)	5.05e+04	1.93	34.2	1.73e+05	0.91
□ QC (QC: Concentration vs Values)	4.4e+04	2.14	35.2	1.6e+05	0.936

Weighting: Fixed

Cooling



4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
○ Ref Std (Ref Std: Concentration vs Values)	6.6e+04	1.93	45.7	2.29e+05	0.977
□ QC (QC: Concentration vs Values)	6.54e+04	1.85	47.6	2.35e+05	0.981

Weighting: Fixed

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Incubation Time Effects

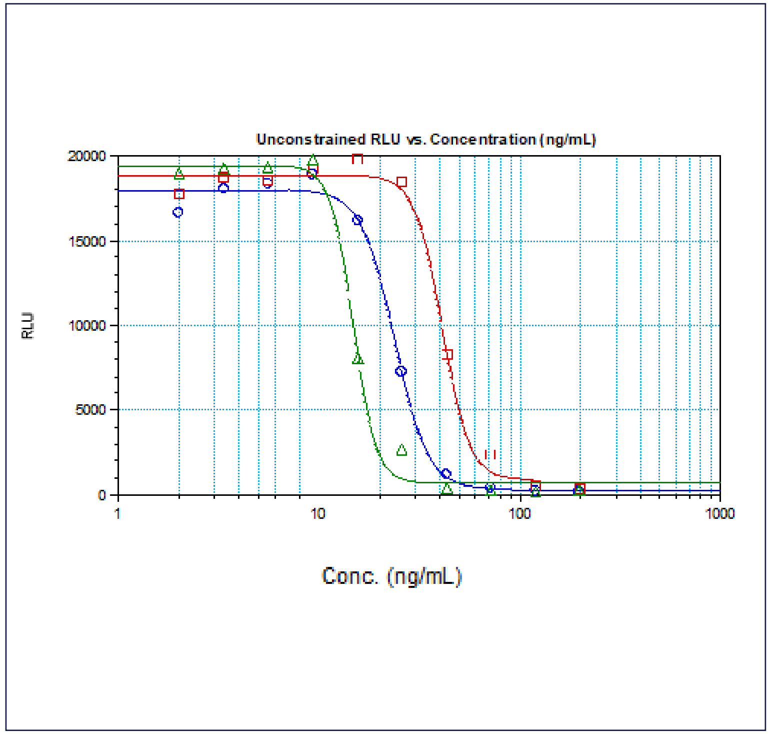
Example

Optimization of System Parameters

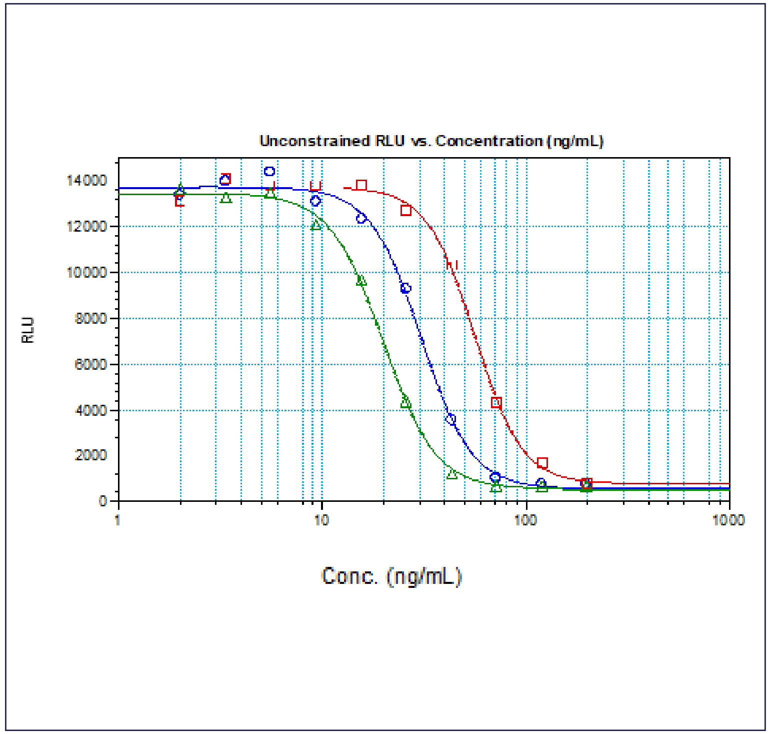
- Length of assay incubation dictated by drug MOA
- Comparison of curve shape across 3-day versus 4-day incubation

Extra day of incubation provides lesser steep curves, more points within hills-slopes, preventing “cobra shapes”, improved parallelism & goodness of fit

3 Day



4 Day



□ 50% ○ 100% △ 150%

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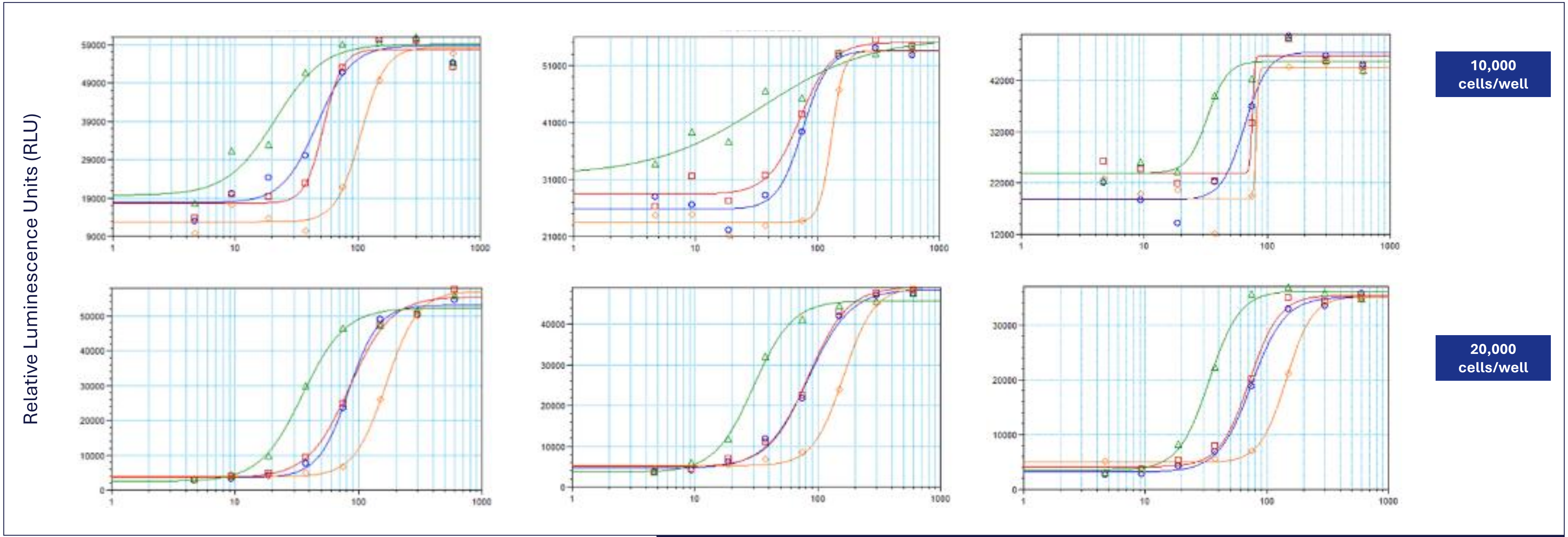
Cell Density Comparison vs Curve Shapes

Example



○ Ref. Standard 🟥 ACS (100%) △ TS1 (200% RS) ◇ TS2 (50% RS)

Simple adjustment of cell density from 10,000 cells/well to 20,000 cells/well makes a difference in the response curves in terms of parallelisms & goodness of fit

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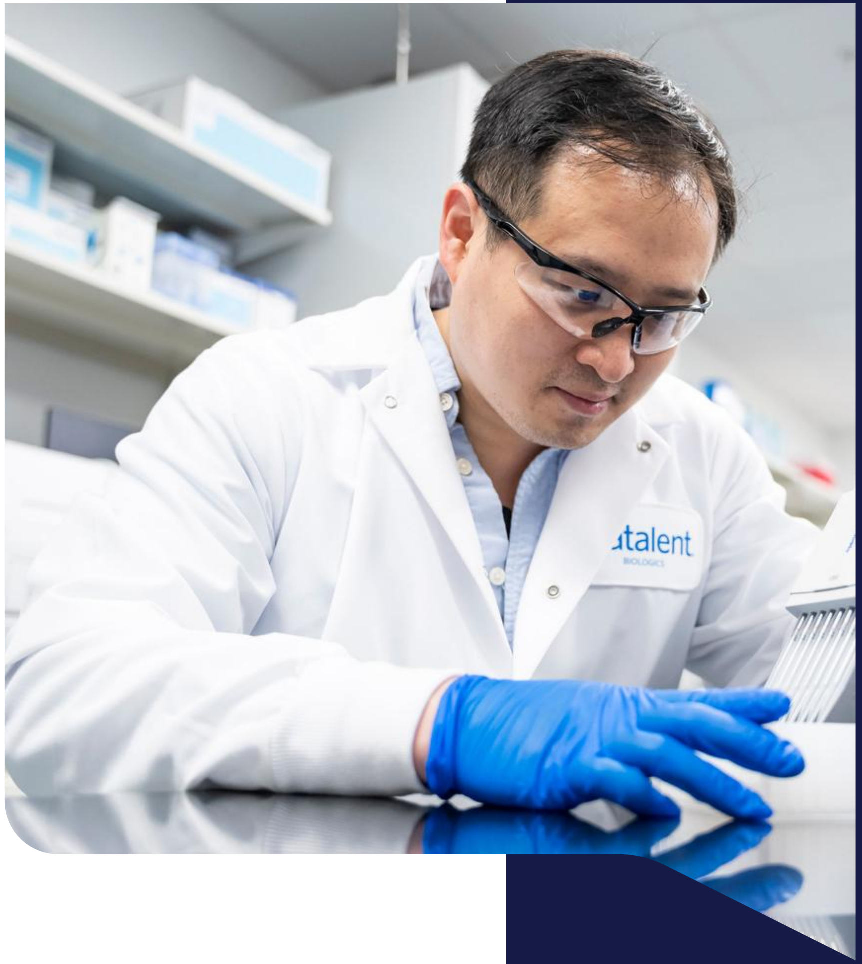

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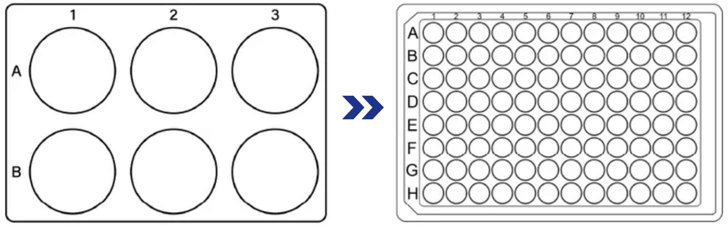
mRNA Transcription as Readout for Cell-Based Potency

Challenge



Initial prototype: 6-well plate required RNA extraction before cDNA synthesis

Limitations:

- Low throughput – Not suitable for GMP assays
- 96-well format still had RNA yield issues
- RNA extraction variability impacted assay robustness

Solution

✓ Optimized direct lysis approach:

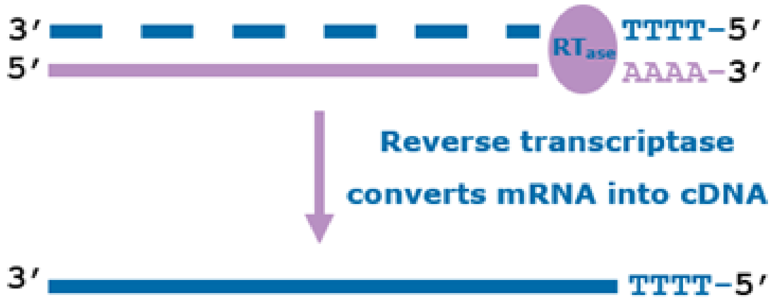
Cell lysis	(~7 min)
Reverse transcription	(~5 min)
Fast real-time PCR	(~35 min)

✓ No RNA extraction → Less variability

✓ Higher throughput & direct qPCR from lysates

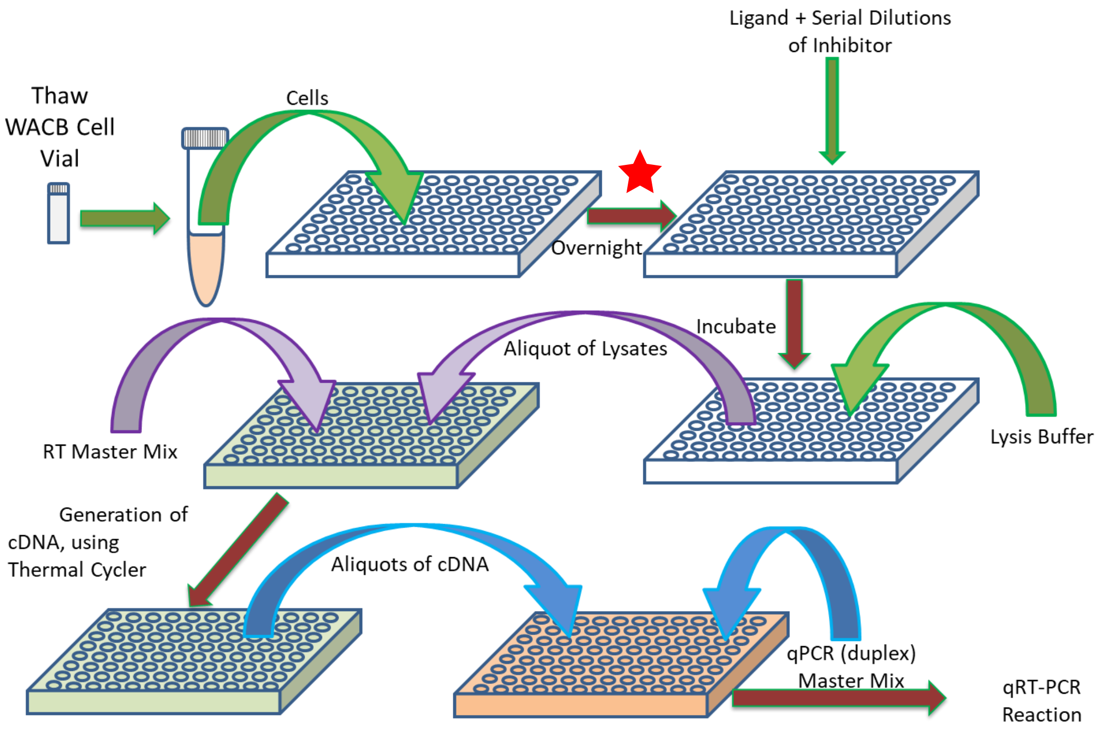
✓ TaqMan multiplexing:

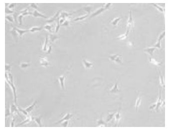
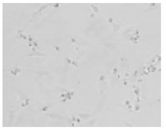
- Allows FAM (reference) vs VIC (target) for well-to-well normalization.



Reverse transcriptase converts mRNA into cDNA

Assay Schematical Overview



Serum-Free supplemented?	Yes	No
		
Dead	No	Yes

★ *Critical step identified – cells are thawed directly on serum-free medium to allow maximization of response to ligand.*

Cells previously treated with plasmid, ligand, or transgene are subjected to direct lysis

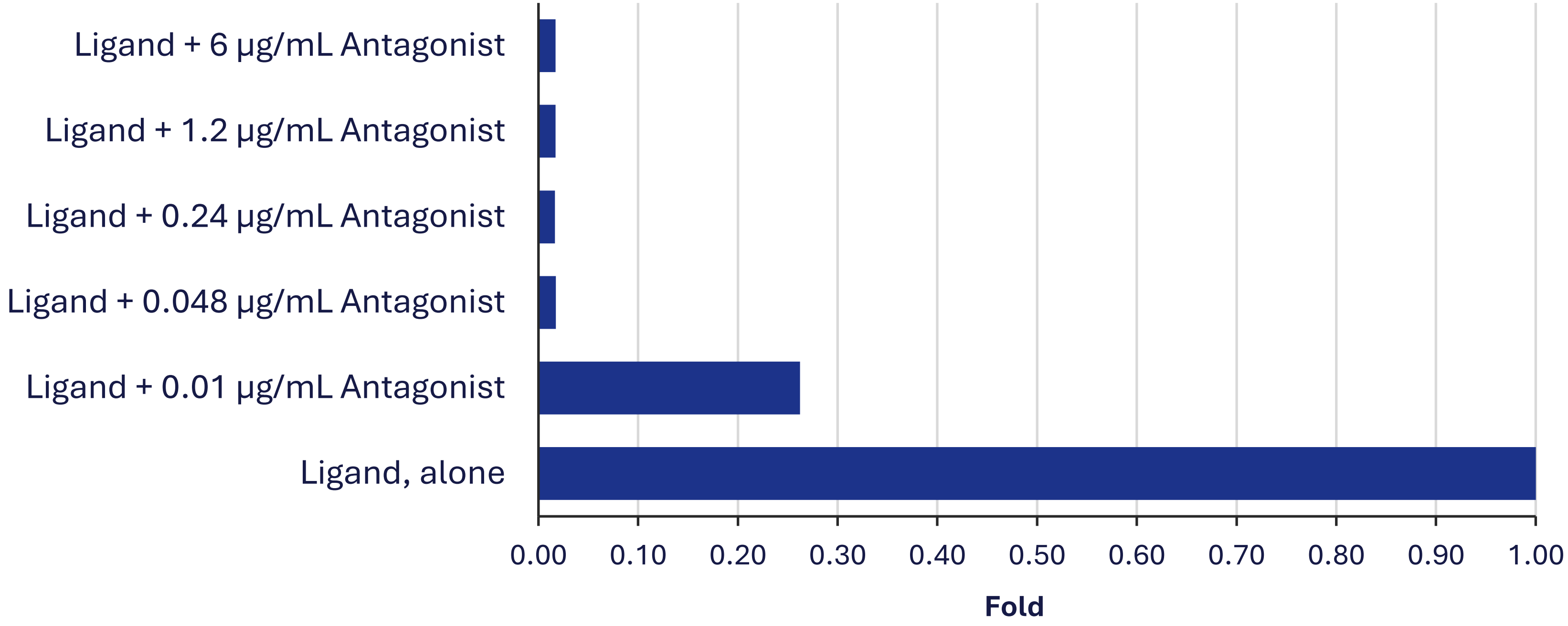
Cell lysate is subjected to reverse transcription to generate cDNA of transcript

The cDNA is then placed into a qPCR duplex qPCR reaction to assess cycle threshold (C_T) of the target & reference transcripts

General Assay Development

Ligand and antagonist concentration titrated to find optimal induction concentration

Fold-increase evaluated using $\Delta\Delta C_T$ Analysis

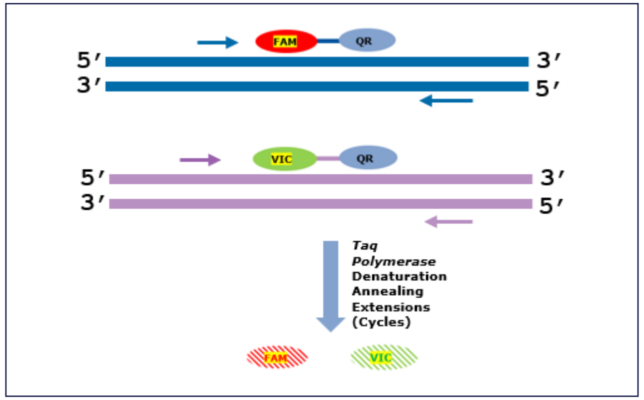


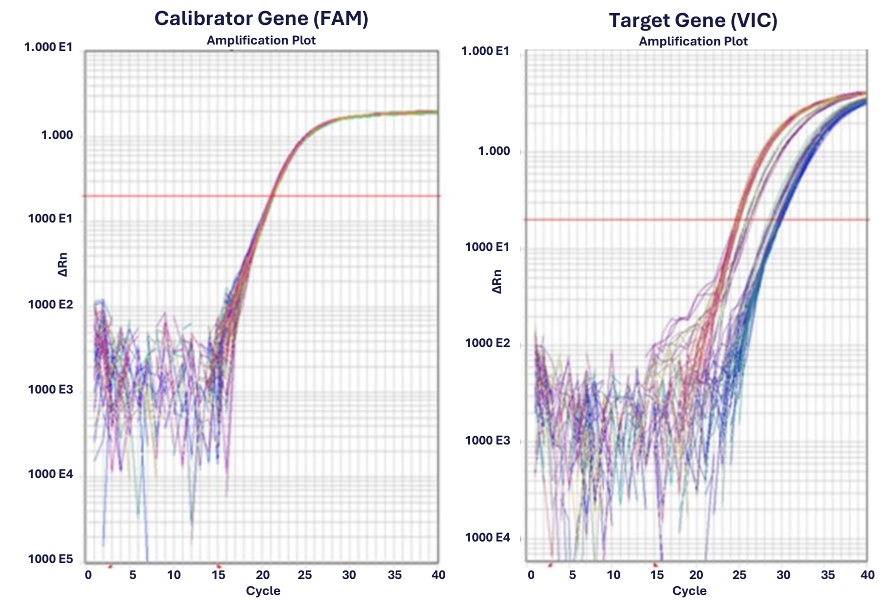
Initial PoC with a limited number of data points. Ligand is tested at a fixed concentration & antagonist tested at titrated concentration to find optimal antagonistic working concentration

mRNA Transcription - ΔCT Analysis

Principle

- qPCR measures C_t for target (VIC) & reference (FAM) genes
- ΔC_t = Target C_t - Reference C_t → Plotted against ligand/ vector dilution
- ΔC_t normalizes well-to-well variability for consistency



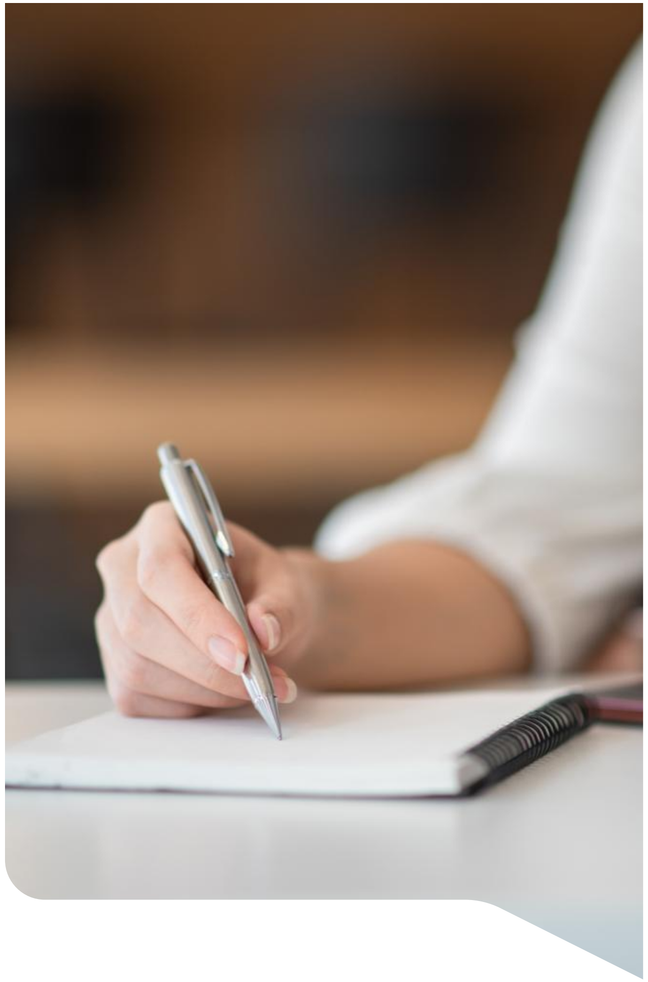


Baseline to determine cycle threshold

Raw Data

- Housekeeping gene (FAM) = Internal control (no treatment effect)
- Earlier C_t crossing → Higher mRNA expression
- Differential detection (FAM vs VIC) allows normalize well-to-well

Qualification Plan



✓ Range of 5 concentrations/test samples tested (dilutional samples):

- 50%, 70%, 100%, 140% and 200%

✓ On each plate, all samples (in 8-point serial dilution), run as pseudo-replicates:

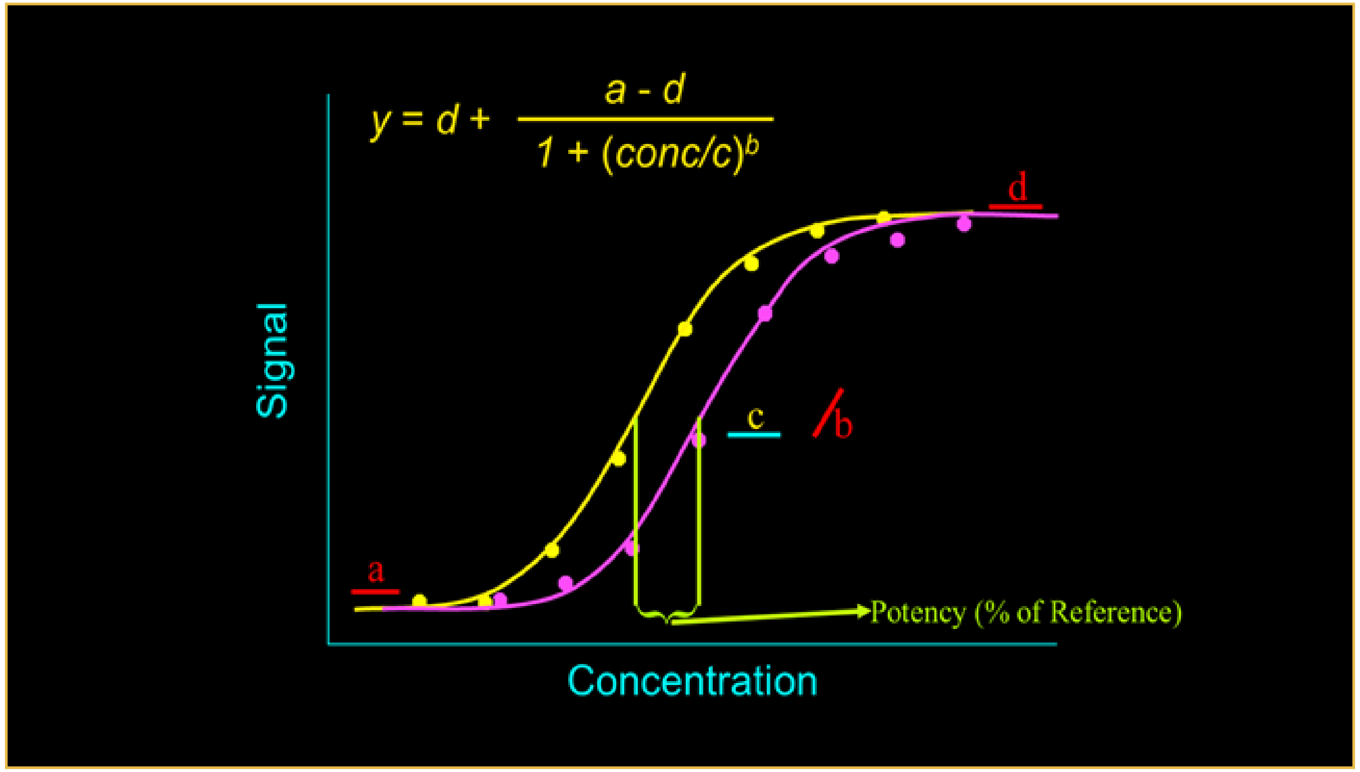
- Reference standard
- 3 independent preparations of the same test sample
- Assay control sample

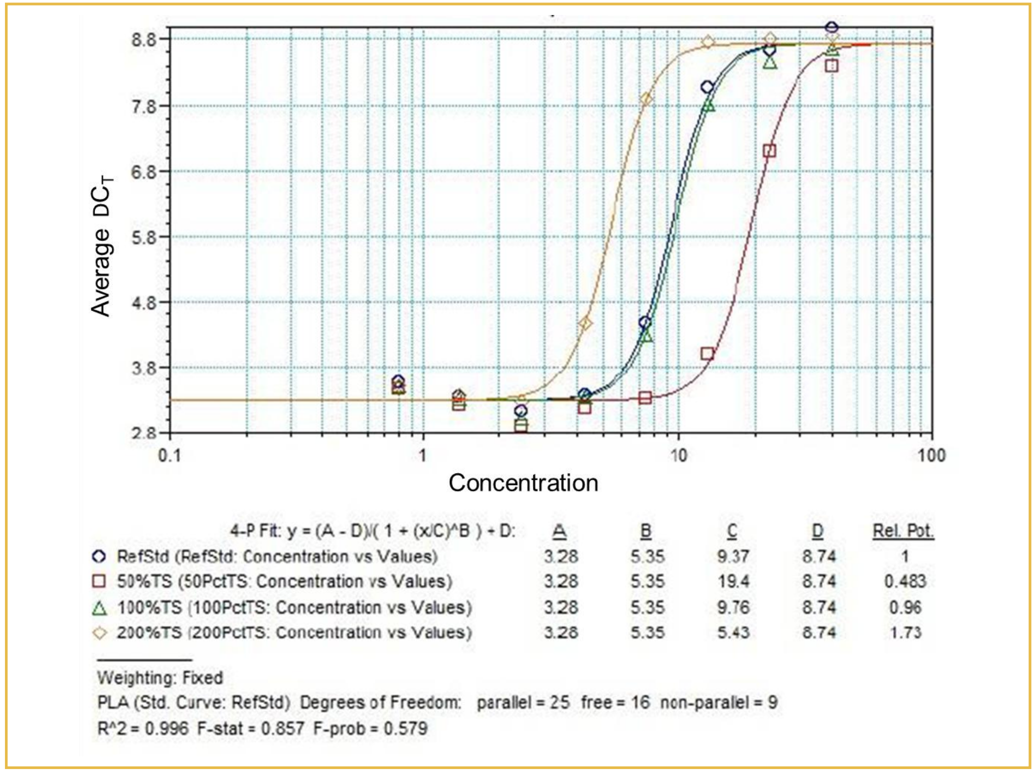
✓ 3 plates run for each test sample (dilutional sample)

✓ 3 plates for 100% sample run by one analyst (for repeatability/intra-assay precision)

✓ 3 additional plates for 100% sample run by alternative analyst & combined with first three 100% sample plates (for intermediate/inter-assay precision)

Four Parameter Fit Analysis





4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	Rel. Pot.
○ RefStd (RefStd: Concentration vs Values)	3.28	5.35	9.37	8.74	1
□ 50%TS (50PctTS: Concentration vs Values)	3.28	5.35	19.4	8.74	0.483
△ 100%TS (100PctTS: Concentration vs Values)	3.28	5.35	9.76	8.74	0.96
◇ 200%TS (200PctTS: Concentration vs Values)	3.28	5.35	5.43	8.74	1.73

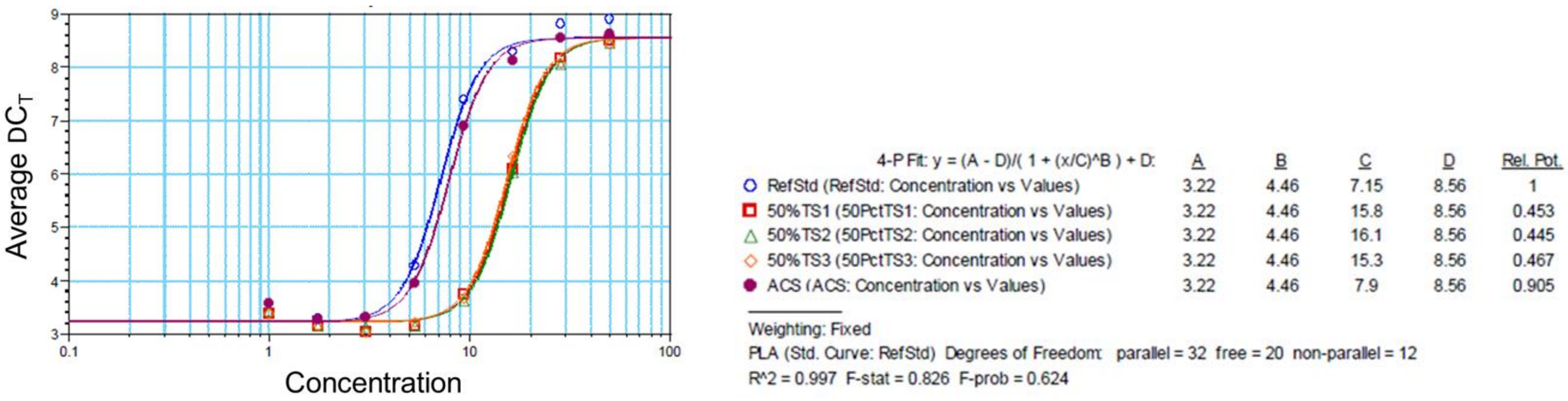
Weighting: Fixed
PLA (Std. Curve: RefStd) Degrees of Freedom: parallel = 25 free = 16 non-parallel = 9
R^2 = 0.996 F-stat = 0.857 F-prob = 0.579

ΔCT is plotted against sample concentration.
Plots shown are 200% and 50% of reference standard and control

Qualification Results: Accuracy

50%

Accuracy Level Test



4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	Rel. Pot.
○ RefStd (RefStd: Concentration vs Values)	3.22	4.46	7.15	8.56	1
□ 50%TS1 (50PctTS1: Concentration vs Values)	3.22	4.46	15.8	8.56	0.453
△ 50%TS2 (50PctTS2: Concentration vs Values)	3.22	4.46	16.1	8.56	0.445
◇ 50%TS3 (50PctTS3: Concentration vs Values)	3.22	4.46	15.3	8.56	0.467
● ACS (ACS: Concentration vs Values)	3.22	4.46	7.9	8.56	0.905

Weighting: Fixed
PLA (Std. Curve: RefStd) Degrees of Freedom: parallel = 32 free = 20 non-parallel = 12
R^2 = 0.997 F-stat = 0.826 F-prob = 0.624

Expected Potency (% Relative Potency)	Observed Potency (% Relative Potency)	Accuracy (% of expected)
50	46.7	93%
70	66.0	94%
100	100.2	100%
140	142.0	101%
200	175.1	88%

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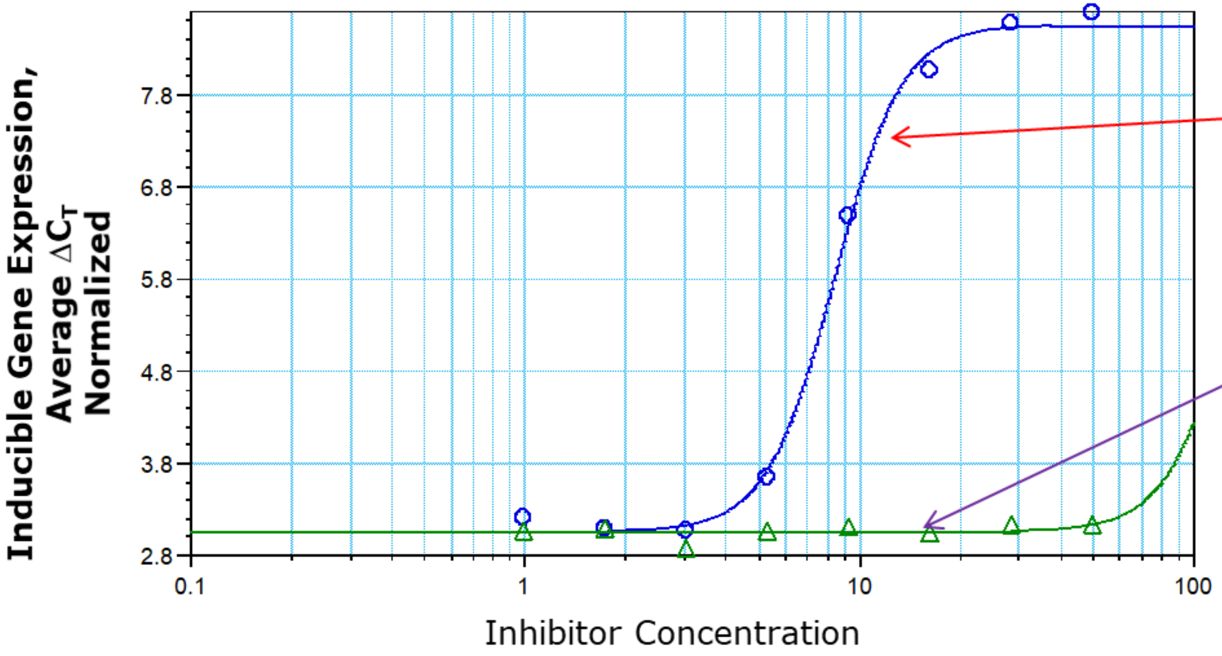
Qualification Results: Precision

	Reportable Potency Values (Mean of 3 independent values from same plate)	Averaged Values across the three plates	Intra-Assay (Repeatability) Precision	Inter-Assay (Intermediate) Precision
Analyst # 1	104.3	100.2	3.3%	4.4%
	100.1			
	96.3			
Analyst #2	103.4	103.2		
	109.3			
	96.9			

Repeatable results with high precision

Qualification Results: Specificity



Nominal Inhibitor

Unrelated Inhibitor (for ligand that works via a different receptor expressed on the same cells)

4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	Rel. Pot.
○ RS (RS: Concentration vs Values)	3.05	4.3	8.42	8.55	1
△ TS (TS - Rep 2: Concentration vs Values)	3.05	4.3	135	8.55	0.0624

These Assays Are Most Powerful When Integrated Into Broader Bioanalytical Workflows

✓

Fits into overall assay panel for products

● ● ● ●

✓

Powerful when used in conjunction with orthogonal assays

e.g., flow cytometry, mass spectrometry

● ● ● ●

✓

Can evaluate performance of genes that impact metabolites

e.g., carbohydrates, proteoglycans, lipids

● ● ● ●

✓

Can be used when gene products are difficult to quantify and coupled with sequencing could be used to verify gene expression editing

● ● ● ●

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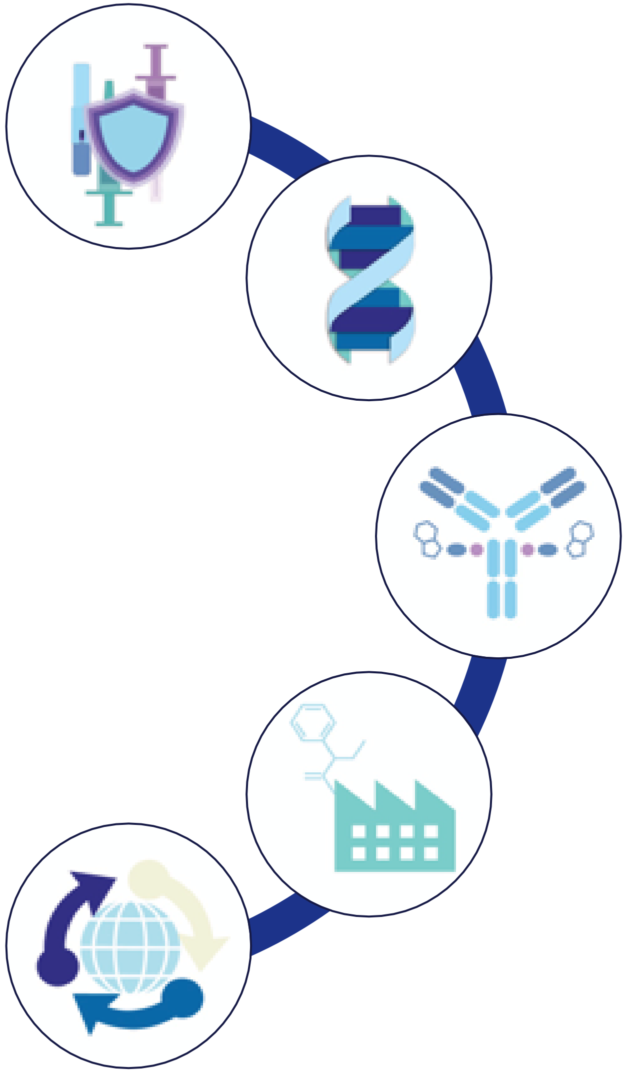
Conclusion



- Assess response at mRNA transcription
- No variability due to RNA extraction & purification
- Well-by-well normalization results in better precision
- Assay has been successfully qualified
- Assay is accurate and precise and suitable for cGMP use
- Additional tool for gene therapy with potential to conversion to ddPCR/dPCR

Thank you

Any questions?

Sonya Banks, MS
Account Director
sonya.banks@catalent.com

Tomorrow, 4/29 @ 12:00 PM
Salons D-G

Visit us at Booth #6!

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Comparison With Other Bioassay Readout Types

	mRNA Transcriptional Assay by qPCR	Reporter Gene Assays
Advantages	Evaluates the mRNA expression of endogenous or exogenously introduced genes	Evaluates response to ligand directly (reporter)
	Because amplification is involved, it is more sensitive to differences in response	Options for off-the-shelf cell lines available
	Allows for analysis of transfected, transduced or edited gene activity	Evaluates functionally by product
Limitations	Analysis requires further transformation of raw data	Requires genetic engineering of cell line of interest

Context for CGT Assays

	ddPCR	mRNA Transcriptional Assay by qPCR	Flow Cytometry
Advantages	Assess absolute gene copy number	Assess inserted or transferred gene construct functionality	Evaluates translational activity
	No standard curve required	Verification of cell transcriptional activity	Determines population of cells expressing transgene
Limitations	Low dynamic range	Requires normalization using a housekeeping gene	No amplification detectability limitations
	Unable to indicate if gene construct is inserted functionally	Multi-step assay	Could be difficult to standardize