Catalent
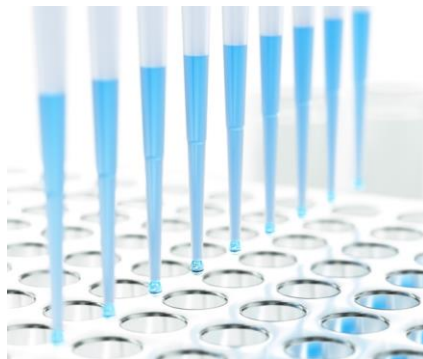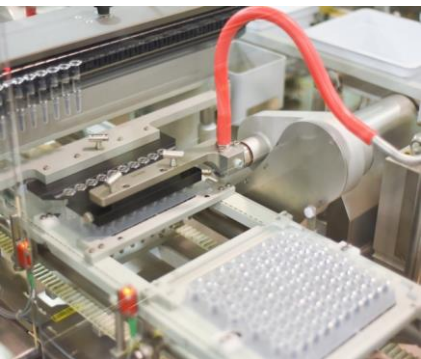## BIOLOGICS

Best Practices, Strategies & Utilization of Novel Biological Responses for Robust Cell-Based Potency Assays

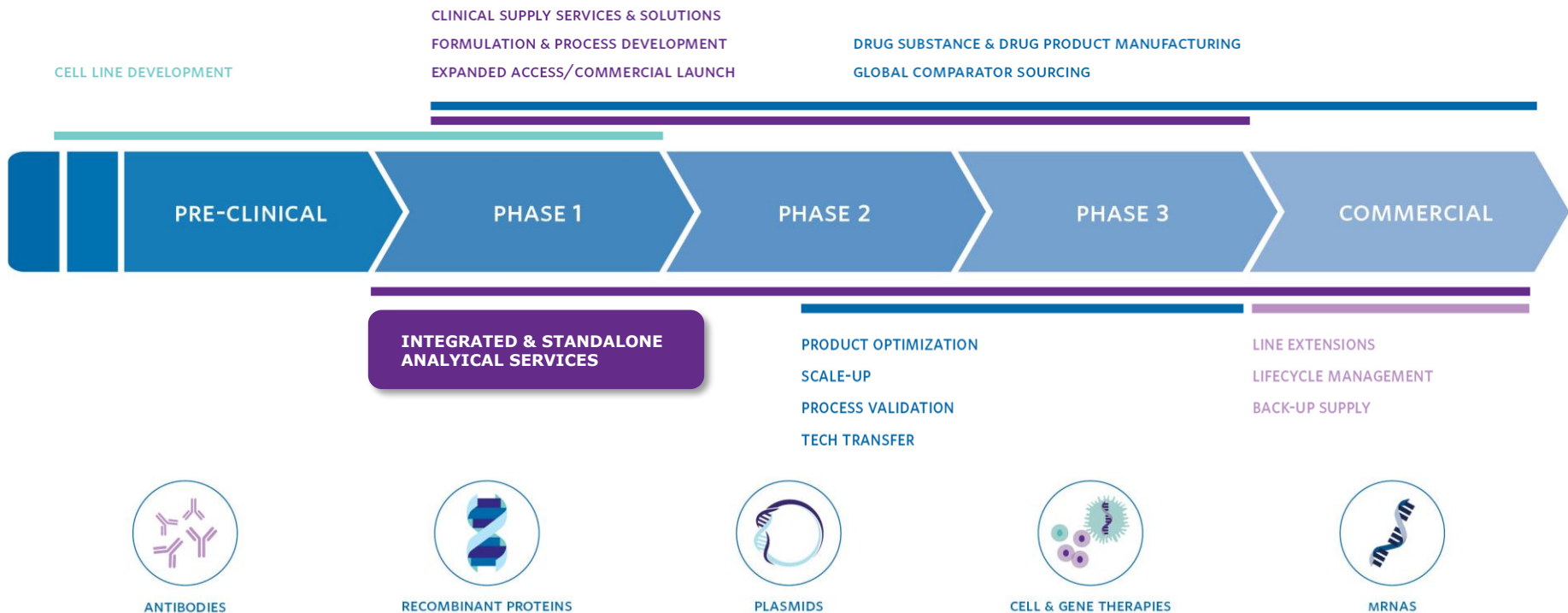**PEDRO MORALES**
DIRECTOR, BIOLOGICS ANALYTICAL

more products. better treatments. reliably supplied.™

0

INTEGRATED & STANDALONE CDMO SOLUTIONS TO ACCELERATE YOUR BIOLOGIC TO MARKET

CLINICAL SUPPLY SERVICES & SOLUTIONS

FORMULATION & PROCESS DEVELOPMENT

DRUG SUBSTANCE & DRUG PRODUCT MANUFACTURING

CELL LINE DEVELOPMENT

EXPANDED ACCESS/COMMERCIAL LAUNCH

GLOBAL COMPARATOR SOURCING

PRE-CLINICAL	PHASE 1	PHASE 2	PHASE 3	COMMERCIAL

INTEGRATED & STANDALONE ANALYICAL SERVICES

PRODUCT OPTIMIZATION

LINE EXTENSIONS

SCALE-UP

LIFECYCLE MANAGEMENT

PROCESS VALIDATION

BACK-UP SUPPLY

TECH TRANSFER

ANTIBODIES

RECOMBINANT PROTEINS

PLASMIDS

CELL & GENE THERAPIES

MRNAS

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Biologics Analytical Services
## Single Source for Integrated & Standalone Capabilities

GMP Analytical Services
- Method development, transfer & optimization
- Phase-appropriate validation
- In-process, release & stability testing
- Binding & cell-based assays
- Extractables & leachables

GLP Support for Clinical Studies

Experience with broad classes of large molecules:

- Monoclonal, polyclonal & bispecific antibodies
- Bioconjugates & ADCs
- Oligonucleotides
- Recombinant proteins
- Fusion proteins

- Pegylated peptides
- Cell and gene therapies
- Aptamers
- Vaccines
- Oligosaccharides
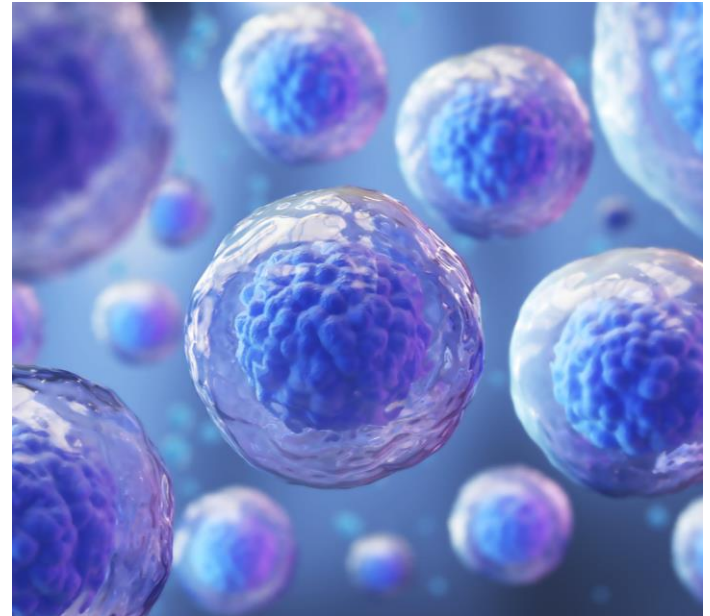
225+ scientists across the sites

100,000+ ft^3 of stability chambers
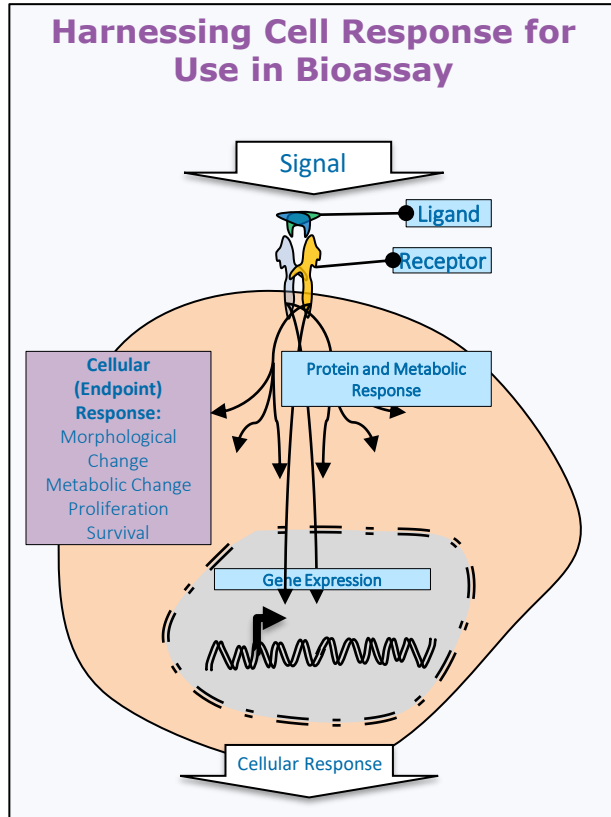
800+ assays/ techniques offered

300+ client programs supported

AGENDA

Introducing Cell-Based Potency Assays

Harnessing Cell Response for Use in Bioassay

Signal

Ligand

Receptor

Cellular (Endpoint) Response:
Morphological Change
Metabolic Change
Proliferation
Survival

Protein and Metabolic Response

Gene Expression

Cellular Response

- **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.

General Aspects to Consider During Cell-Based Potency Method Development

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

AGENDA

1 Introduction & Considerations

2 **Readouts**

3 Strategy

4 Roadmap Visualization

5 Examples: Dose Response Curves

6 mRNA Transcription Assay Development & Qualification

7 Q&A

Bioassays Readout - Where Biology Meets Chemistry!

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

Cyto-toxicity	Enzymatic	Reporter	Metabolic	Nucleic Acid

Examples

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

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

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

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

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

AGENDA

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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, etc.)
- Convert continuous culture to "thaw and use"
- Randomize distribution of replicates across plate
- Maximize throughput (horizontal vs vertical)
- Inclusion and trending assay control sample

R=Ref. Std.   S=Test Sample 1   S=Test Sample 2   C=Control Sample
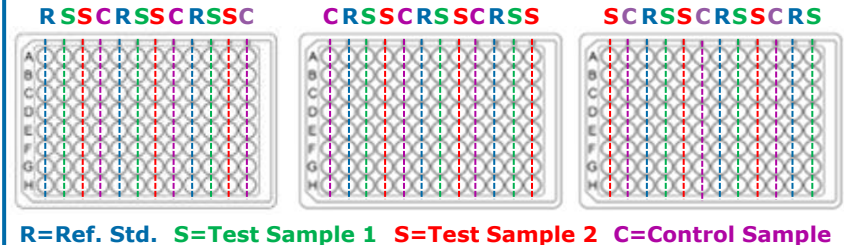
Pre-Validation Activities

- 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
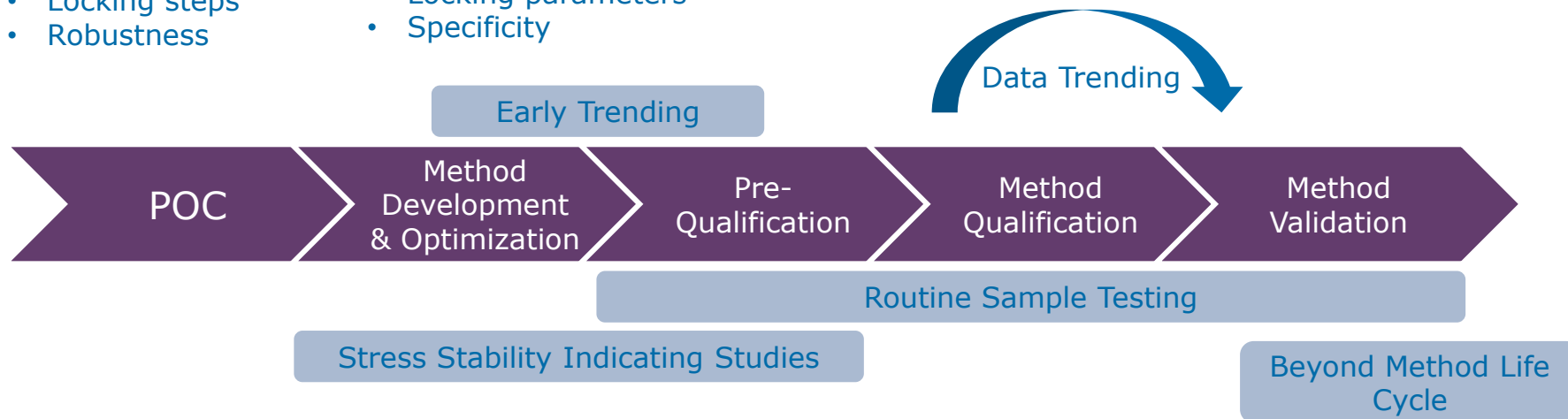- Based on client needs and minimum requirements of Catalent SOPs

AGENDA

Roadmap to a Validated Cell-Based Assay

Aspects to Consider

- Incubation times
- Critical reagents
- Training
- DS/DP scope
- Trending
- System suitability
- Locking steps
- Robustness

- Number of plates
- Critical steps
- Assay parameters
- True ACS lot
- Assay acceptance criteria
- Specifications
- Locking parameters
- Specificity

Data Trending

Early Trending

POC → Method Development & Optimization → Pre-Qualification → Method Qualification → Method Validation

Routine Sample Testing

Stress Stability Indicating Studies

Beyond Method Life Cycle

AGENDA

Bioassay Dose Response Curves- Example of Optimal

- Drug dilution series generate biological responses
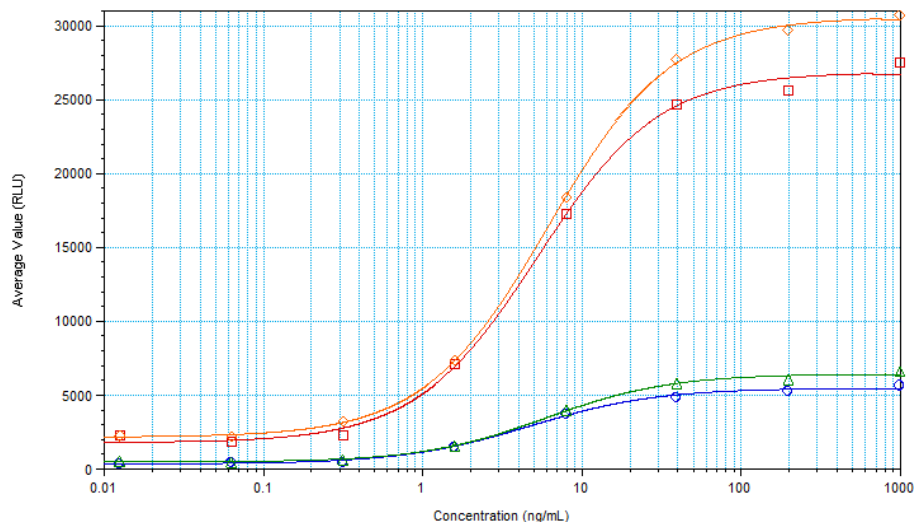
- Accuracy/Precision across range of method
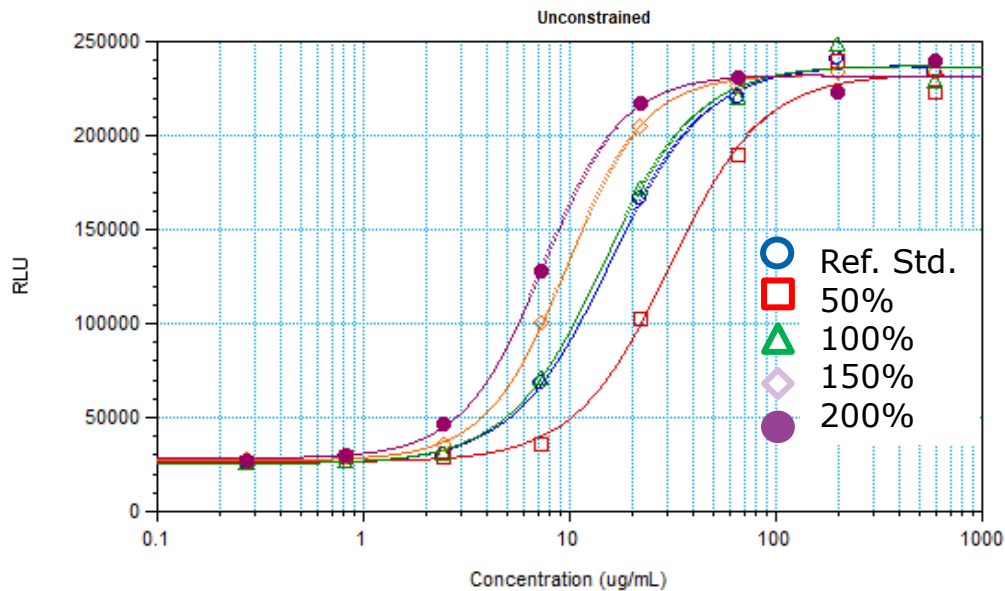
Example of well-balanced curves, with defined upper and lower asymptotes, more than one point at the linear range, good parallelism and responses across the assay range

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Examples – Alternative Optimized Reagents

Optimization of System Parameters

- Detection reagent choice: traditional versus optimized reagent
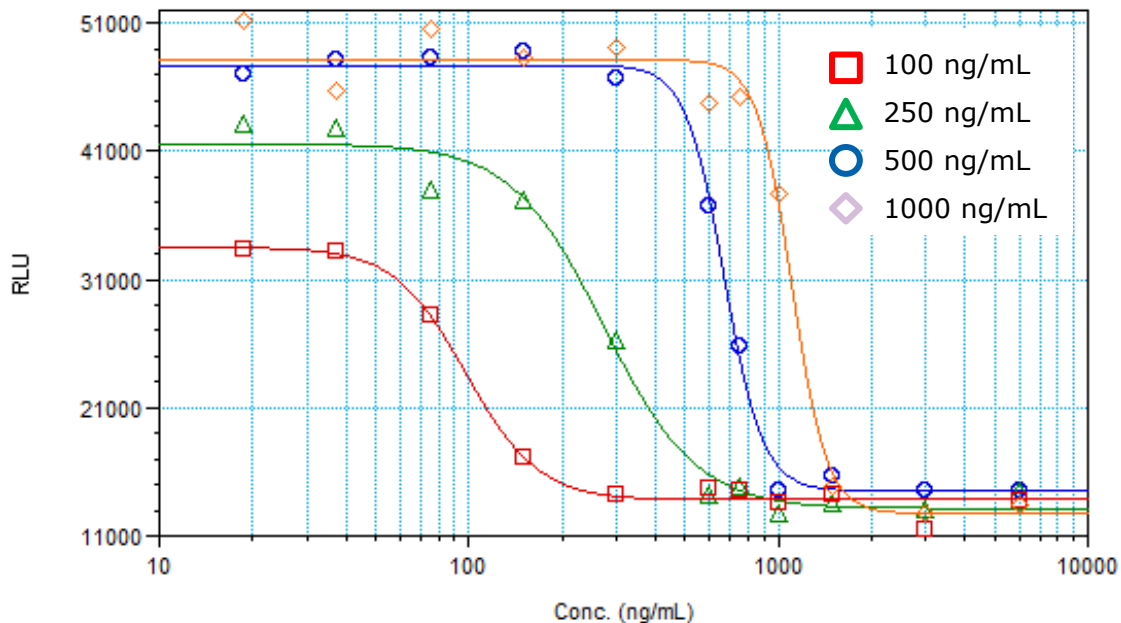

Optimized Reagent

Traditional Reagent

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

Examples – Ligand Activity Optimization

Optimization of System Parameters

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

Examples – Incubation Conditions During Reactions

**Optimization of incubation with Luciferase substrate**

- Identical dilutions used on two 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).
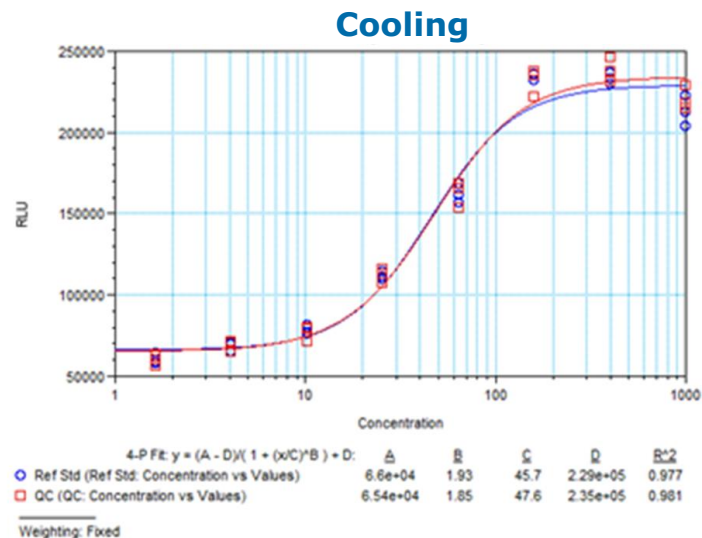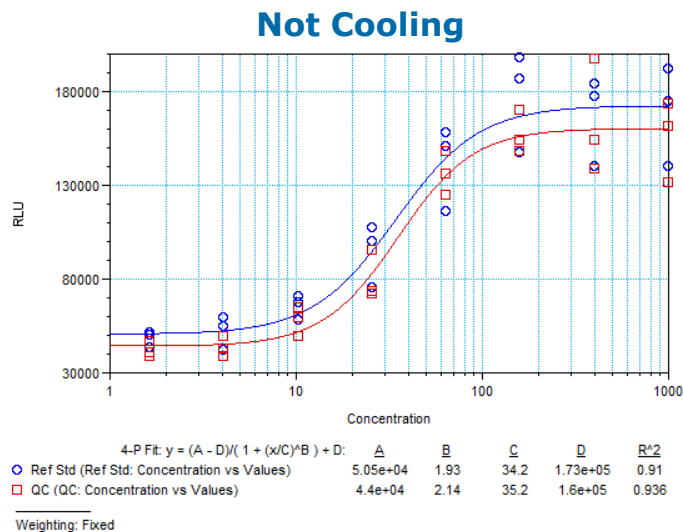
Examples – Incubation Time Effects

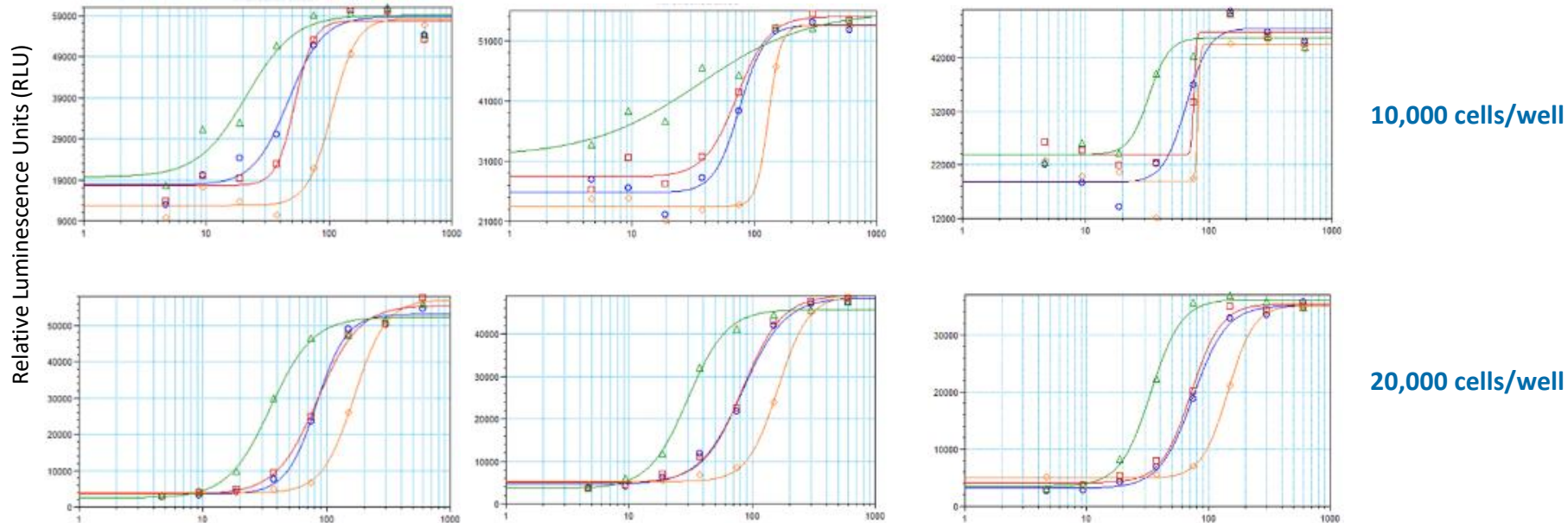**Optimization of System Parameters**

- Length of assay incubation dictated by drug MOA

- Comparison of curve shape across 3-day versus 4-day incubation
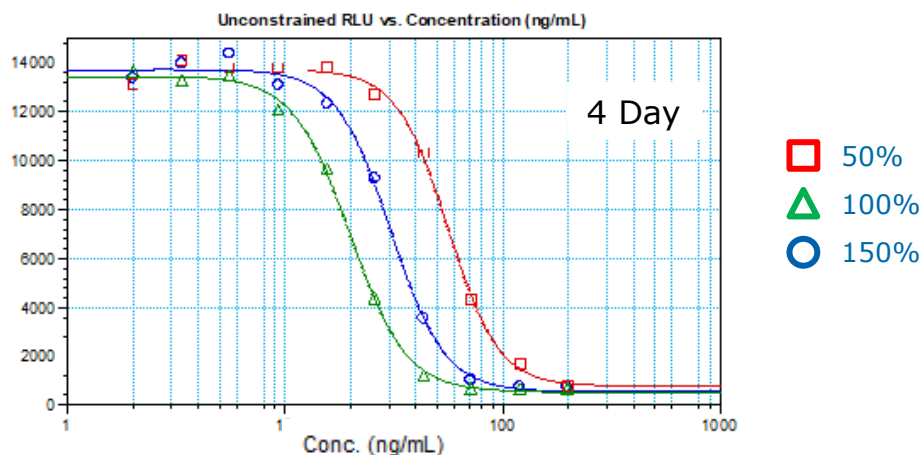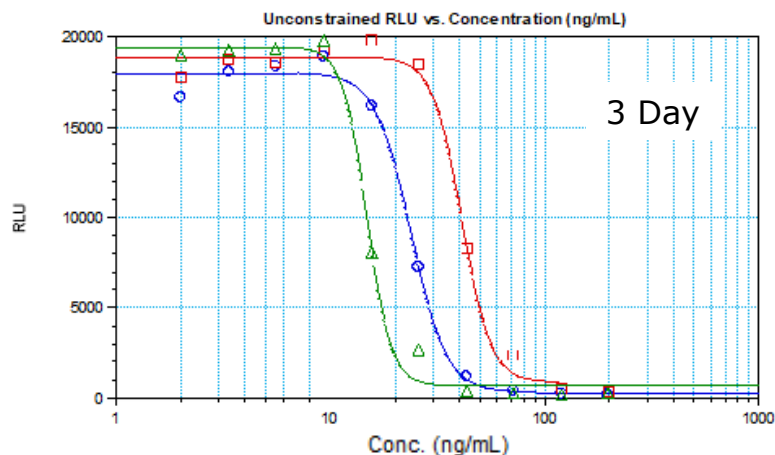
Extra day of incubation provides lesser steep curves, more points within hills-slopes, preventing "cobra shapes", improved parallelism and goodness of fit

Example - Cell Density Comparison vs Curve Shapes

O RS □ ACS Δ TS1 (200% RS) ◊ TS2 (50% RS)

10,000 cells/well

20,000 cells/well

Relative Luminescence Units (RLU)

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 and goodness of fit
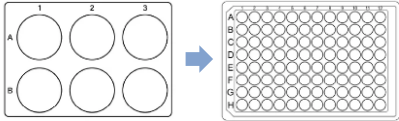
AGENDA

1. Introduction & Considerations

2. Readouts

3. Strategy

4. Roadmap Visualization

5. Examples: Dose Response Curves

6. **mRNA Transcription Assay Development & Qualification**

7. Q&A

# mRNA Transcription as Readout for Cell-base Potency

Challenge

- The available prototype was a 6-well plate requiring RNA extraction prior cDNA synthesis

- Transcription regulation was required to recapitulate MOA of the product

- 6-well plate not feasible for GMP assay due to low throughput for all replicates, controls, and full curve

- However, a 96-well plate requiring RNA extraction will not provide enough RNA yield for downstream activities

- RNA extractions efficiencies accounts for variability, thus impacting the robustness of the assay
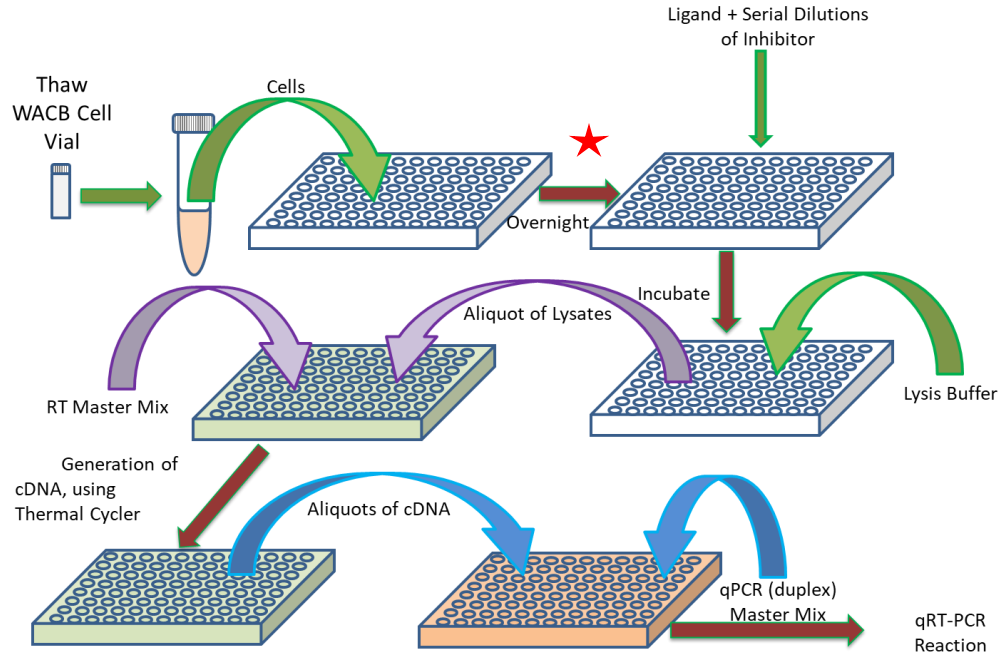
Solution

- An optimized direct lysis method
  - Cell Lysis (~7 min total)
  - Reverse Transcription (~5 min)
  - Fast Real-Time PCR (~35 min)
- Faster direct cell lysis after stimulation of response
- No RNA extraction, no variability between extractions
- Higher throughput
- qPCR directly from lysates using TaqMan chemistries
- RNA can be easily converted to cDNA using the lysates and then amplified by PCR:
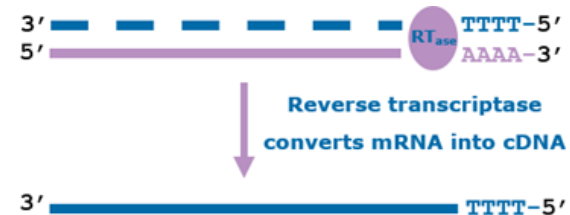
- TaqMan chemistries allows for multiplexing in same reaction (FAM vs VIC, for example), allowing well to well signal normalization
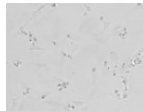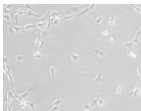
Assay Schematical Overview

Ligand + Serial Dilutions of Inhibitor

Thaw WACB Cell Vial

Cells

★

Overnight

Incubate

Aliquot of Lysates

RT Master Mix

Lysis Buffer

Generation of cDNA, using Thermal Cycler

Aliquots of cDNA

qPCR (duplex) Master Mix

qRT-PCR Reaction

Serum-Free supplemented?

Yes   No

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

Dead   No   Yes

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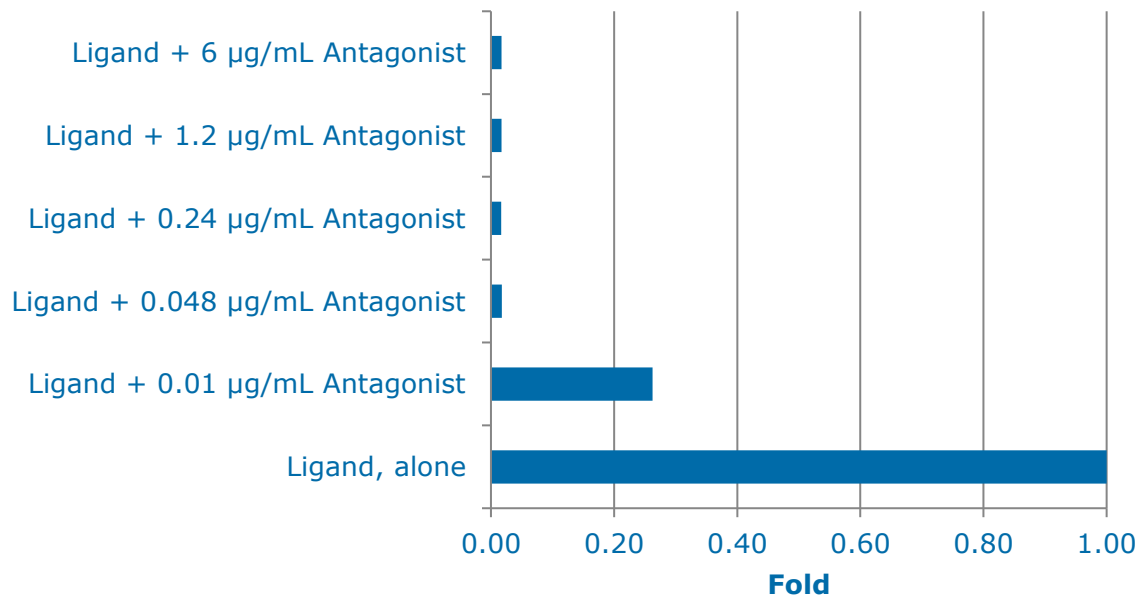
Initial proof of concept with a limited number of data points.
Ligand is tested at a fixed concentration and antagonist tested at titrated concentration to find optimal antagonistic working concentration

mRNA Transcription - ΔC_T Analysis

Principle

- Each well tested on qPCR is evaluated for cycle threshold (C_T) for the target sequence & the reference sequence

- Calculate ΔC_T (difference between C_T values of target & reference sequences) against concentration of the ligand or dilution factor of transgenic vector, and plot it

- Using ΔC_T is advantageous because each well is normalized to each reaction. Small variations in total cDNA content among replicates becomes negligible

Raw Data

Baseline to determine cycle threshold
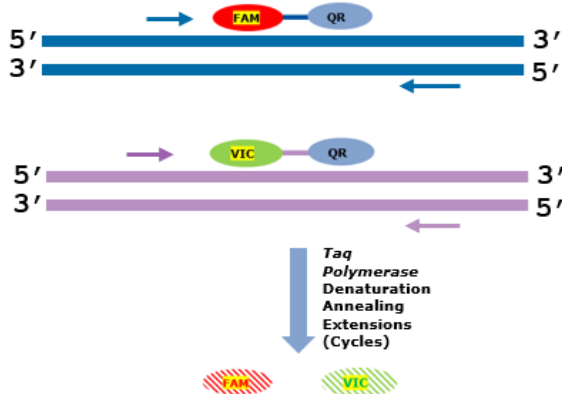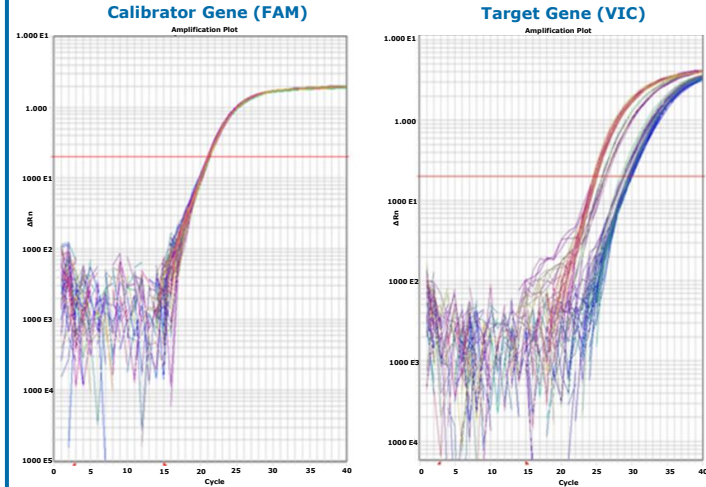
- Calibrator gene = housekeeping gene with consistent transcriptional activity (no change with treatments)

- Amplicons (mRNA) that cross the cycle threshold earlier are more abundant or highly expressed after treatments

- Differential detection (FAM vs VIC) allows normalize well-to-well

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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, and combined with first three 100% sample plates (for intermediate/inter-assay precision)

Four Parameter Fit Analysis

$$y = d + \frac{a - d}{1 + (conc/c)^b}$$
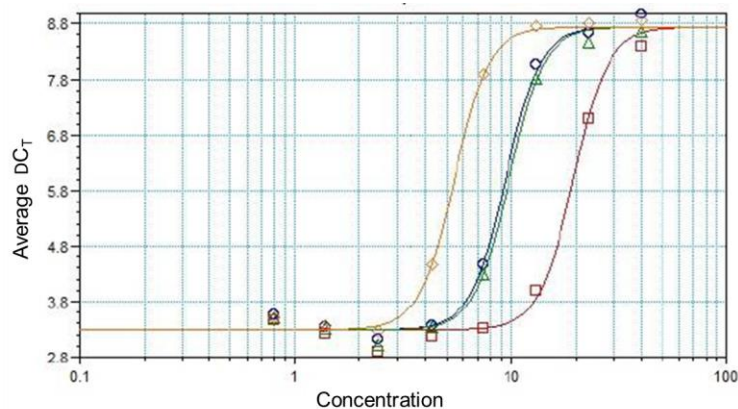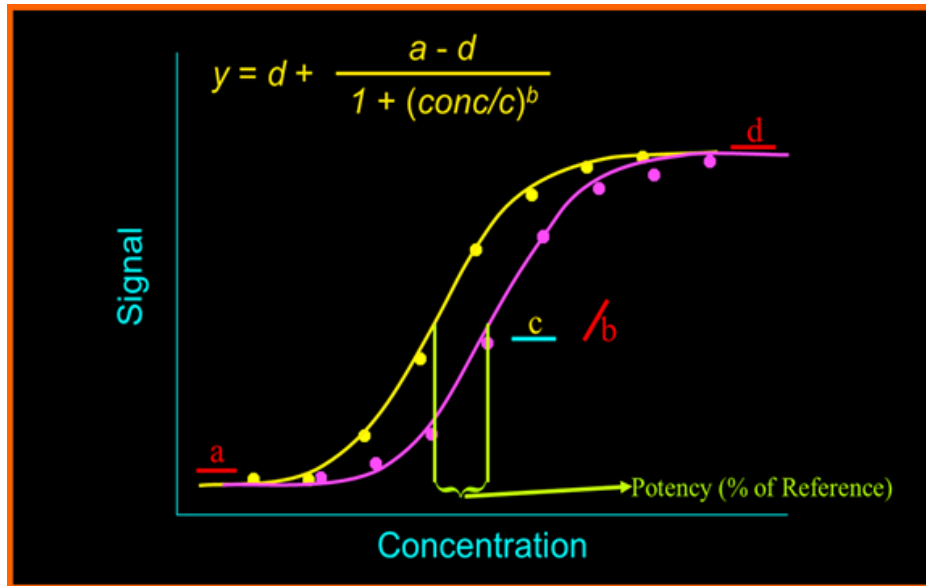
4-P Fit: $y = (A - D)/(1 + (x/C)^B) + D$:

	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: $y = (A - D)/(1 + (x/C)^B) + D$:
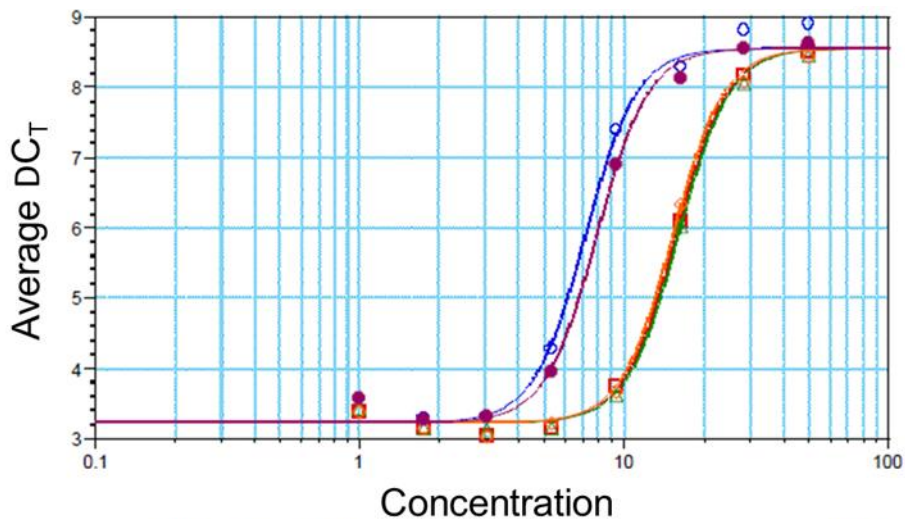
	A	B	C	D	Rel. Pot.
○ RefStd (RefStd: Concentration vs Values)	3.22	4.46	7.15	8.56	1
□ 50%TS1 (50PctTS1: Concentration vs Values)	3.22	4.46	15.8	8.56	0.453
△ 50%TS2 (50PctTS2: Concentration vs Values)	3.22	4.46	16.1	8.56	0.445
◇ 50%TS3 (50PctTS3: Concentration vs Values)	3.22	4.46	15.3	8.56	0.467
● ACS (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
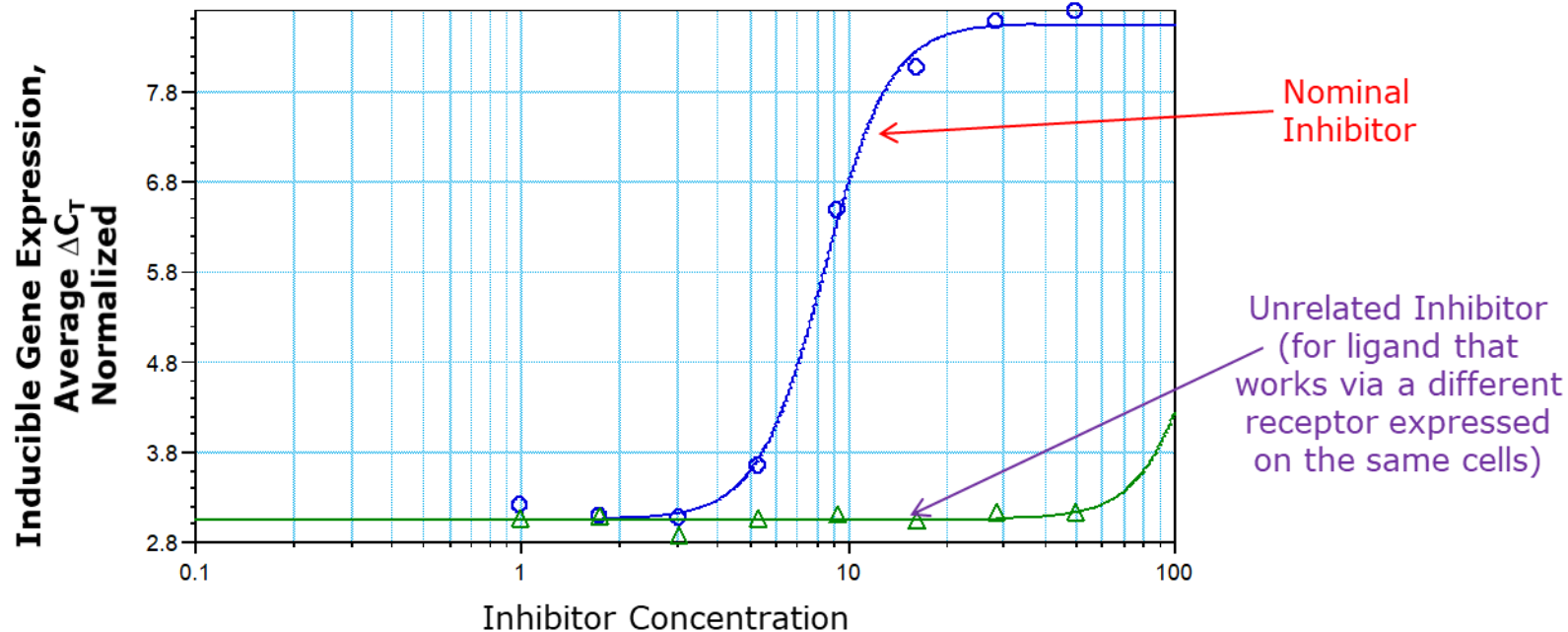
$R^2 = 0.997$ F-stat = 0.826 F-prob = 0.624

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%

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			
	100.1	100.2	3.3%	
	96.3			
Analyst #2	103.4			4.4%
	109.3	103.2		
	96.9			

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

Comparison With Other Bioassay Readout Types

Assay Type	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 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 Cell and Gene Therapy Assays

Assay Type	ddPCR	mRNA Transcriptional Assay by qPCR	Flow Cytometry
Advantages	Assess absolute copy number transduced	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	Difficult to standardize

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

Context For Cell and Gene Therapy Assays

Fits into overall assay panel for products

Powerful when used in conjunction with orthogonal assays (e.g., flow cytometry, mass spectrometry)
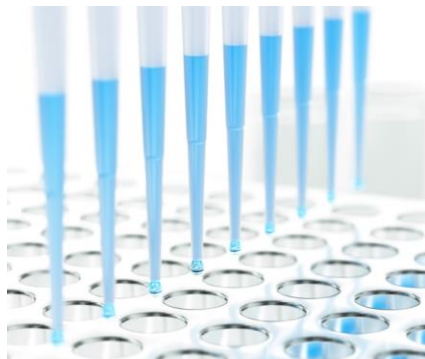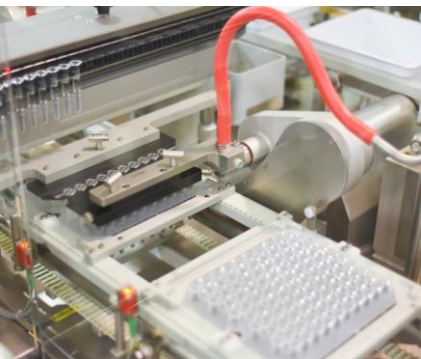
Can evaluate performance of genes that impact metabolites (e.g., carbohydrates, proteoglycans, lipids)

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

Q&A

more products. better treatments. reliably supplied.™