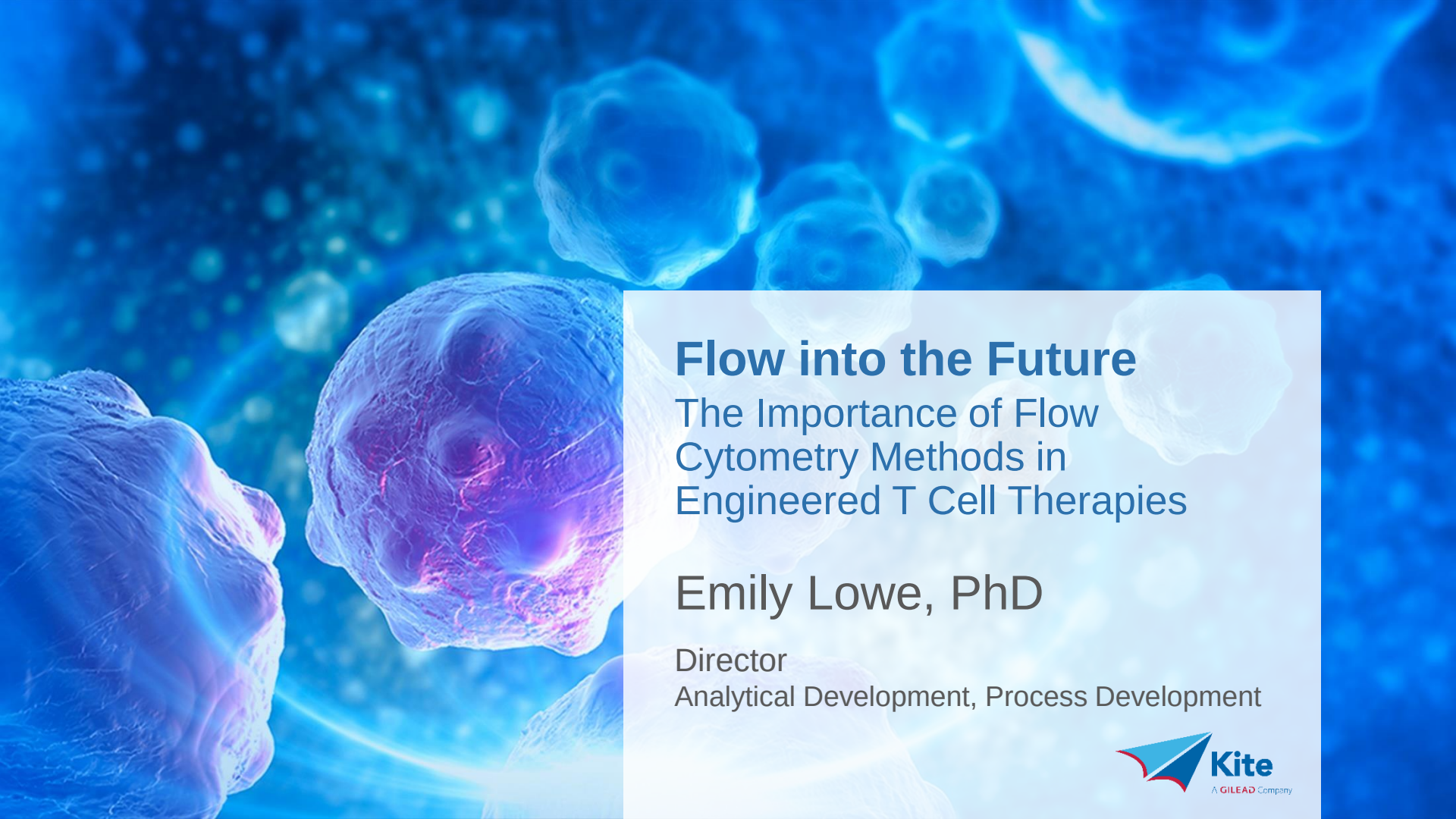

Flow into the Future

The Importance of Flow Cytometry Methods in Engineered T Cell Therapies

Emily Lowe, PhD

Director
Analytical Development, Process Development

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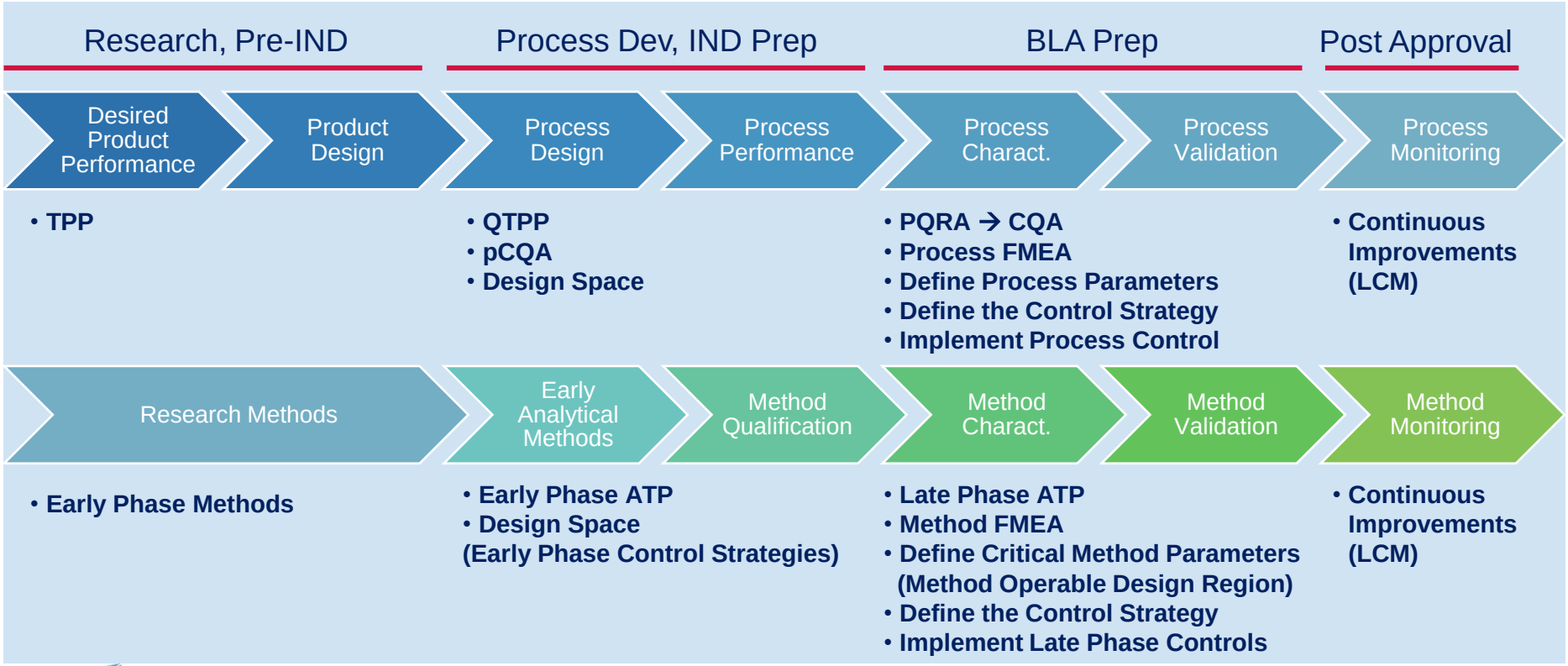
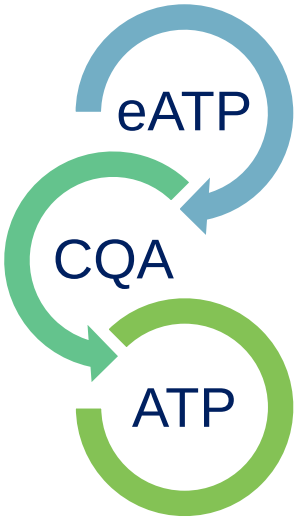
Life Cycle Approach to Cell Therapy Product Design



TPP: Target Product Profile; QTPP: Quality Target Product Profile; CQA: Critical Quality Attribute; pCQA: potential Critical Quality Attribute; FMEA: Failure Mode Effects Analysis; LCM: Life Cycle Management; ATP: Analytical Target Profile

The Challenges of QbD in Engineered T Cell Therapies

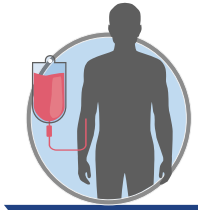
- **The ATP/CQA Dilemma**: The product's CQA should drive the design, development and validation of appropriate analytical methods **but CQAs are defined fairly late in the process!**
- Due to product complexity, CQA's are often not known in early development & CMC quality control can be challenging (high batch-to-batch variability)
- Thorough Analytical characterization is key to understand, identify, and develop appropriate quality control strategy for various phases of clinical development through commercial
- **Defining CQA's by correlational analysis requires a broad range of cell characterization and potency assays**



QbD: Quality by Design; CQA: Critical Quality Attribute; ATP: Analytical Target Profile; eATP: Early Analytical Target Profile

Analytical Toolbox For Engineered T Cell Products Enables Product Quality

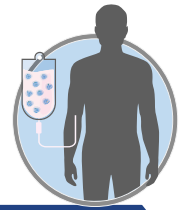
Collect patient's white blood cells



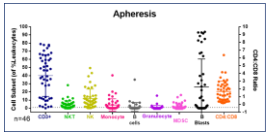
Apheresis Manufacturability
Deep dive of cellular attributes to identify biomarkers (MQAs) to understand and predict manufacturing success

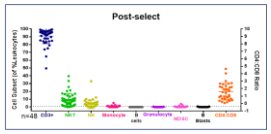
Product Understanding
Deep dive of final product attributes to identify biomarkers linked to patient outcome

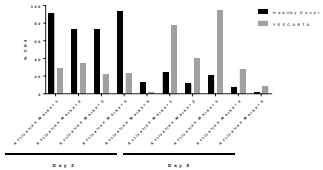
Infuse patient with engineered T cells

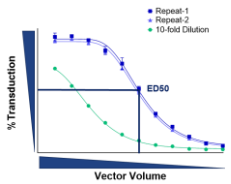


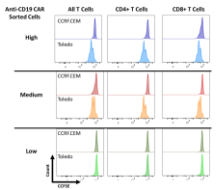
Apheresis → PBMC/T cell Selection → T Cell Activation → RVV Transduction → T Cell Expansion → Formulation

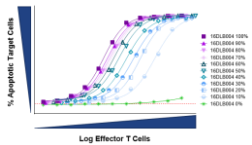












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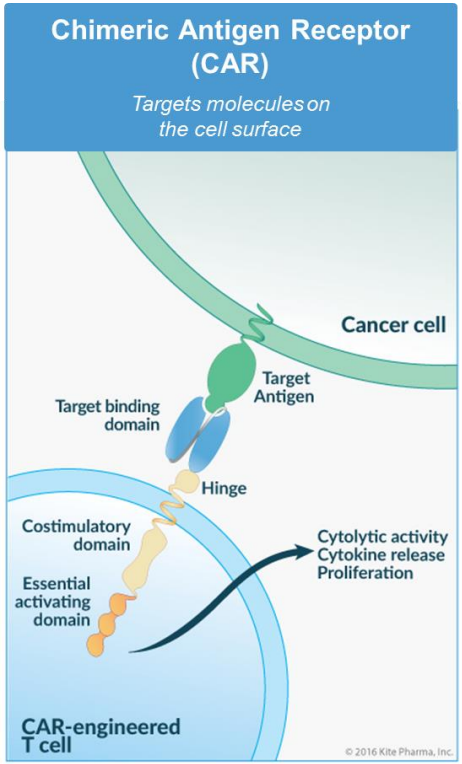
Engineered Protein Receptors' Mechanisms of Action



- Cell-based potency assays are essential for engineered cell therapy products to demonstrate final product activity is linked to biological critical quality attributes
  - **CAR/TCR surface expression is critical for potency**
- Identifying and controlling variability is one of the biggest challenges in designing and executing cell-based potency assays
- Poorly controlled and highly variable assays:
  - Increase invalid and re-test rates (compliance risks)
  - May cause a manufacturing process to appear out of control
  - May result in final product to appear unstable

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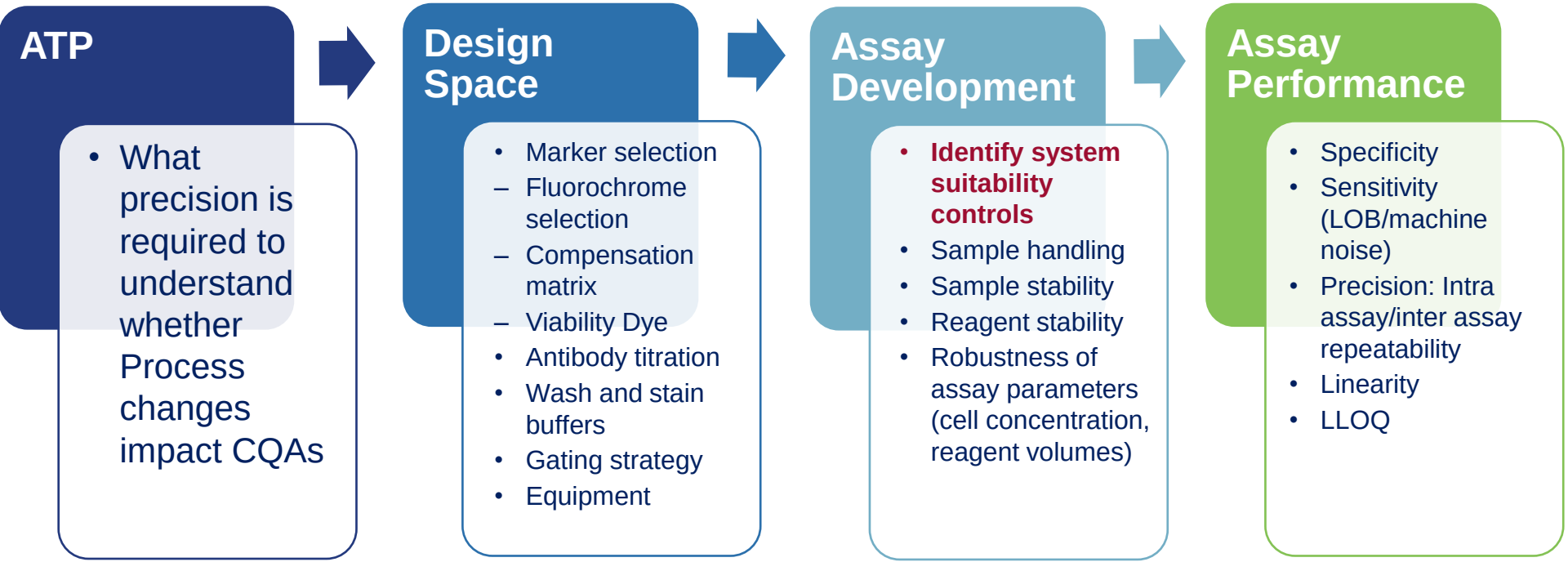
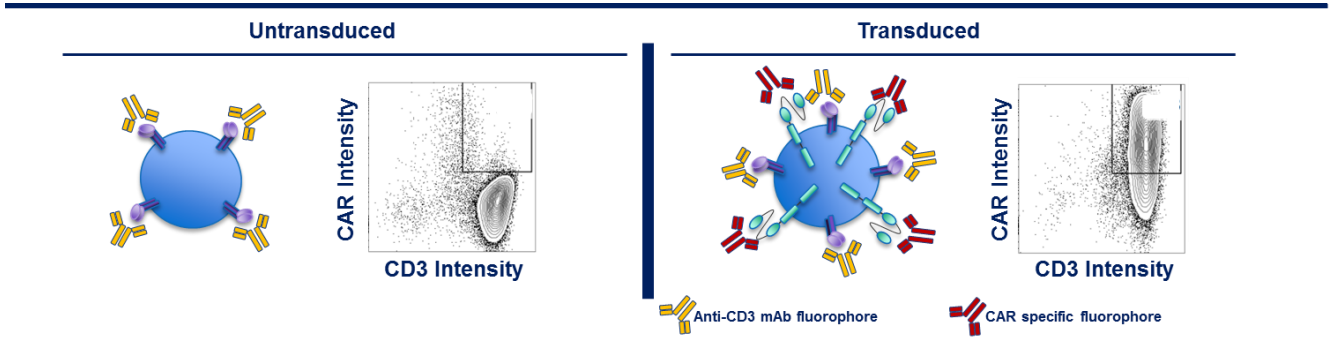
A QbD Approach to Flow Cytometry Analytical Method Development



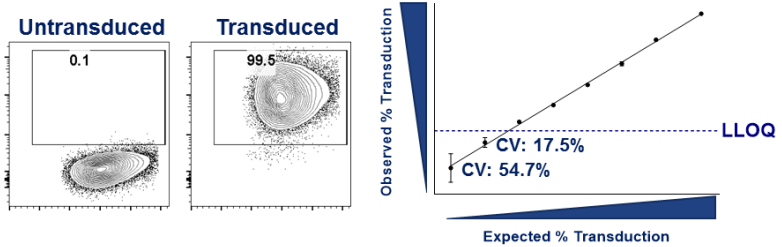
Flow Cytometry Quantitates % T Cells Expressing The CAR:
% CAR T Cells x Total Number Of Viable Cells Enables Dosing

Flow Cytometry methods can be validated for commercial lot release

- Specificity
- Linearity
- Range: LOQ – 100%
- Accuracy by dilutional linearity
- Precision
- Robust (identify critical method parameters)
- Assay validity control: establish 100% sorted, single cell expanded cell banks then make various blends



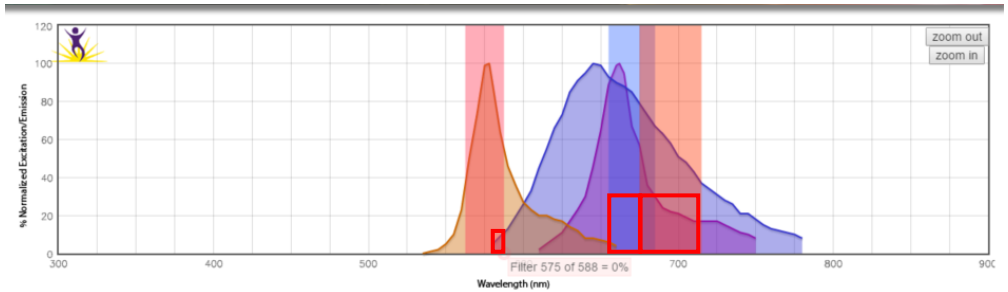
- Linearity and accuracy can be assessed by mixing sorted transduced and untransduced cells
- LOQ can be assessed by ~ N x SD and background (untransduced cells)



Considerations for Flow Cytometry Based Methods

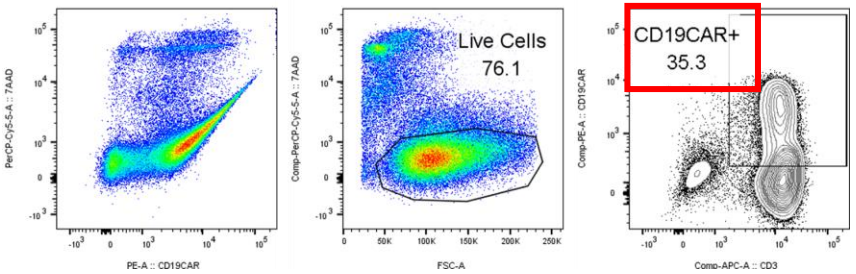
Choose antibody-fluorophore combinations to reduce spectral overlap

Research Method

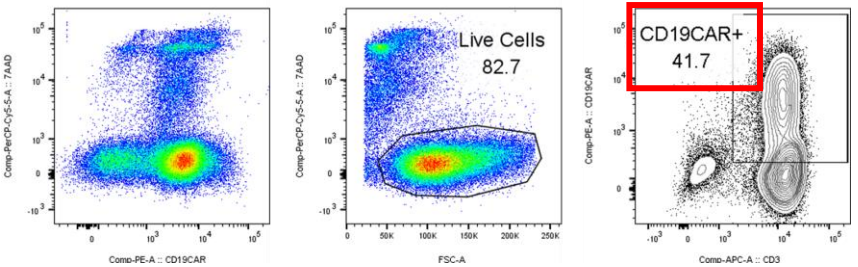


- Poorly compensated data by inexperienced operators will increase day-to-day variability, increase invalid rates and potentially increase OOS

Poor Compensation



Corrected Compensation



Considerations for Flow Cytometry Based Methods

Choose antibody-fluorophore combinations to reduce spectral overlap

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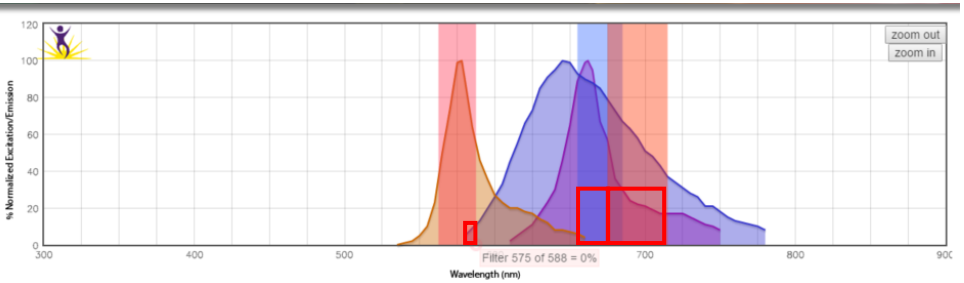
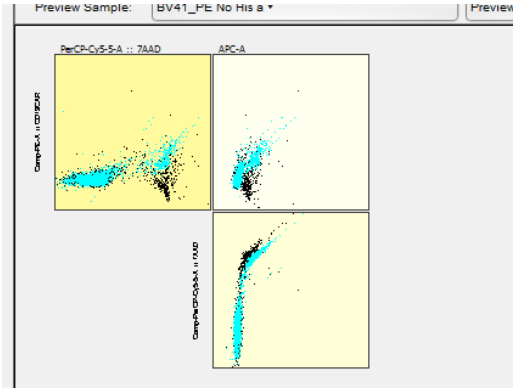
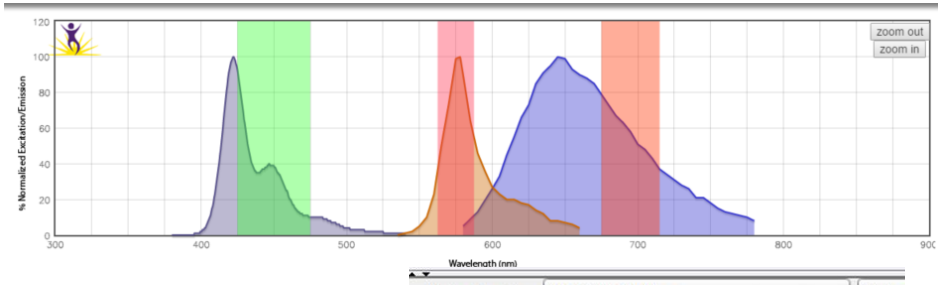
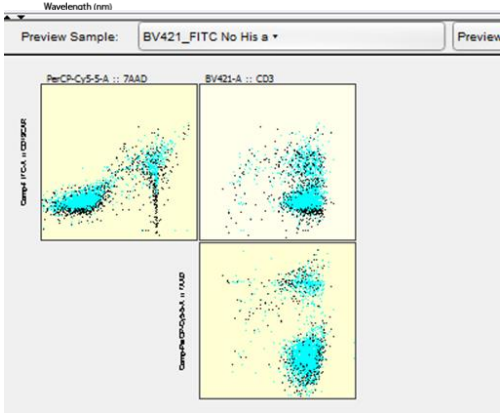




Analytical Optimized Method

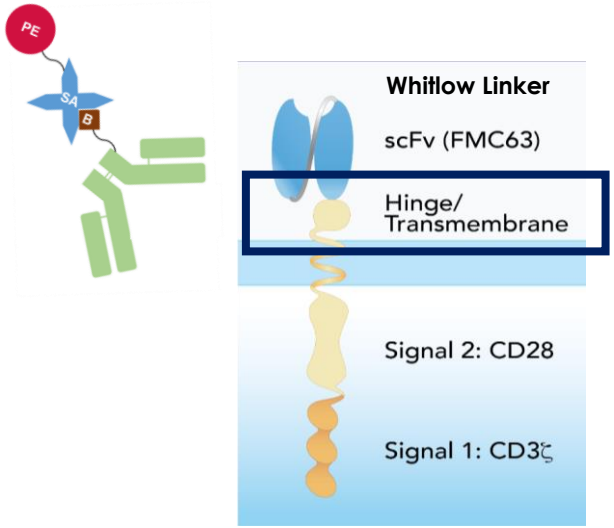




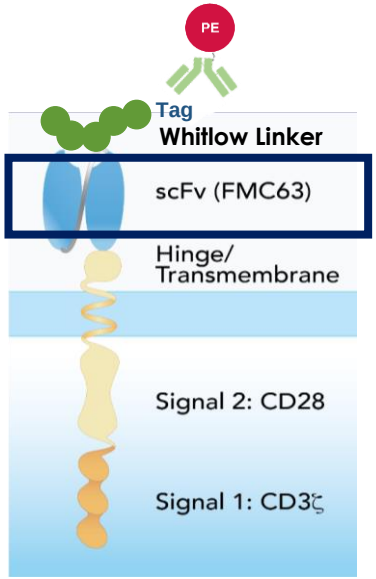
- Keep release methods simple (≤ 5 colors)
- Take advantage of each laser to reduce spectral overlap and eliminate the need for compensation

Orthogonal Percent Transduction Analytical Methods
Each Detects a Different Part of the CAR

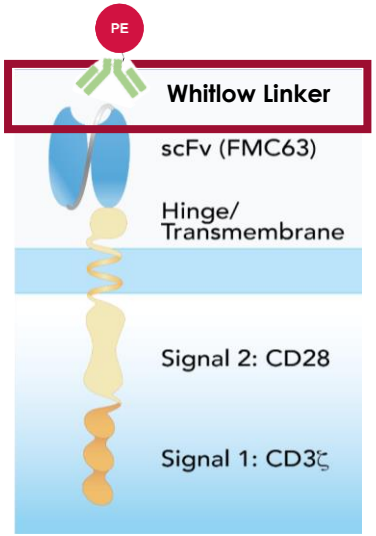
Phase I "Fab"



Phase II/Commercial rhCD19-Tag

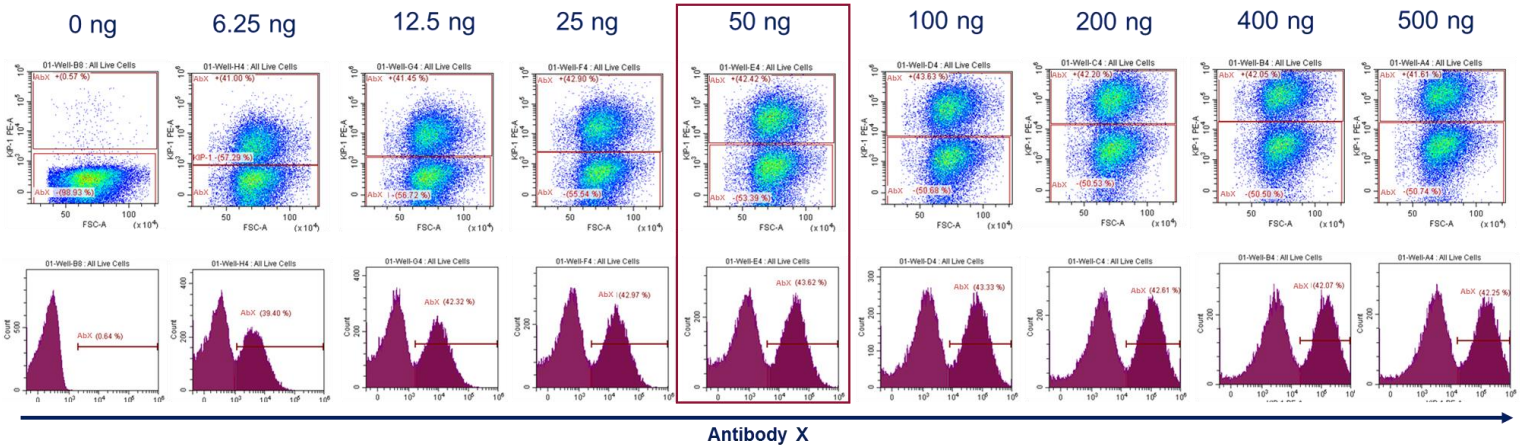


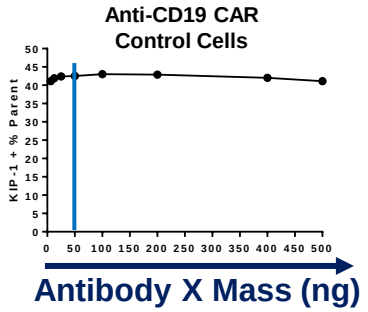
Characterization "Antibody X"

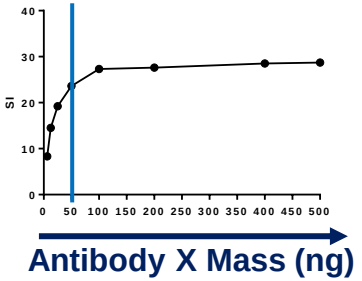


Antibody X Titration Reveals First Surprise

Negative population shifts with positive population

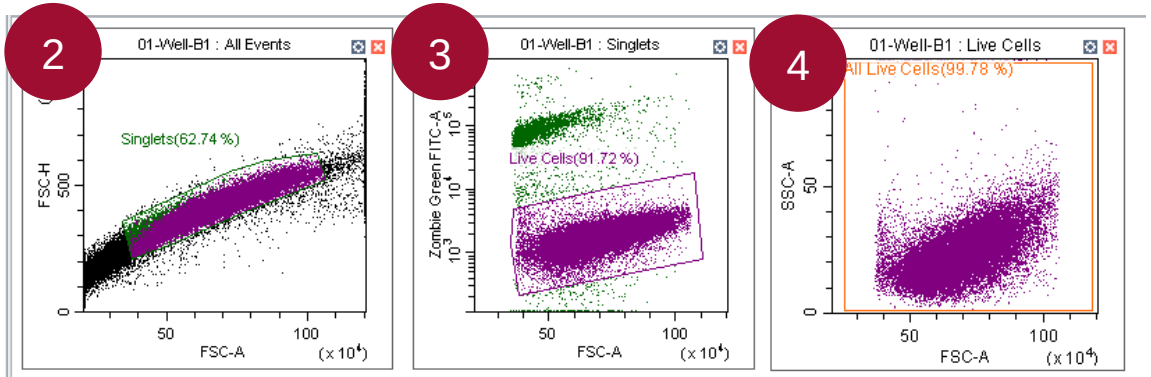


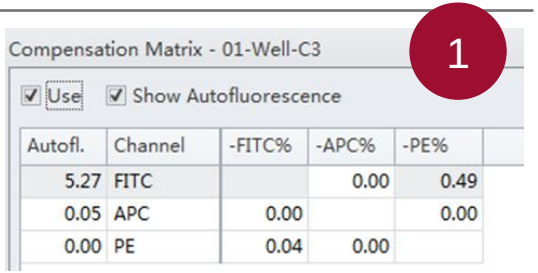




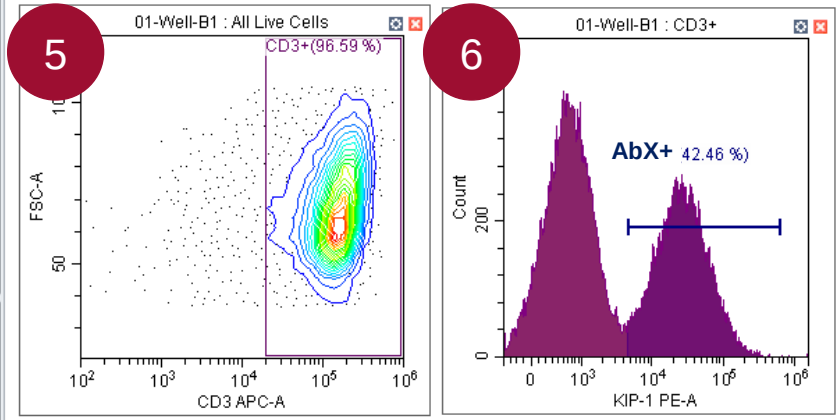
FMO / Isotype gating is not appropriate for this kind of antibody

New Chzn Method Gating Strategy Includes Autogating Feature for Reduced Subjectivity





Autofl.	Channel	-FITC%	-APC%	-PE%
5.27	FITC		0.00	0.49
0.05	APC	0.00		0.00
0.00	PE	0.04	0.00	



1. Choose fluorochrome-laser configuration to reduce spectral overlap
2. Remove doublets
3. Analyze live cells
4. Remove autofluorescence
5. Gate on T cells (be careful!)
6. Autogate algorithm identifies bimodal distribution
   - *Not showing extensive algorithm development in Phase 2 of method development*

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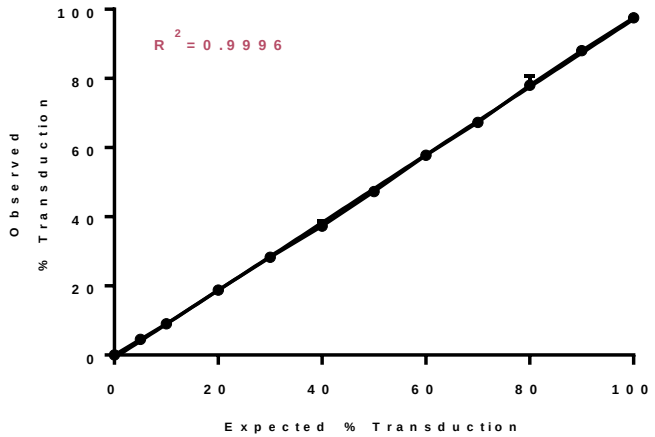
The New Chzn Method is Linear: 4 Independent Assays by 3 Analysts and 2 Flow Cytometers



SUMMARY OF LINEARITY

Expected %Td	100% CD19 cells/well	Untransduced cells/well	Observed Percent Transduction					AVG Recovery	AVG CV
			Assay 1	Assay 2	Assay 3	Assay 4	AVG		
			Analyst 1	Analyst 2	Analyst 3	Analyst 2			
			EQT 1	EQT 1	EQT 2	EQT 2			
100%	1.0×10^6	0	97%	97%	98%	98%	97%	97%	5.65%
90%	0.9×10^6	0.1×10^6	87%	87%	88%	90%	88%	98%	0.42%
80%	0.8×10^6	0.2×10^6	76%	76%	78%	82%	78%	97%	1.33%
70%	0.7×10^6	0.3×10^6	67%	66%	69%	67%	67%	96%	2.18%
60%	0.6×10^6	0.4×10^6	57%	57%	59%	58%	58%	96%	2.04%
50%	0.5×10^6	0.5×10^6	47%	46%	48%	48%	47%	95%	2.47%
40%	0.4×10^6	0.6×10^6	36%	36%	39%	38%	37%	93%	4.20%
30%	0.3×10^6	0.7×10^6	28%	28%	29%	28%	28%	94%	2.29%
20%	0.2×10^6	0.8×10^6	18%	19%	20%	18%	19%	94%	5.32%
10%	0.1×10^6	0.9×10^6	9%	9%	9%	9%	9%	91%	2.11%
5%	0.05×10^6	0.95×10^6	5%	4%	5%	4%	4%	88%	11.46%
0%	0	1.0×10^6	0	0	0	0	0%	NA	NA

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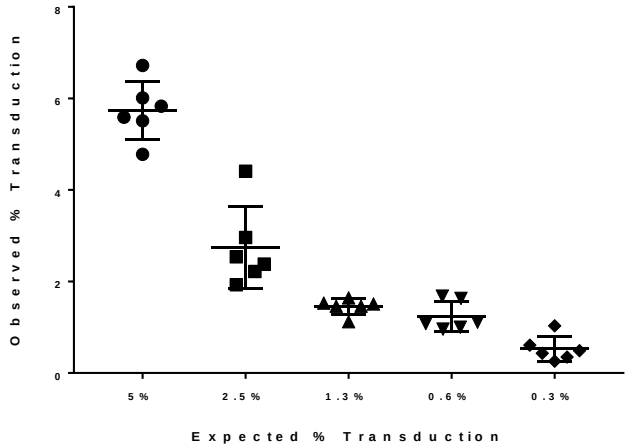
The New Chzn Method LLOQ is 5%: The Limitation Stems from the Autogate Algorithm*

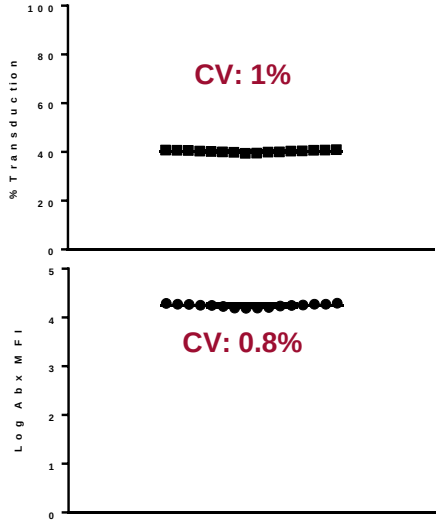


Expected %Td N=6 Replicates	Number 100% cells/well	Number Utd cells/well	Observed %Td	%Recovery	%CV
5%	0.25×10^5	4.75×10^5	5.7%	114%	18%
2.5%	0.125×10^5	4.88×10^5	2.7%	110%	32%
1.25%	0.0625×10^5	4.94×10^5	1.5%	116%	12%
0.63%	0.0315×10^5	4.97×10^5	1.2%	197%	26%
0.31%	0.0155×10^5	4.98×10^5	0.5%	171%	52%

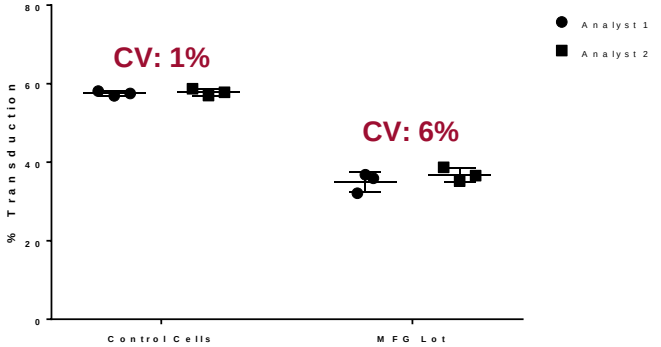
** Phase 1 algorithm*

The New Chzn Method is Highly Repeatable
Intra-plate precision ≤ 1%, Inter-plate precision ≤ 6%

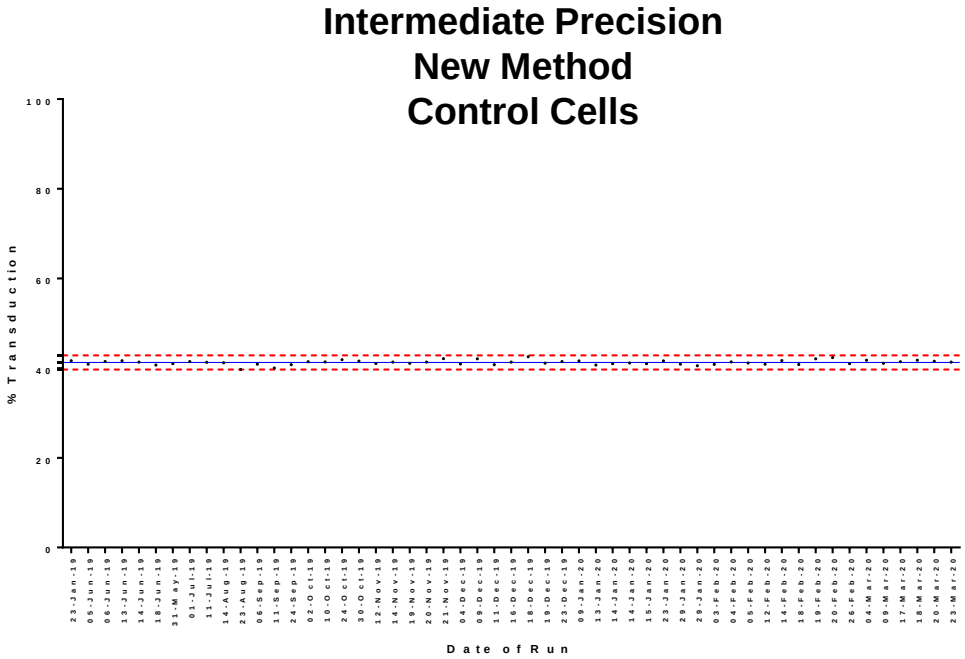
- **Repeatability: intra-plate**
  - One control sample was plated into 16 wells of one plate, stained and acquired



- **Repeatability: inter-sample, inter-plate**
  - Two analysts assayed three independent vials of control cells and three independent vials of one MFG lot one on day



Intermediate Precision of the New Chzn Method
>1 year in Analytical Services: ~ 1% CV, 0 Invalids



Control Cells (Range: 39.7% – 42.8%)	
Number of values	53
Minimum	40%
Maximum	43%
Mean	**41%**
Std. Deviation	0.5%
%CV	**1.3%**

Time Spent Assessing and Controlling Sources of Variability Pay Off in Robust Assay Performance

- Sample handling pre-stain for increased antigen stability
- Cell seeding density
- Antibody mass
- Mass, staining volume, incubation time, temperature
- Antibody X stability
- Viability dye
- Dilution, staining volume, incubation time, temperature
- Number of wash steps
- Staining buffer
- Post-staining hold time, temperature

Method Parameter	Acceptance Criteria
LOQ	5%
Dilutional Linearity	$R^2 > 0.9$
Accuracy (% Recovery)	80%-120%
Repeatability	<10% CV
Intermediate Precision	<10% CV

All performance parameters met commercial method's validation criteria

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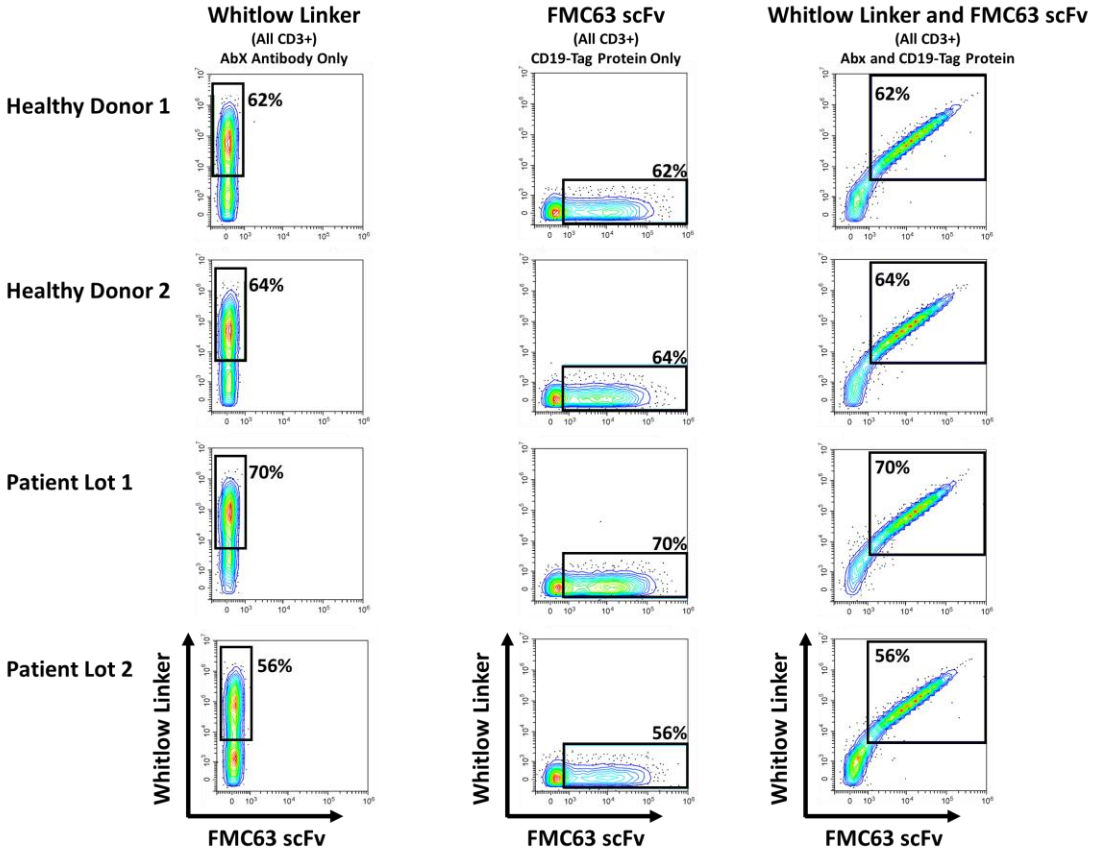
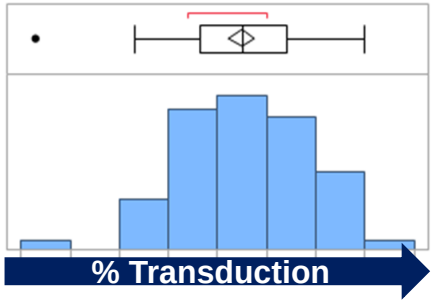
Are the Two Method Orthogonal?
Yes, Antibody X Co-stains with rhCD19

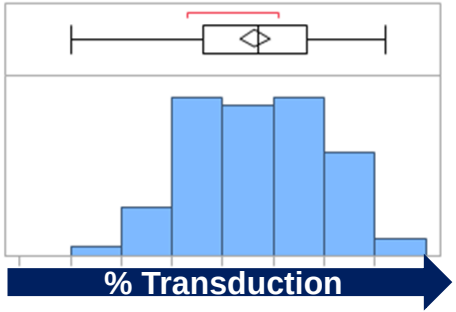


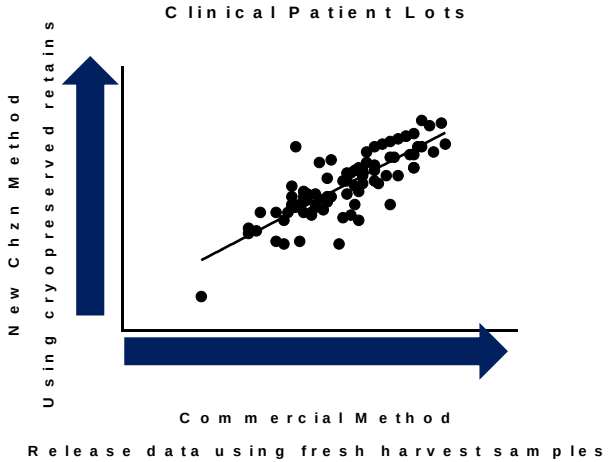
Experiments in Analytical Development Show Promise for Equivalence

New Chzn Method



Commercial Method





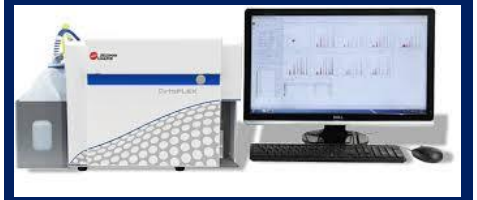
Correlation and Linear Regression	
Pearson r	0.818
R squared	0.6691
P(two-tailed)	<0.0001
Significant? (alpha=0.05)	Yes
Number of XY Pairs	82

Considerations for Flow Cytometry Based Methods

- Platform, platform, platform!
  - Assay Conditions
- Gating strategy and gating controls
- Assay validity and method monitoring controls
- Acquisition EQT: digital with 21 CFR part 11 compliant software
- Automation friendly (assay and analysis)
- Your clinical method will likely be more complex than your commercial method – to support equivalence, plan your studies early
- Increase speed from pipeline to IND
- Improved method understanding, qualification/validation strategies

High Throughput Flow Cytometry

- **Automated acquisition and analysis**
- **21 CFR Part 11 Compliant**
- **Small footprint**
- **Robust and Repeatable**
- **Easy to execute in QC**



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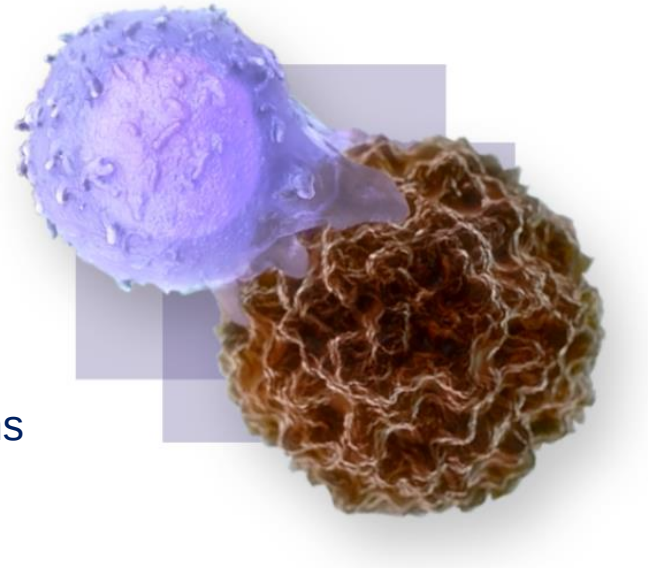
Conclusions

- Engineered cell therapies are still very new modalities, have complex modes of action and large inherent sources of variabilities
- Using a QbD approach to Process and Method development ensures the focus is on Product Quality from the beginning
- Flow cytometry is a relatively new analytical tool that can be used to determine transgene expression for lot release
  - A well planned eATP will help to identify the right controls ensuring confidence in data through early and late phase
  - Late phase method/ATP changes can impact specifications set during pivotal and require extensive bridging/comparability studies
  - Platform early and keep methods simple for lot release



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Acknowledgements

- The patients, family, friends, and caregivers
- The Process Development Department under the leadership of Vijay Chiruvolu
- The Analytical Development Group under the leadership of Max Tejada
- Aparna Subramanian, Iska Morales and Nick Rice (Bioassay)
- Stuart Sievers and Arianne Perez (Cell Biology, Research)

Questions?


May your ligands bind, sequences amplify and proteins fluoresce
-Dream Team 2016