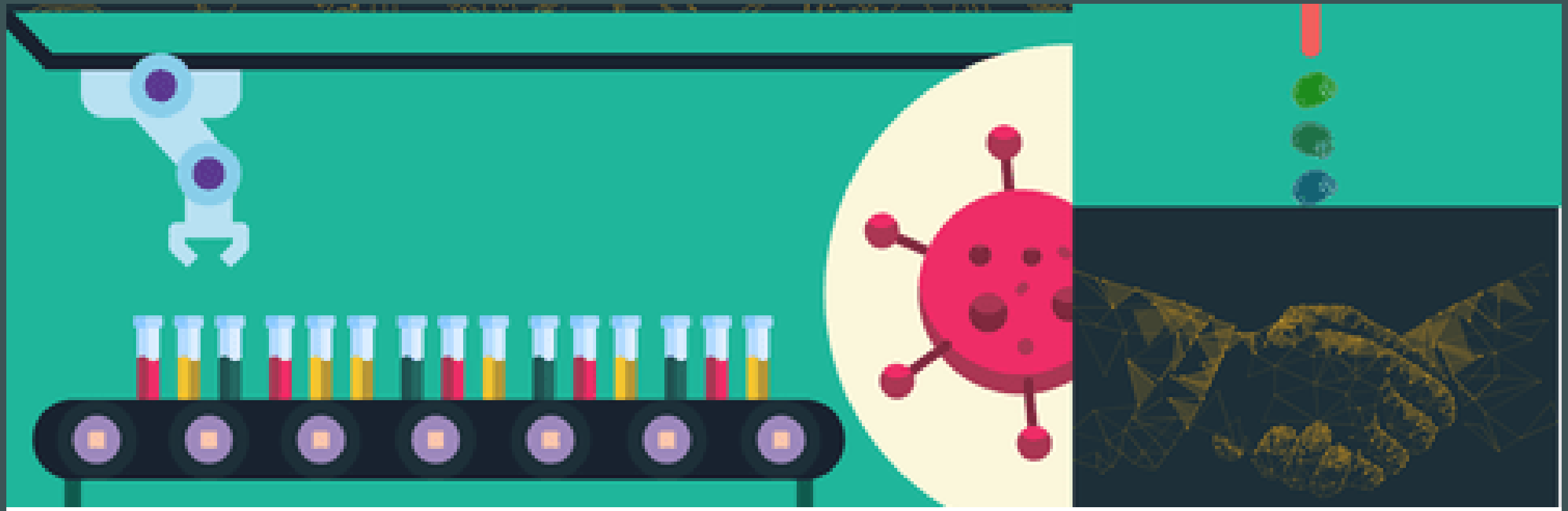


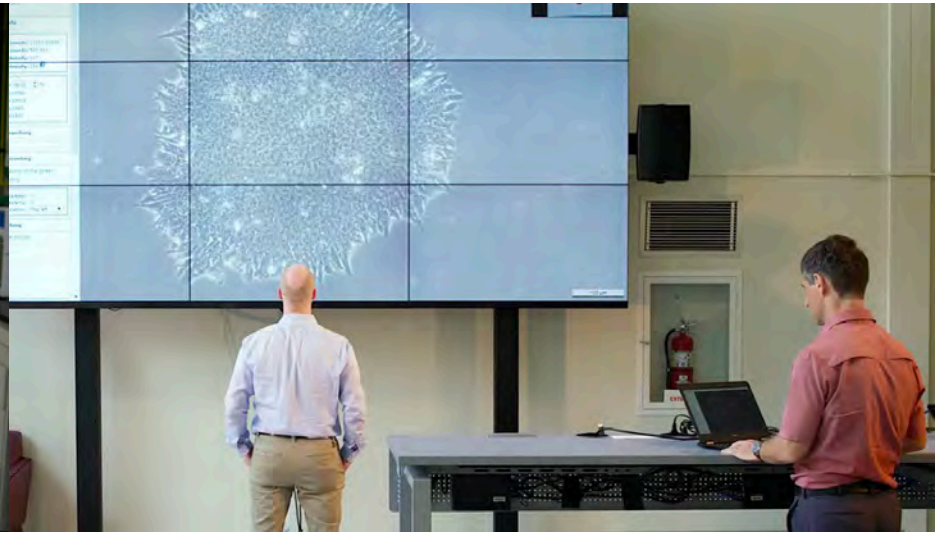
Welcome and NIST Overview

Sheng Lin-Gibson
Chief, NIST Biosystems and Biomaterials Division

Feb 16, 2021

NIST Mission

To promote U.S. innovation and industrial competitiveness by advancing measurement science, standards, and technology in ways that enhance economic security and improve our quality of life



Industries of the Future: Biotechnology

https://www.nist.gov/speech-testimony/industries-future

Building the Next Generation Biometrology and Engineering Biology Capabilities to support U.S. Biotechnology Enterprise and Bioeconomy

Living Measurement Systems Foundry

Advanced Biometrology

Unprecedented measurement capabilities to quantify complex *living* systems and processes

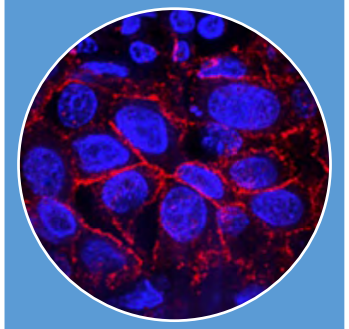
Design-Build-Test-Learn

Tools, platforms, and data/knowledge to predictively engineer biological systems to accelerate innovation in R&D and to advance biomanufacturing

Standards

Standards and related infrastructure to accelerate technology development and translation/clinical use

Advanced Biometrology: Core Measurement Capabilities



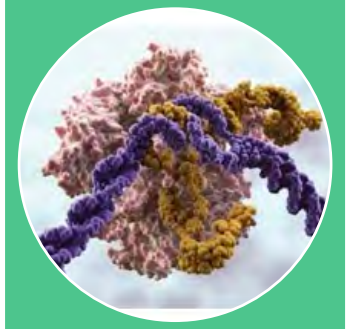
qPCR/dPCR

Next-Gen sequencing

Transcriptome

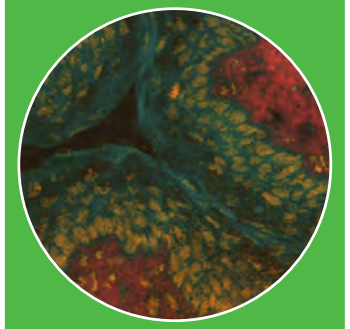
Proteome

Metabolome

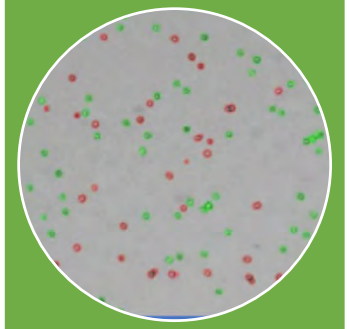


Assays for genome editing

Flow cytometry

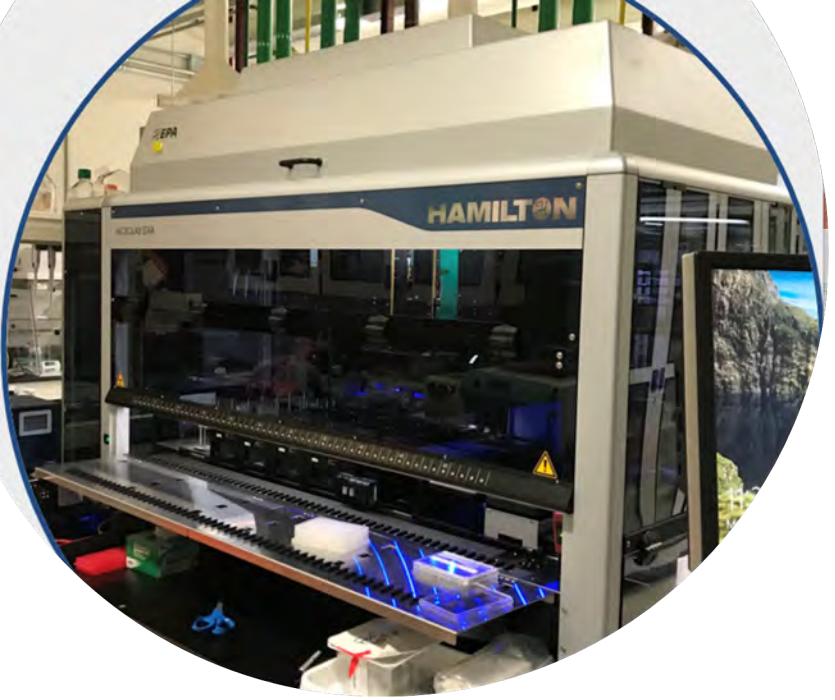


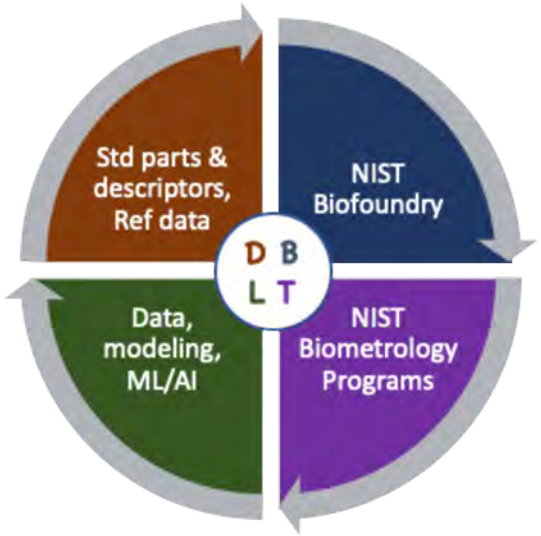
Quantitative spectroscopy, microscopy, & imaging



Cell count, viability and function/fitness

Measurement Assurance



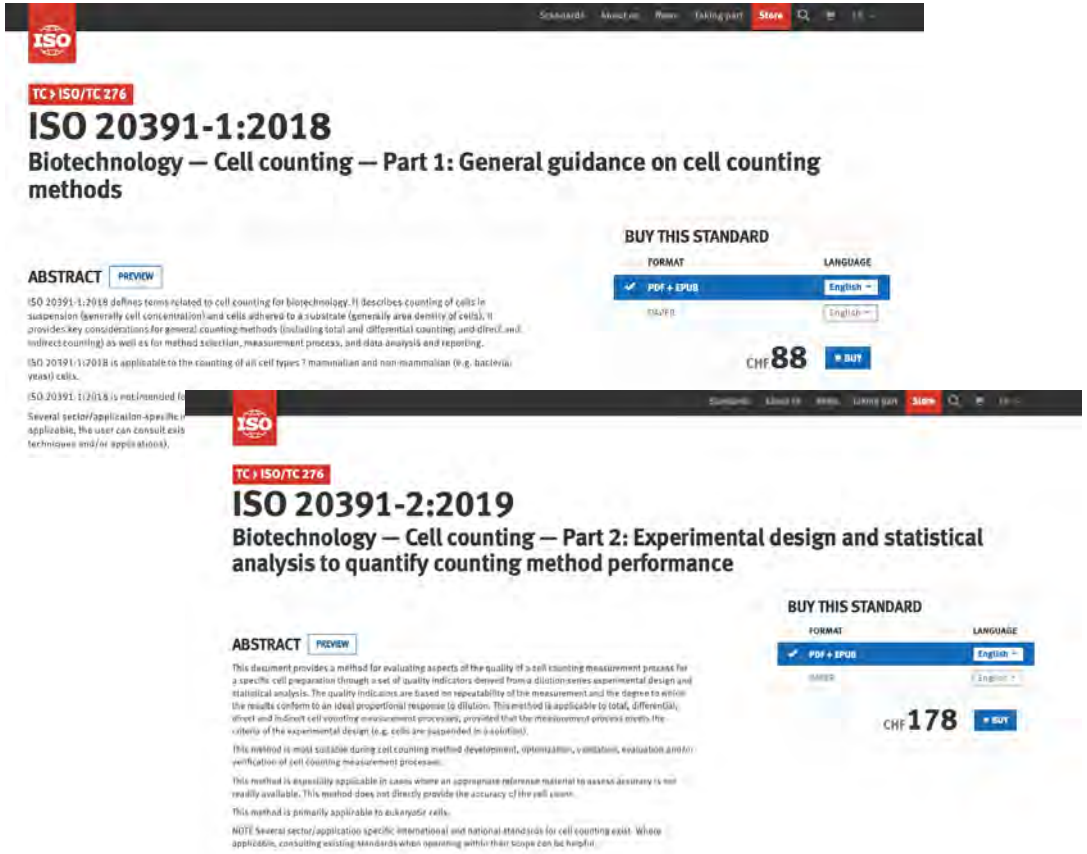


NIST promotes engineering biology and bioeconomy through advances in well-defined design-build-test-learn (DBTL) cycle for biological systems.

Specifically, we aim to

- demonstrate standardized parts and descriptors, reference data, and design principles to facilitate "**predictive design**;"
- accelerate "**build**" including biomanufacturing through NIST biofoundries;
- support "**test**" through the NIST biometrology programs;
- and "**learn**" including process improvements via state-of-the-art data sciences, including ML/AI efforts.

Standards to Accelerate R&D, Product Translation, and Commercialization of Biotechnology Products



Documentary Standards: can specify definition of terms; classification of components; delineation of procedures; specifications; test methods and sampling procedures; or descriptions of fit and measurements of size or strength

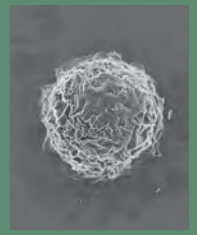
Reference Materials: Key requirements – Homogeneity and Stability; Fitness-for-purpose

Selected Biological Reference Baterials

Genome in a Bottle (GIAB)*

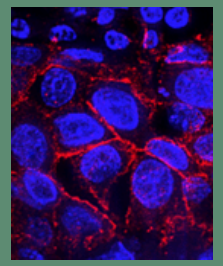
RMs for flow cytometry and imaging measurements*

NISTmAB*



Jurkat cells with different VCNs**

Lentiviral vector**



Fully consented cancer & normal cells

* Currently available

** High value material donation via MTA from an industry leader

NIST-FDA Collaborations on Standards: Leveraging unique expertise

NIST engages in discussions and collaborates with industry and others on pre-competitive technologies

NIST expertise in measurement sciences address specific analytical challenges

FDA scientific and regulatory expertise ensure that standards

- do not conflict with FDA regulation and policy

- address significant regulatory challenges that recur across the field

ELSEVIER

Cytotherapy

Volume 20, Issue 6, June 2018, Pages 779-784

Reports

FDA and NIST collaboration on standards development activities supporting innovation and translation of regenerative medicine products

Judith A. Arcidiacono [1], Steven R. Bauer [1], David S. Kaplan [2], Clare M. Allocca [3], Sumona Sarkar [4], Sheng Lin-Gibson [4]

Show more

https://doi.org/10.1016/j.jcyt.2018.03.039

- Standards Development
- Workshops and Public Meetings
- Research Collaborations

Coordination of Standards Development

- ISO TC/276: *Biotechnology***
- *ASTM F04: Medical and Surgical Materials and Devices*
- *American National Standards Institute (ANSI) accredited SDOs: CLSI, ATCC, PDA*

- *Professional organizations: ISCT, ISSCR*
- *Accreditation bodies: AABB, FACT*
- *ICH, USP, etc.*
- *Industry consortia*

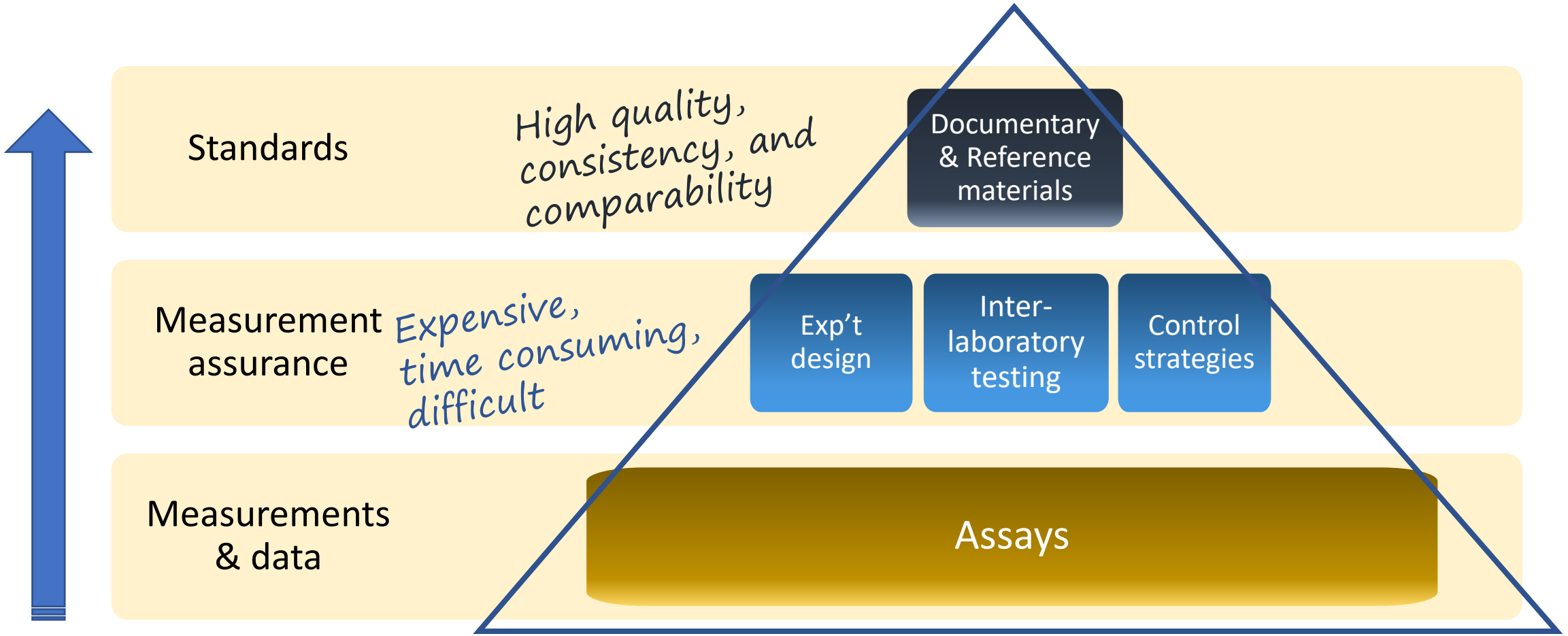
Coordination is required to avoid conflict and duplication

NIST FDA

STANDARDS COORDINATING BODY
REGENERATIVE MEDICINE

** NIST Chairs the Analytical Methods Working Group & the US Mirror Technical Committee

Measurement Assurance



Demonstration of measurement assurance strategies:
https://www.nist.gov/mml/bbd/measurement-assurance-strategies

NIST Consortia

NIST GENOME IN A BOTTLE (GIAB) CONSORTIUM

Provides authoritative characterization of benchmark human genomes

NIST GENOME EDITING CONSORTIUM

Addresses the measurements and standards needed to increase confidence and lower the risk

NIST FLOW CYTOMETRY QUANTITATION CONSORTIUM**

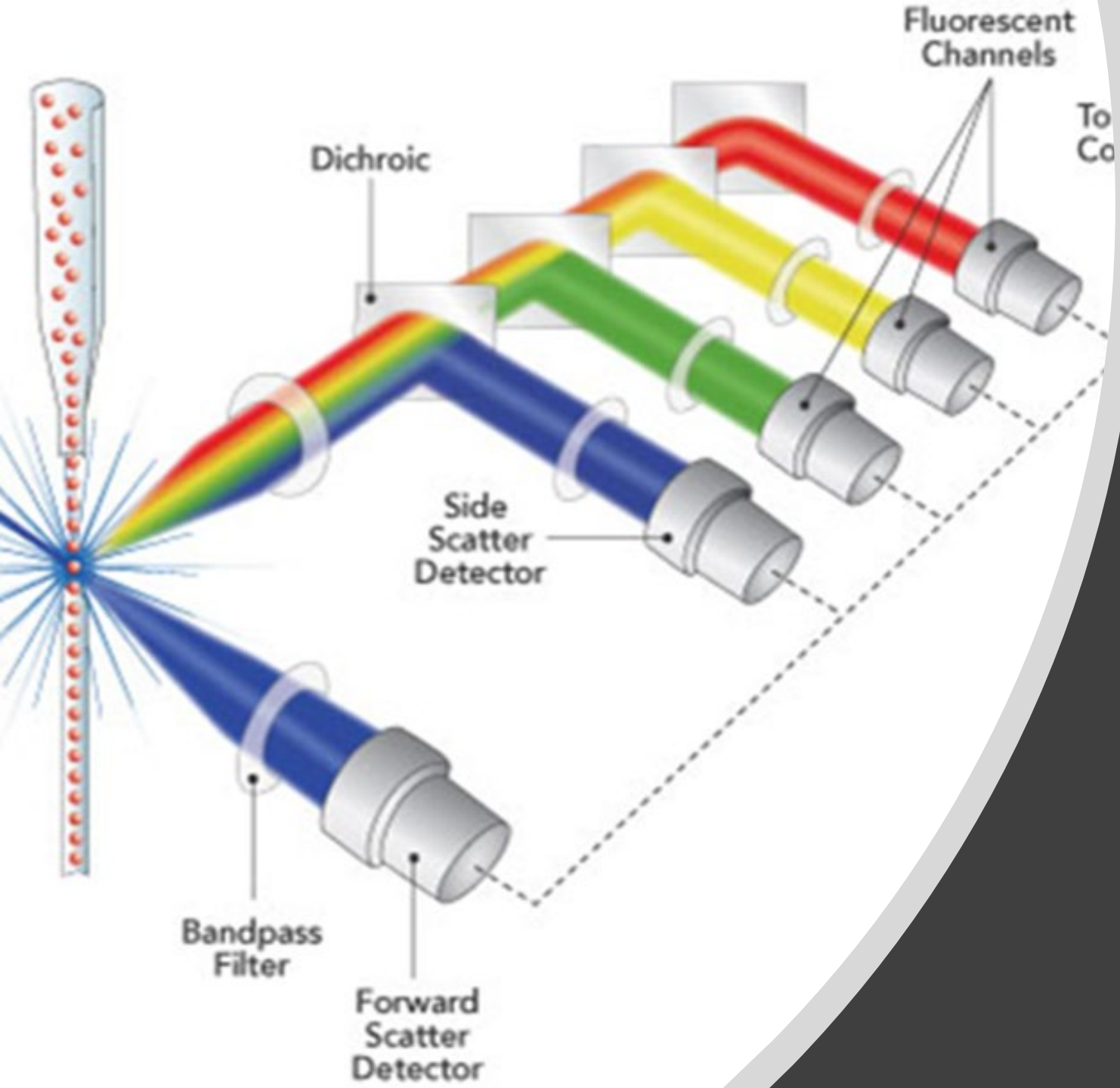
Accelerates the adoption of quantitative flow cytometry in biomanufacturing

NIST RAPID MICROBIAL TESTING METHODS CONSORTIUM

Addresses the measurements and standards needed to increase confidence and lower the risk

Workshop Goals

- Launch and inform future directions of the Flow Cytometry Standards Consortium.
- State-of-the-art in flow cytometry applications
- Identify pressing measurement challenges and standards needs
- Provide initial feedback on the proposed directions of the Consortium.



NIST Flow Cytometry Program Overview

Paul DeRose, Linhua Tian, Elzafir Elsheikh, Sarah Inwood, Adolfas Gaigalas, Hua-Jun He, and Lili Wang

Biosystems and Biomaterials Division, NIST

Flow Cytometry Standards Consortium

https://www.nist.gov/programs-projects/nist-flow-cytometry-standards-consortium

Quantitative Flow Cytometry Measurements

https://www.nist.gov/programs-projects/quantitative-flow-cytometry-measurements

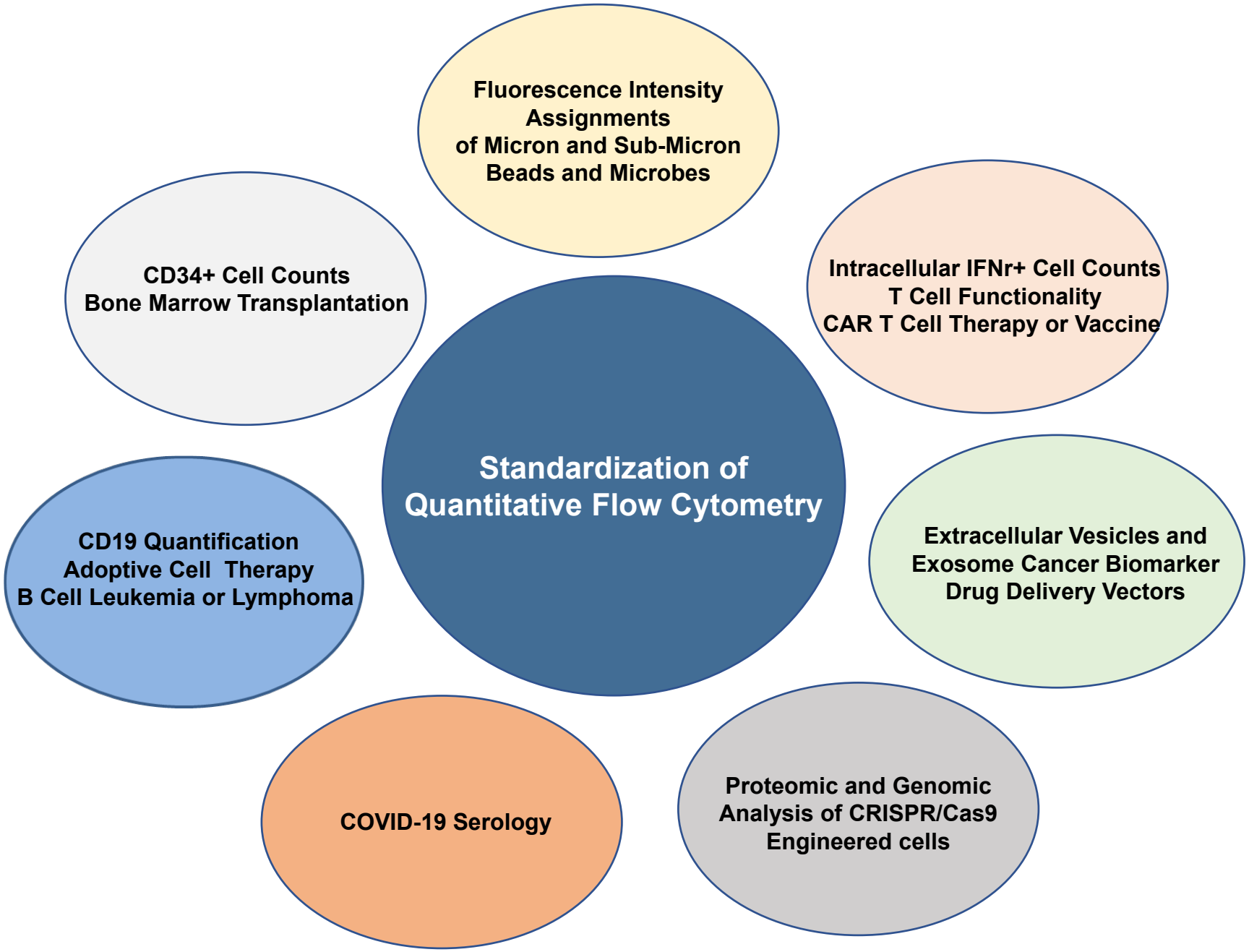
Serology and Neutralization Assays for COVID-19

https://www.nist.gov/programs-projects/serology-and-neutralization-assays-covid-19

NIST Flow Cytometry Lab

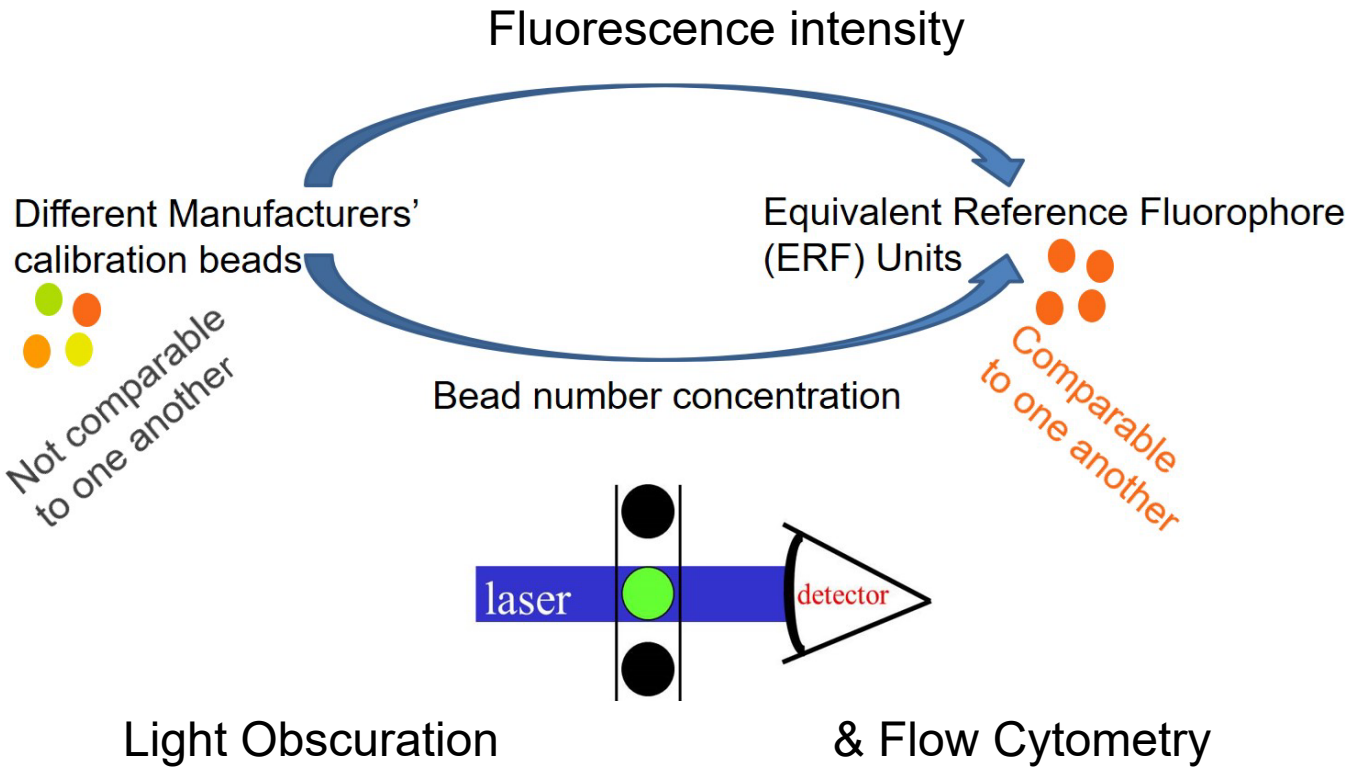
https://www.nist.gov/programs-projects/nist-flow-cytometry-lab

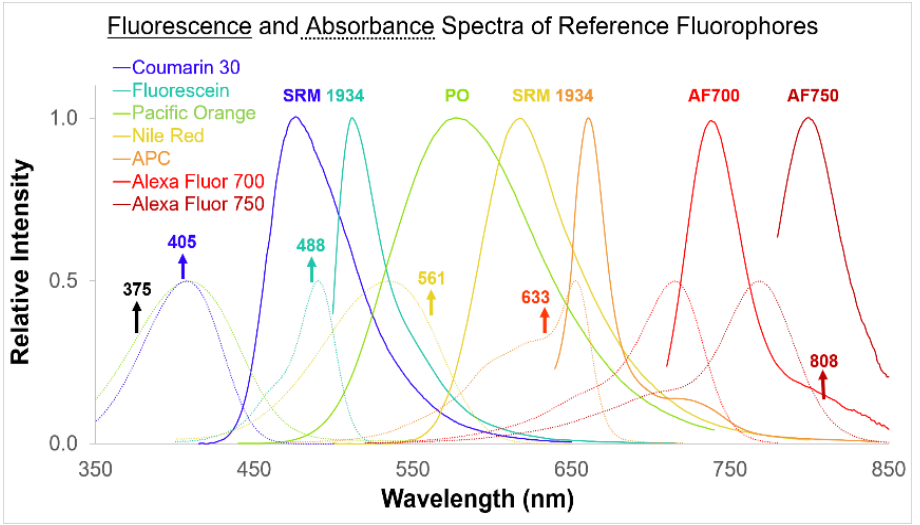
Flow Cytometry Projects



NIST Flow Cytometry Quantitation Consortium

Assignment of ERF Units – SI Traceable Calibration Beads





NIST enables comparable and traceable flow cytometry:

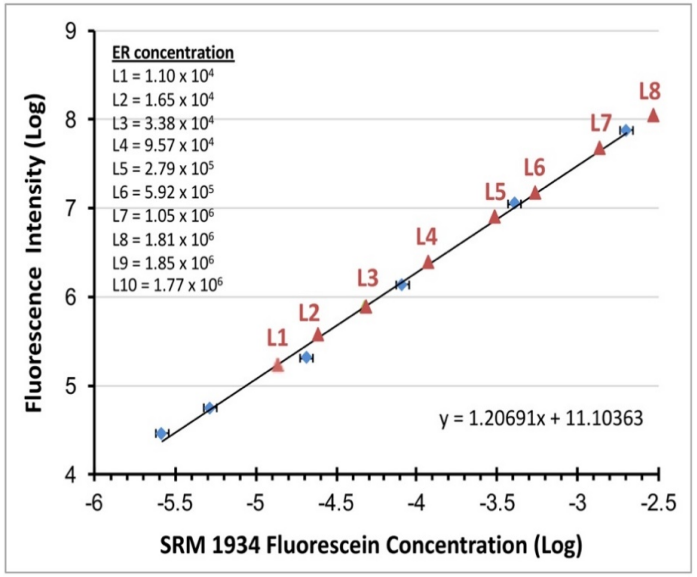
- We provide measurement service to calibrate the fluorescence signal from microsphere/calibration beads in terms of a unit of equivalent number of reference fluorophores (ERF)

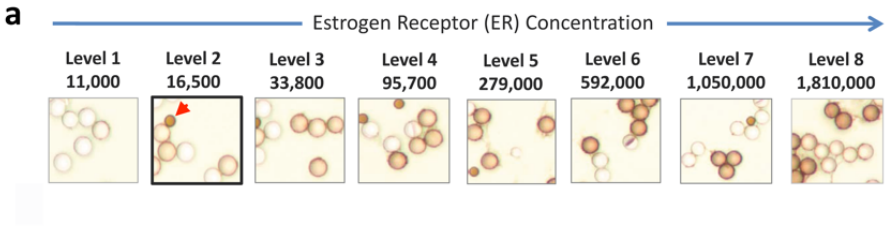
QUANTIFYING THE LIMIT OF DETECTION OF ESTROGEN RECEPTORS USING AN SI-TRACEABLE FLUORESCENCE INTENSITY SCALE

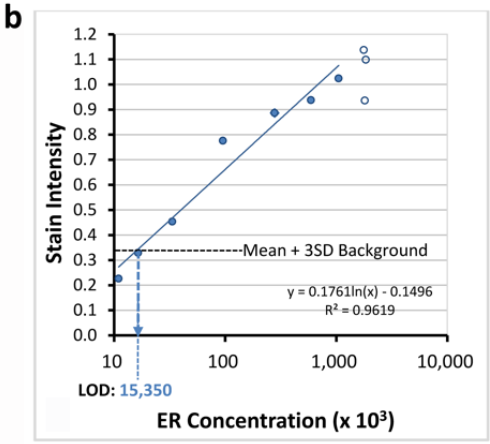
Clinical testing lab

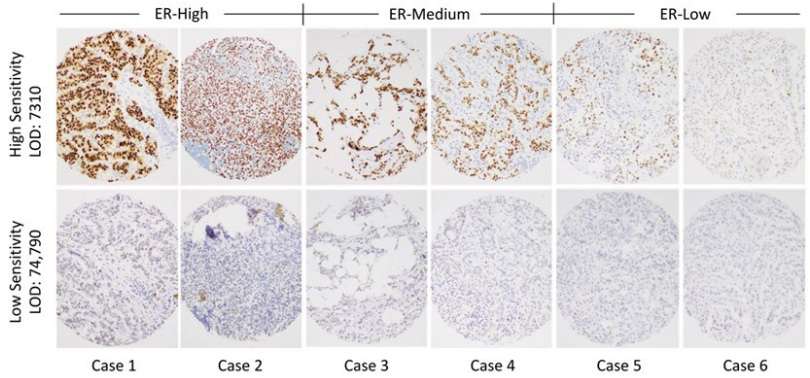
Pathology/IHC

clQc









Clin Chem (in press)

- Use SI-traceable intensity scale to assign estrogen receptor (ER) concentrations to glass bead calibrators
- Calibrating the stain intensity of beads measured on a fluorescence microscope to the ER concentration

REFERENCE MATERIAL 15/272: ENUMERATION OF CYTOKINE POSITIVE T LYMPHOCYTES

Journal of Immunology and Regenerative Medicine 9 (2020) 100032

Contents lists available at ScienceDirect

Journal of Immunology and Regenerative Medicine

journal homepage: www.elsevier.com/locate/regen

An accurate and rapid single step protocol for enumeration of cytokine positive T lymphocytes

Deepa Rajagopal [a], Linhua Tian [b], Shiqiu Xiong [c], Lili Wang [b], Jonathan Campbell [c], Luisa Saraiva [a], Sandrine Vessillier [a,*]

[a] Biotherapeutics Division, National Institute for Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, UK
[b] Biosystems and Biomaterials Division, National Institute of Standards and Technology (NIST), Gaithersburg, MD, 20899, USA
[c] Health Science & Innovation, LGC Ltd., Queens Road, Teddington, Middlesex, TN11 0LY, UK

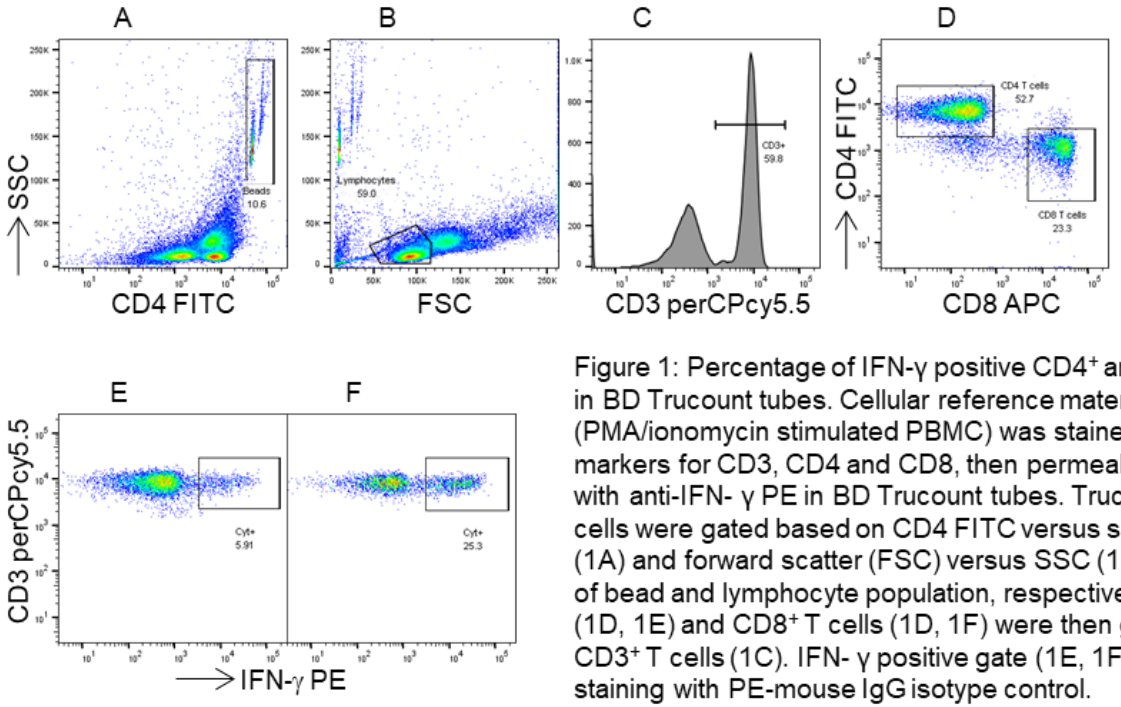


Figure 1: Percentage of IFN-γ positive CD4⁺ and CD8⁺ T cells in BD Trucount tubes. Cellular reference material 15/272 (PMA/ionomycin stimulated PBMC) was stained with surface markers for CD3, CD4 and CD8, then permeabilized and stained with anti-IFN- γ PE in BD Trucount tubes. Trucount beads and cells were gated based on CD4 FITC versus side scatter (SSC) (1A) and forward scatter (FSC) versus SSC (1B) for determination of bead and lymphocyte population, respectively. The CD4⁺ (1D, 1E) and CD8⁺ T cells (1D, 1F) were then gated as subsets of CD3⁺ T cells (1C). IFN- γ positive gate (1E, 1F) was set based on staining with PE-mouse IgG isotype control.

B CELL REFERENCE MATERIAL: ENABLING MEASUREMENTS OF B CELL MARKER CD19, CD20 AND CD22 EXPRESSION LEVELS

Establishing CD19 B-cell Reference Control Materials for Comparable and Quantitative Cytometric Expression Analysis

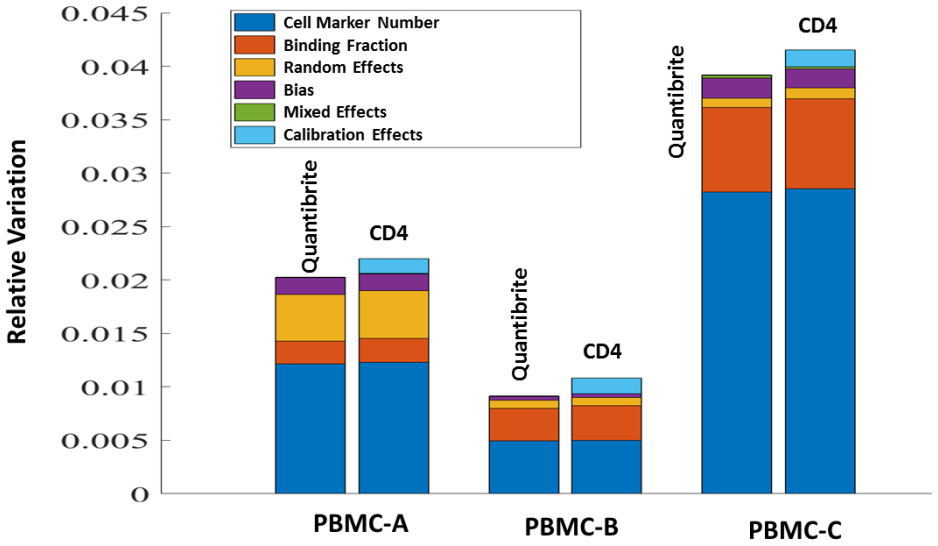
Lili Wang[1*], Rukmini Bhardwaj [2], Howard Mostowski[2], Paul N. Patrone [3], Anthony J. Kearsley[3], Jessica Watson[4], Liang Lim [4], Jothir Pichaandi [4], Olga Ornatsky [4], Daniel Majonis [4], Steven Bauer [2], and Heba Degheidy[2*]

[1] Biosystems and Biomaterials Division, National Institute of Standards and Technology (NIST), Gaithersburg, MD,

[2] Office of Tissues and Advanced Therapies, Center for Biologics Evaluation and Research, Food and Drug Administration (FDA), Silver Spring, MD,

[3] Applied and Computational Mathematics Division, NIST, Gaithersburg, MD,

[4] Fluidigm Canada, 1380 Rodick Road, Markham ON, L3R 4G5, Canada



Analysis of Cross Platform, Inter-Laboratory, Intra-Laboratory, and Antibody Lot-to-Lot Variability in Flow Cytometric Quantification of Antigen Expression and Development of Reference Controls for Across Instrument Standardization

Aaron R. Nelson*, Linhua Tian^, Linda S. Weaver*, Hao-Wei Wang*, Constance M. Yuan*, Lili Wang^, Maryalice Stetler-Stevenson*

*Laboratory of Pathology, NCI, NIH, Bethesda, MD. ^Biosystems and Biomaterials, NIST, Gaithersburg MD

QUANTITATIVE MEASUREMENT OF EXTRACELLULAR VESICLES

JOURNAL OF EXTRACELLULAR VESICLES
2020, VOL. 9, 1816641
https://doi.org/10.1080/20013078.2020.1816641

Taylor & Francis
Taylor & Francis Group

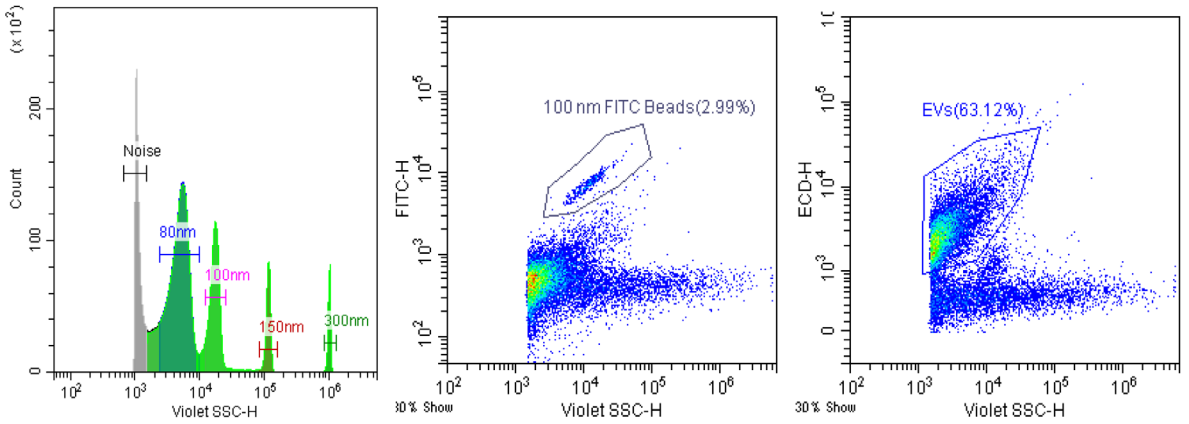
REVIEW ARTICLE

OPEN ACCESS Check for updates

Towards defining reference materials for measuring extracellular vesicle refractive index, epitope abundance, size and concentration

Joshua A. Welsh[a], Edwin van der Pol[b,c,d], Britta A. Bettin[b,d], David R. F. Carter[e], An Hendrix[f,g], Metka Lenassi[h], Marc-André Langlois[i,j,k], Alicia Llorente[l], Arthur S. van de Nes[m], Rienk Nieuwland[b,d], Vera Tang[i,j], Lili Wang[n], Kenneth W. Witwer[o] and Jennifer C. Jones[a]

	Technique	Diameter measurement	Phenotyping	Concentration measurement	Refractive index measurement	Full EV population detection	Quantitative detection limit	High-throughput
Single Particle Detection Assay	Nanoparticle tracking analysis	✓	✓	✓	✓	x	x	x
	Flow cytometry (single EV)	✓	✓	✓	✓	x	✓	✓
	Electron microscopy	✓	✓	x	x	✓	✓	x
	Resistive pulse sensing (coulter)	✓	x	✓	x	x	✓	x
	SP-IRIS (sizing & fluorescence)	✓	✓	✓	x	x	x	x
	Super resolution microscopy	✓	✓	✓	x	✓	✓	x
Bulk Detection Assay	Flow cytometry (bead-assay)	x	✓	x	x	x	✓	x
	Dynamic light scattering	✓	x	x	x	x	x	x
	Refractometer	x	x	x	✓	x	✓	x
	ELISA	x	✓	x	x	x	✓	x
	Western Blot	x	✓	x	x	x	✓	x



Flow Cytometry Quantitation Consortium Members

Stakeholder representative organizations:

NIH National Institutes of Health *Turning Discovery Into Health*

FDA

ISAC International Society for Advancement of Cytometry

ISTH

International Society for Extracellular Vesicles

Calibration bead vendors:

BD

Beckman Coulter

ThermoFisher Scientific

Bangs Laboratories, Inc. BEADS ABOVE THE REST

Spherotech Let the Possibilities Flow

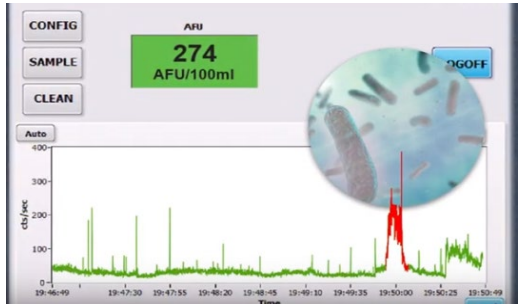
BCS Boston Cell Standards LLC

Other stakeholders:

Clinical testing labs

clQc

BioPharma – Water
>16 BioPharmas



Quantitative FC Applications

BioLegend®

FLUIDIGM

NIBSC Confidence in Biological Medicines

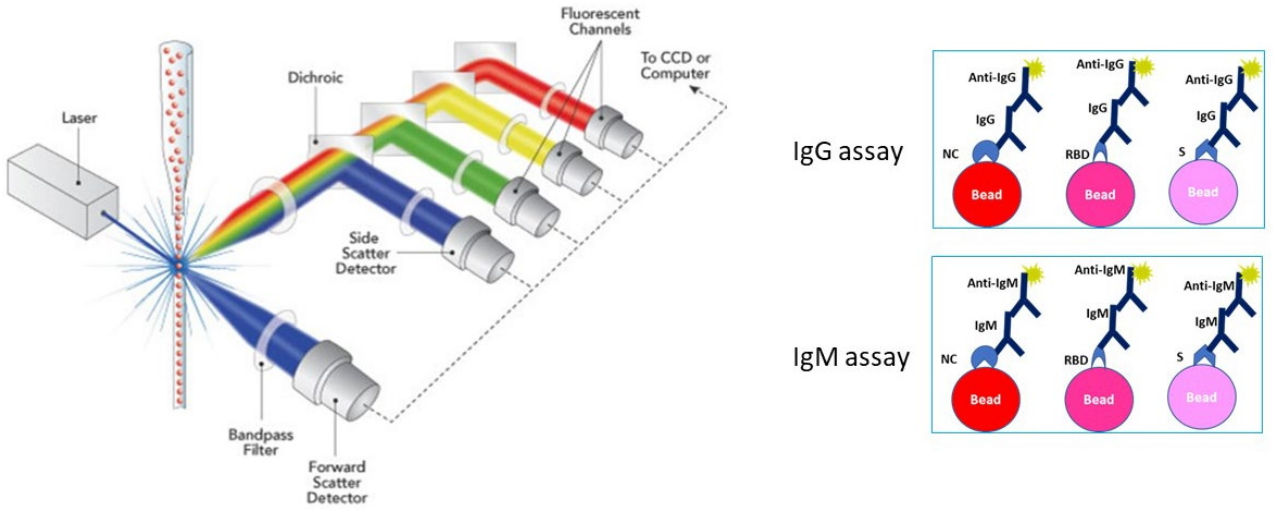
LGC

Documentary standards:

- **CLSI H62 Guidance Document-Validation of Assays Performed by FC**
- **USP Chapter <127> Flow Cytometric Enumeration of CD34+ Cells**

COVID-19: Serology

Multiplexed Flow Cytometry Serology Assays

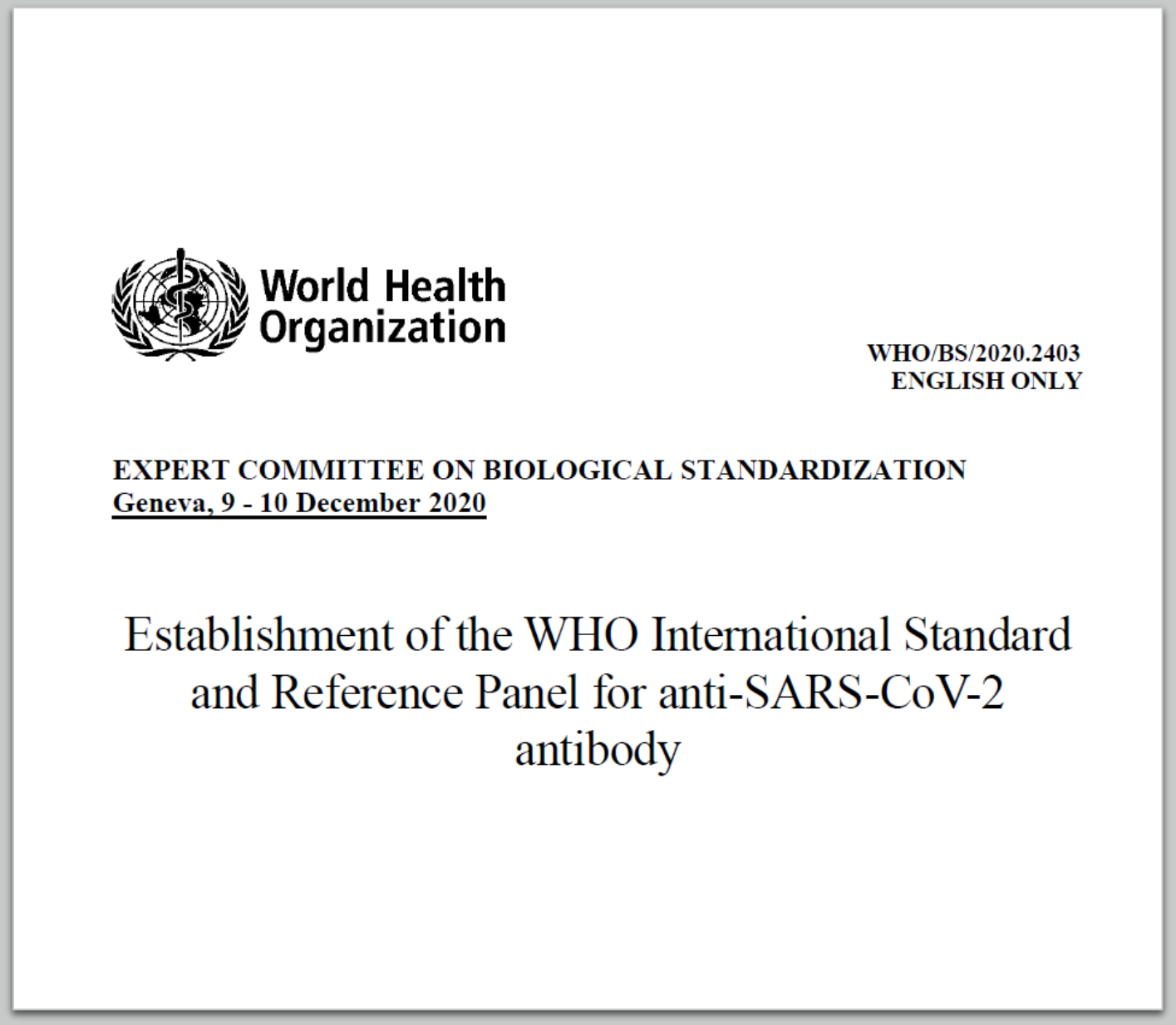


Quantitative and robust serology assays are critical measurements underpinning global COVID-19 response with respect to diagnostic, surveillance, and vaccine development. We have developed quantitative, multiplexed flow cytometry based serological and neutralization assays.

NIST Participation in WHO-NIBSC Serology Study

Develop serological antibody standard

- assess the suitability of different antibody preparations
- characterize the antibody preparations in terms of reactivity/specificity
- assess each preparation's potency and commutability
- recommend to the WHO ECBS, the suitable antibody preparation(s) as the standard



World Health Organization

WHO/BS/2020.2403
ENGLISH ONLY

EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 9 - 10 December 2020

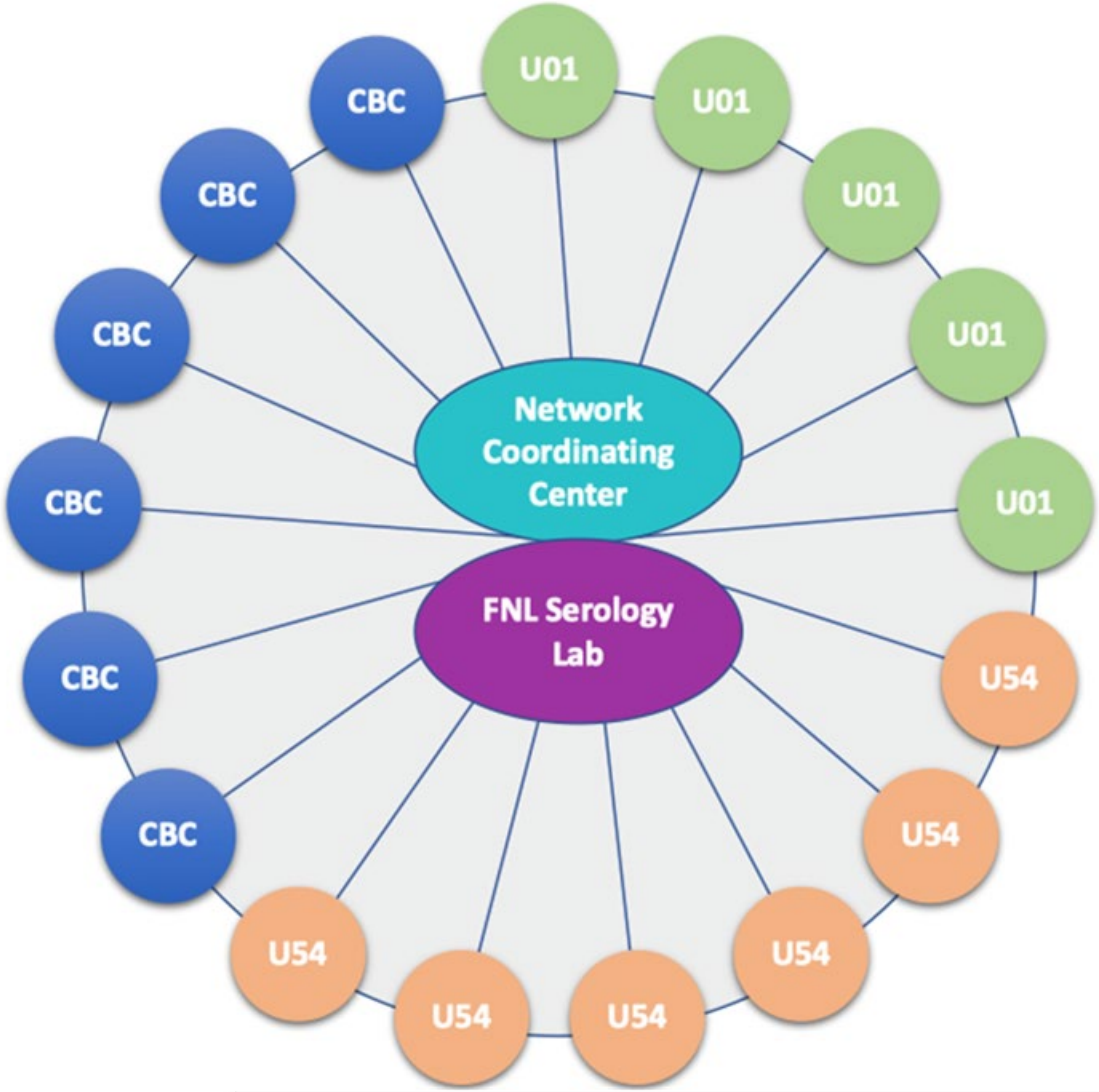
Establishment of the WHO International Standard and Reference Panel for anti-SARS-CoV-2 antibody

NIST Contribution to NCI's Serology Sciences Network

- NIST, CDC, NIAID, and FDA to participate as ancillary partners

- Develop qualified assay standards for the serology community
  - Reference antigens
  - Reference antibodies for assay quantification
  - Positive controls
  - Antibody panels

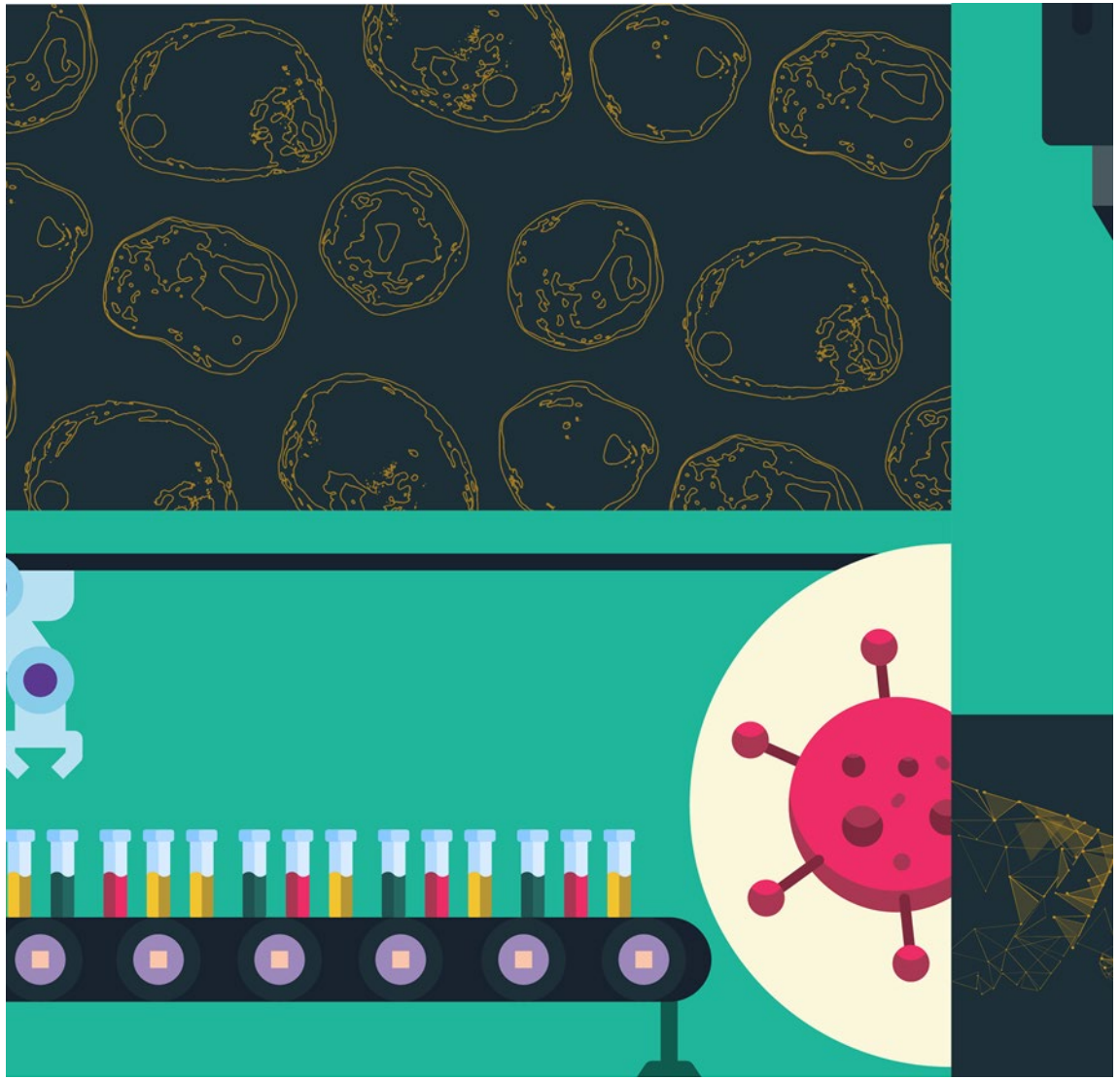
- Help evaluate/validate panel used for FDA approval process



NIST Consortia: Flow Cytometry Standards Consortium

We launched the NIST Flow Cytometry Standards Consortium to accelerate the adoption of quantitative flow cytometry in biomanufacturing of cell and gene therapies.

The consortium will develop standards for flow cytometry applications and reference materials for instrument calibrations.



NIST Consortium: RMTM

The NIST Rapid Microbial Testing Methods (RMTM) Consortium has been established to address the need for measurements and standards, including reference materials, to increase confidence in the use of rapid testing for microbial contaminants in regenerative medicine and advanced therapy products.

https://www.nist.gov/programs-projects/nist-rapid-microbial-testing-methods-consortium

Flow Cytometry Documentary Standards

Virginia Litwin, PhD

NIST Flow Cytometry Standards Consortium Workshop

Virtual Event

February 16-17, 2021

Presentation Overview

- Clinical and Laboratory Standards Institute (CLSI)
- CLSI H62 1st Edition, Validation of Assays Performed by Flow Cytometry
- Gaps and Next Steps
- Resources

CLSI

- Not-for-profit membership organization
- Brings together the global laboratory community for a common cause

  to foster excellence in laboratory medicine

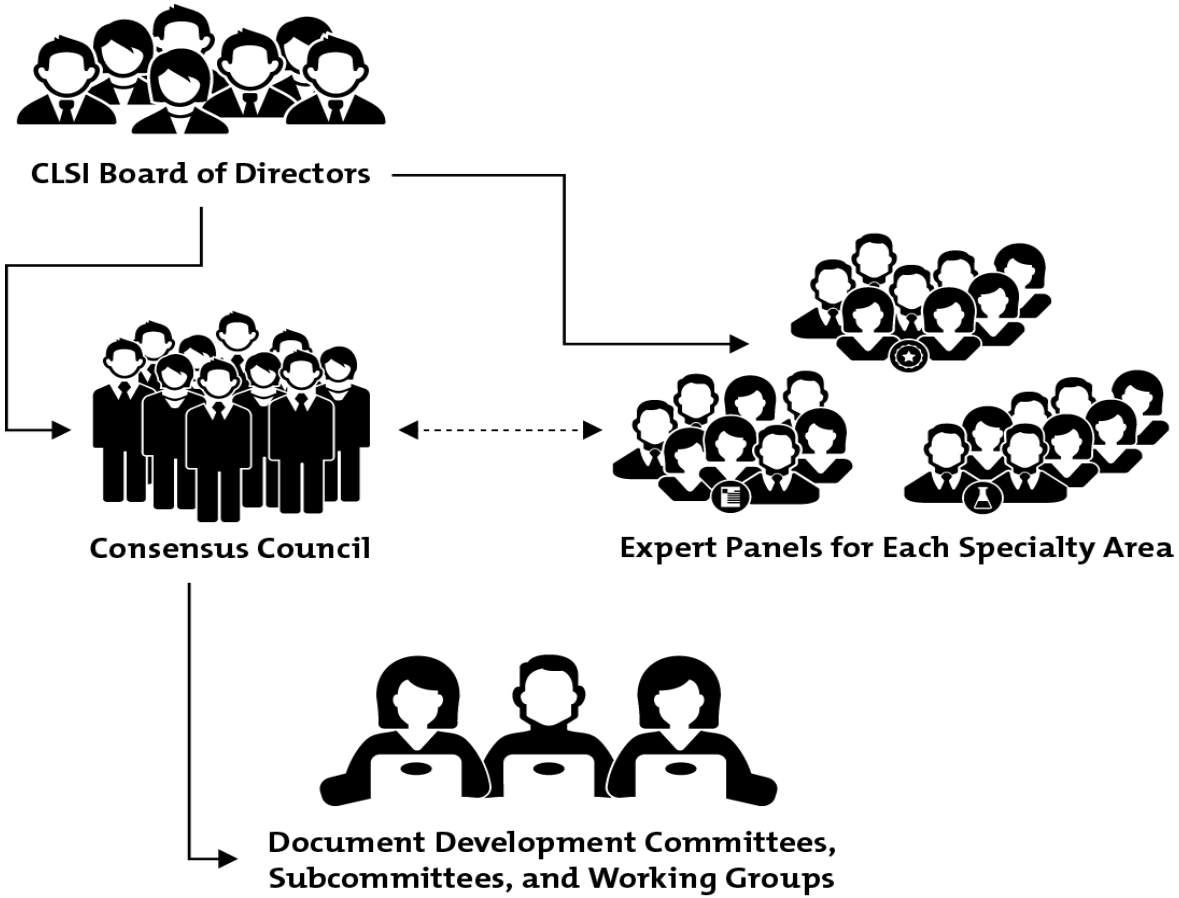
Mission

- Develop clinical and laboratory practices and promote their use worldwide

Vision

- Setting the standard in laboratory medicine for a healthier world

Consensus Document Teams



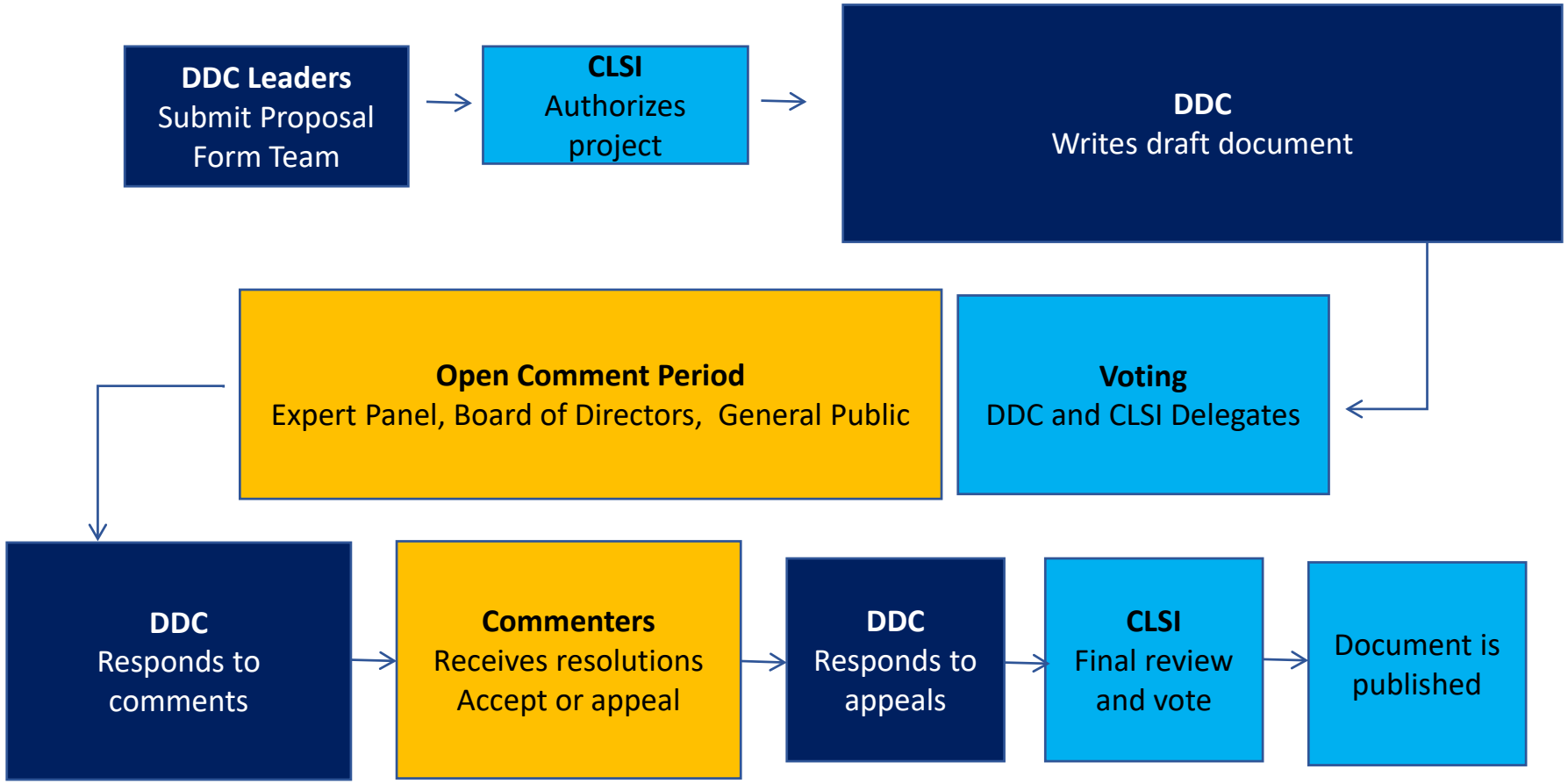
Document Development Committee (DDC)

- Subject matter experts
- Consist of voting and non-voting contributors
- A minimum of 2 members from each constituency
  - Government
  - Industry
  - Professional

Responsibility

- Draft consensus-based documents
- Scientific accuracy, practicality, comprehensibility
- Evaluate and address comments during each phase of the process

Consensus Document Process



CLSI H62 1st Edition

Validation of Assays Performed by Flow Cytometry

- **Constituencies**
  - Government (NIST, FDA)
  - Industry (Pharma, Manufacturers)
  - Professional (Clinical labs, CRO, NIH)
- **Alignment with Scientific Societies**
  - International Clinical Cytometry Society (ICCS)
  - American Association of Pharmaceutical Scientists (AAPS)
  - College of American Pathologists (CAP)
  - European Society for Clinical Cellular Analysis (ESCCA)
  - International Society for the Advancement of Cytometry (ISAC)
- **Provenance**
  - Canada
  - Germany
  - Switzerland
  - UK
  - USA

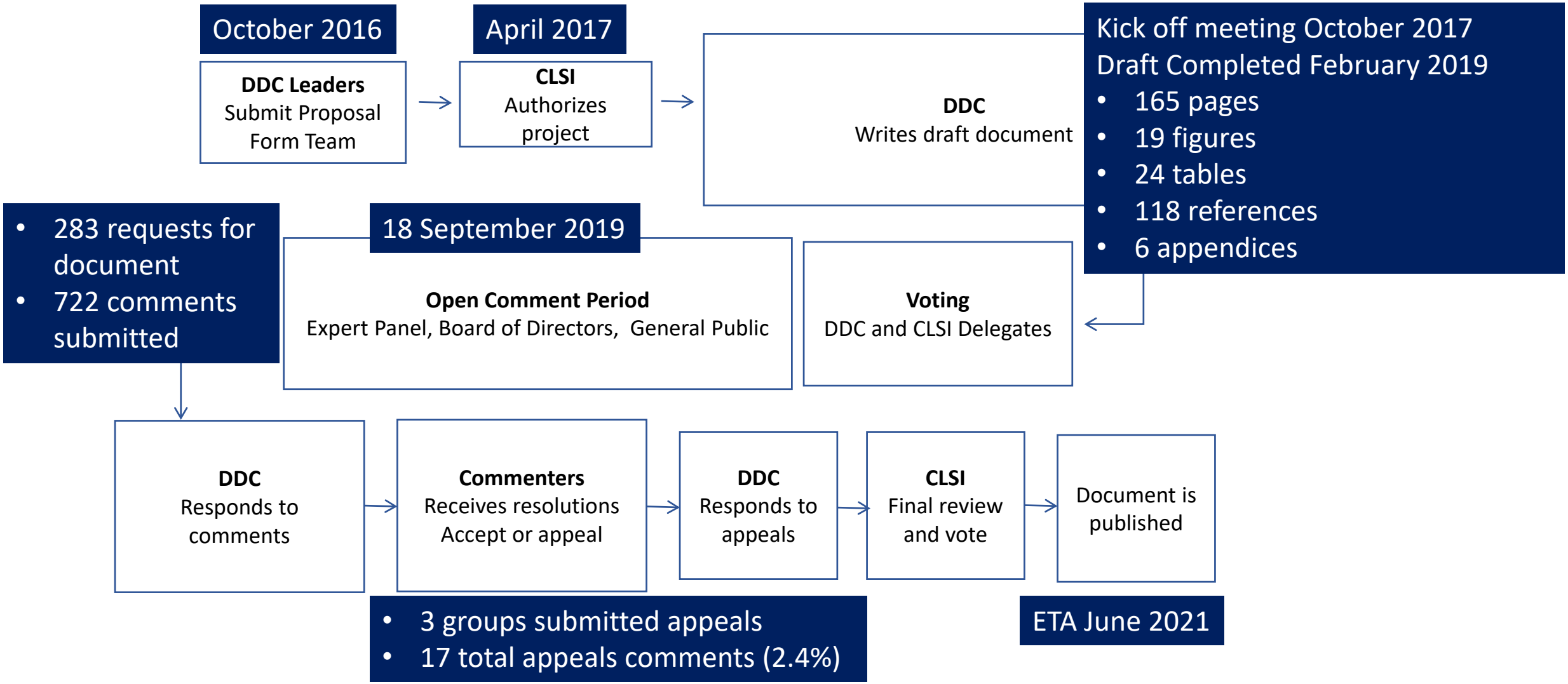
- **Leadership**
  - Virginia Litwin, Chair
  - Teri Oldaker, Vice Chair
  - Raul Louzoa, Secretary
  - Dave Sterry, CLSI Standards Director
- **Voting Members (10)**

  David Barnett, Jacqueline Cleary, Tom Denny, Cherie Green, Mike Keeney, Wolfgang Kern, Virginia Litwin, Teri Oldaker, Jennifer Stewart, Lili Wang

Contributors (27)

Elena Afonina, Ahmad Al Samman, Tony Bakke, Fiona Craig, Bruce Davis, Lorella Di Donato, Steve Eck, Nancy Fine, Ben Hedley, Shuguang Huang, Jerry Hussong, Andrea Illingworth, Cassie Jiang, Natalia Kokorina, Raul Louzoa, Sarah Maremont, Laura Marszalek, Kathy Muirhead, Andy Rawstron, John Schmitz, Alan Stall, Maryalice Stetler-Stevenson, Horacio Vall, Alessandra Vitaliti-Garami, Paul Wallace, Brent Wood, Yuanxin Xu

Consensus Document Process



CLSI H62 Scope

Within Scope

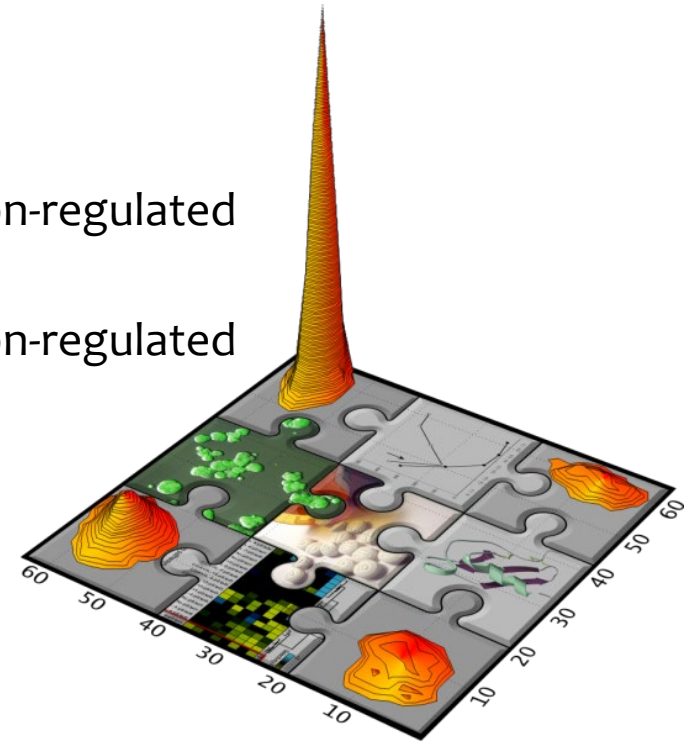
- Comprehensive recommendations
- Practical Instructions
- Current best practices
  - Summarize recent white papers and scientific advances

Out of Scope

- Individual cell type-specific assay development
- The validation of flow cytometric assays for soluble analytes
- Third-party software and LIS interface validation
- Specific instrument types

CLSI H62 Target Audience

- **Basic Science Laboratories**
  - Non-regulated
- **Clinical Laboratories**
  - Regulated US and ex US
- **Pharmaceutical and Biotechnology**
  - Drug discovery
    - Non-regulated
  - Drug development
    - Regulated and non-regulated
  - Clinical development
    - Regulated and non-regulated
  - Manufacturing
    - Regulated
- **Manufacturers**
  - Reagents
  - Assays
  - Instruments
- **Regulatory agencies**



Why

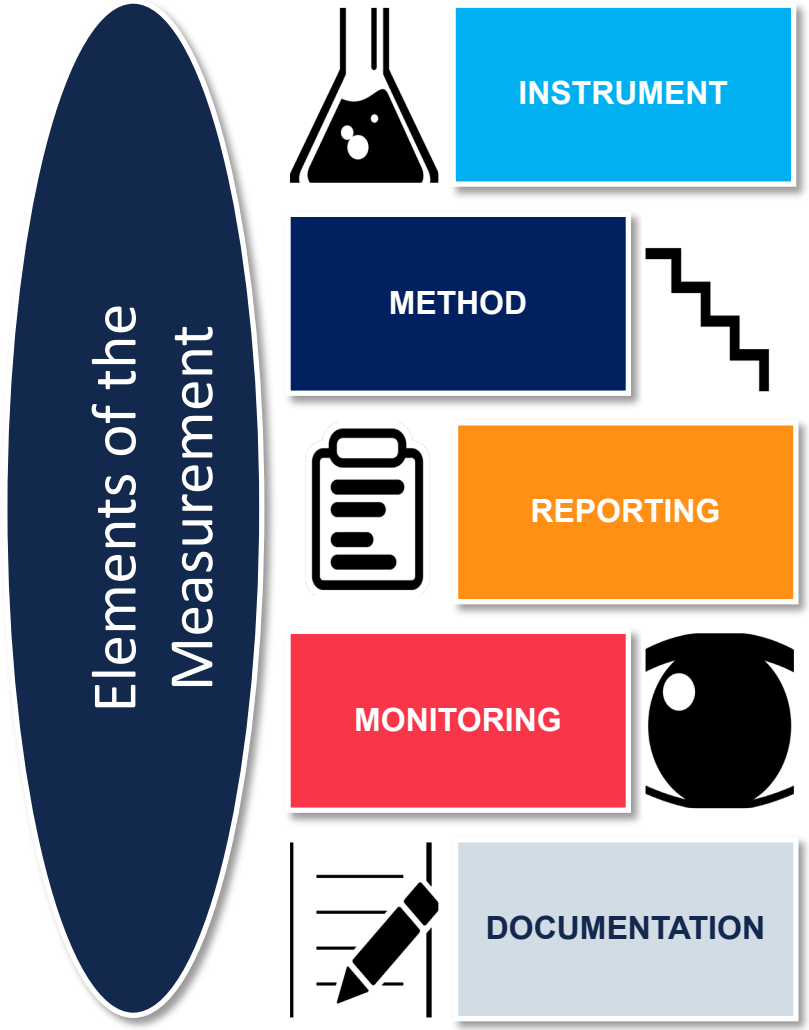
Why Do We Need a Flow Specific Document?

- Existing recommendations for other technologies are not fully applicable
- Unique challenges associate with flow cytometry
  - The complexity of cellular analytes/measurands
  - Increased complexity of cellular analytes in disease state samples
  - Highly complex and configurable instrumentation
  - The rate of technological advances and reagents
  - The rapid rate of biological discoveries
  - The lack of TRUE reference material
  - The fact that data are not derived from a calibration curve

Why CLSI?

- Extensive review process for consensus documents
- American National Standards Institute (ANSI) accredited
- Alignment with International Organization for Standardization (ISO)
- CLSI serves as the ANSI-appointed Secretariat for the ISO Technical Committee 212 (ISO/TC 212)
- Regulatory agencies often recognize CLSI guidelines

CLSI H62 Document Outline



Chapter 1	Scope
Chapter 2	Quality System Essentials
Chapter 3	Fit for Purpose / Iterative Approach
Chapter 4	Instrument Qualification, Setup, Standardization
Chapter 5	Assay Development and Optimization
Chapter 6	Assay Validation
Chapter 7	Examination Phase/ Post-Examination Phase

CLSI H62 Details

INSTRUMENTATION (Chapter 4)	NEW ASSAY (Chapter 3)	DEVELOPMENT (Chapter 5)	VALIDATION (Chapter 6)	QA (Chapters 2 and 7)
IQ, OQ, PQ	Identify the Need	Panel Design	Identify Required Parameters (Appendix A)	Instrument and Reagents
Linearity and Dynamic Range	Type of Assay	Panel Optimization	Validation Plan	Examination phase
Electronic Noise	Bioanalytical Data Category	Preliminary Characterization	Validation Experiments	Postexamination phase
Detector Efficiency and Background	Context of Use	Evaluate QC Material	Validation Report	Records
Resolution	Regulatory Requirements	Optimize		Training/Competency
Carryover	Assay Risk Category	Lock-down Assay		Accreditation
Inter-Instrument Standardization				EQA/PT

Assay Development and Optimization

The output(s) for the assay be defined

How will you define your population of interest?
- Positive selection antigens
- Negative selection antigens

In what matrix?

Number and type of lasers

Number of detectors

The filter configuration should be determined relative to the fluorochromes to be detected

Volumetric cell counting capability?

A reagent strategy should be defined that efficiently generates each of the assay outputs

Reagents should be specified, e.g. mAb clone, manufacturer, fluorophore

Titration should be performed

Specimen processing steps should be defined, e.g. wash, lyse, fix sequence

Panel performance should be confirmed

Manual and/or automated, supervised and/or unsupervised

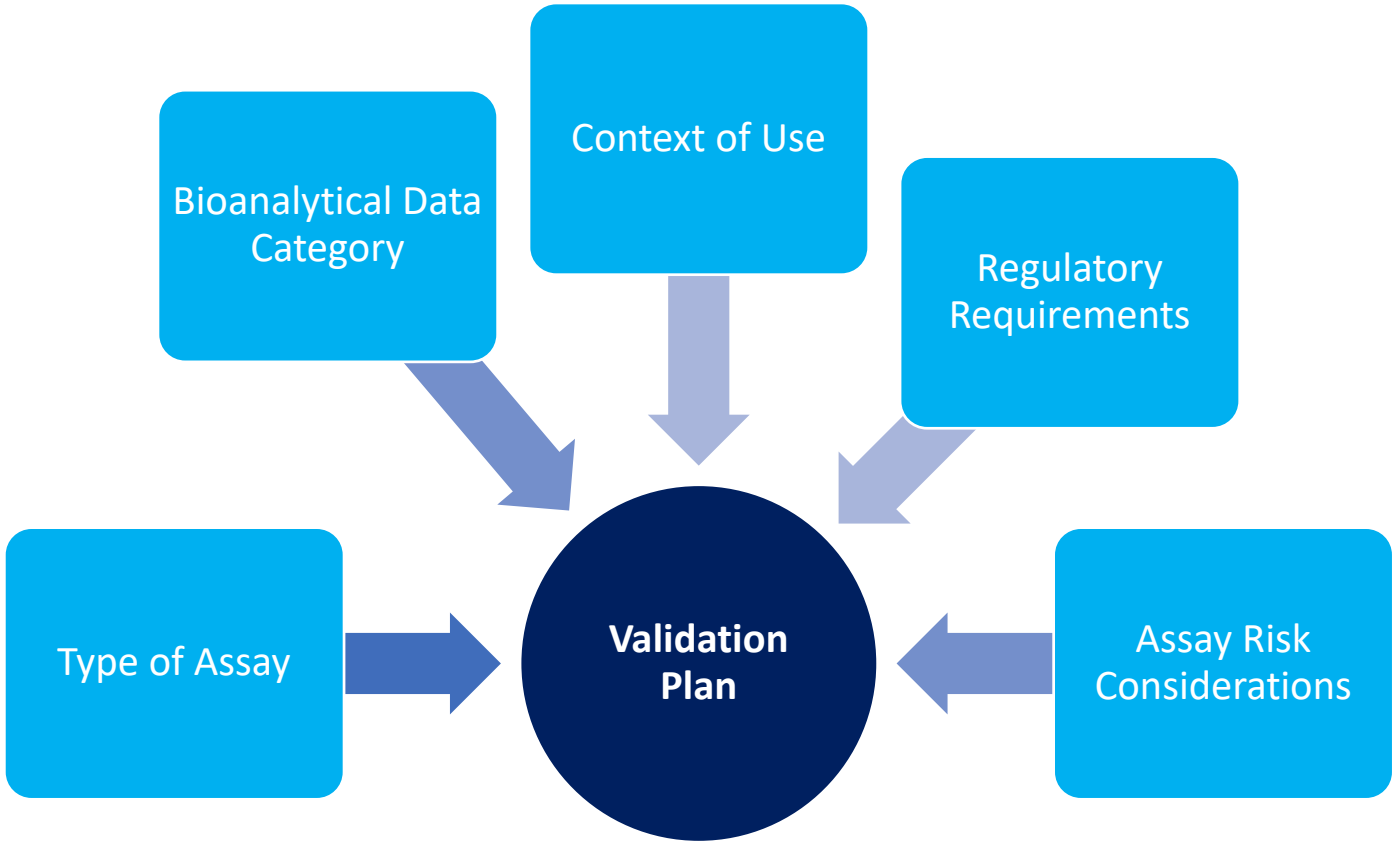
Define gates to exclude doublets, debris, non-viable cells or non-specific events

Define gates to identify population(s) of interest and any downstream markers

Identify reportable results/outputs (eg, %, MFI, cells per µL)

Assay Validation

Tiered Approach Based on Context of Use



Assay Risk Considerations

Clinical Risk	Intended Use of Data
Low	• Basic research assay • Drug discovery assay • End points in clinical trials not related to patient care or treatment
Moderate	• LDT used as an aid to diagnosis in a medical laboratory
High	• LDT used alone for diagnosis in clinical laboratory • Clinical trial biomarker assay (enrollment criteria) • Complementary diagnostic • Combination product/CDx • Assays manufactured as IVD/CE marked devices

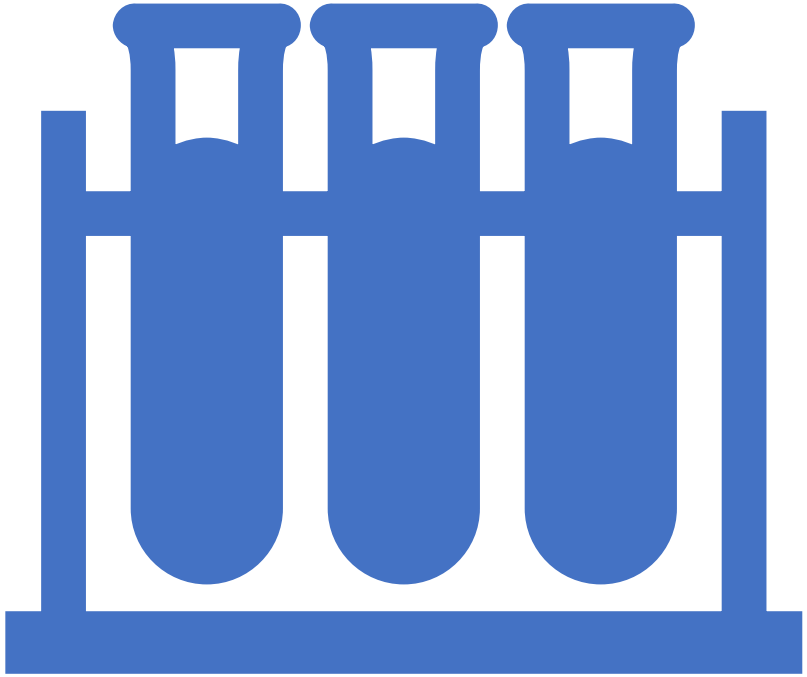
Validation Scenarios

Regulatory Setting	Intended Use	Assay Type	Validation Strategy
Nonregulated	Basic research	Novel assay	FFP validation type 1
	Drug discovery		
	Exploratory end point clin trial		
Nonregulated (GCLP recommended)	Secondary endpoint clin trial	Novel assay	FFP validation type 2
Clinical laboratory (CAP/CLIA/ISO15189)	Patient care and/or treatment	IVD	Verification
		Qualitative LDT assay	CLIA/IMDRF qualitative
			CLIA/IMDRF quantitative
		Lab- initiated revision	Lab initiated assay revision
GLP, GCLP	Primary endpoint clin trial	Novel assay	Analytical validation type 1
Manufacturing (GMP/ISO13485)	New diagnostic test		Analytical validation type 2
Manufacturing (GMP/ISO13485)	CDx		

Validation Strategies (8)

Validation Parameters to Assess

- ✓ Accuracy/trueness
- ✓ Selectivity/Interference
- ✓ Detection Capability
  - LOD/LLOQ
- ✓ Precision
  - Repeatability (Intra-assay)
  - Reproducibility (Inter-assay)
  - Between-operator precision
  - Between-instrument
- ✓ QC evaluation and QC ranges
- ✓ Linearity
- ✓ Stability
- ✓ Specimen
- ✓ Carryover
- ✓ Reference intervals
- ✓ Documentation
  - Validation plan /Validation report/QA review



How to Assess Them

- ✓ Type of samples
- ✓ Number of samples
- ✓ Number of analytical runs
- ✓ Statistical calculations

Gaps and Next Steps

CLSI H62

- Important milestone
- Still more work to do

Instrument Qualification, Setup, Standardization

- Address mainly PMT instruments but basic principles would be applicable to ADP and other new instruments
- NIST ERF beads will help facilitate more robust and streamlined platform standardization

Assay Development and Optimization

- Greater emphasis on preanalytical variables

Gaps and Next Steps, cont.

Assay Validation

- Validation for rare events (DOI: 10.1002/cyto.b.21949)
- Method transfer (DOI: 10.1002/cyto.b.21971)
- Validation of CAR-T cell assays (DOI: 10.1002/cyto.b.21985, DOI: 10.1002/cyto.b.21891)
- Validation specific to assay modifications (ICCS planned)
- Validations of precious samples (CSF, vitreous) (ICCS planned)

Reference Material

- Fluorophore standards
- Leukemia/lymphoma controls
- CAR-T cells

Relevant CLSI Flow Cytometry and Immunology Documents

Document #	Title
H20	Reference Leukocyte (WBC) Differential Count (Proportional) and Evaluation of Instrumental Methods
H21	Collection, Transport, and Processing of Blood Specimens for Testing Plasma-Based Coagulation Assays and Molecular Hemostasis Assays
H26	Validation, Verification, and Quality Assurance of Automated Hematology Analyzers
H42	Enumeration of Immunologically Defined Cell Populations by Flow Cytometry
H43	Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells
H52	Red Blood Cell Diagnostic Testing Using Flow Cytometry
H62	Validation of Assays Performed by Flow Cytometry
I/LA26	Performance of Single Cell Immune Response Assays
I/LA28	Quality Assurance for Design Control and Implementation of Immunohistochemistry Assays
I/LA30	Immunoassay Interference by Endogenous Antibodies
I/LA34	Design and Val of Immunoassays for Assessment of Human Allergenicity of New Biotherapeutic Drugs

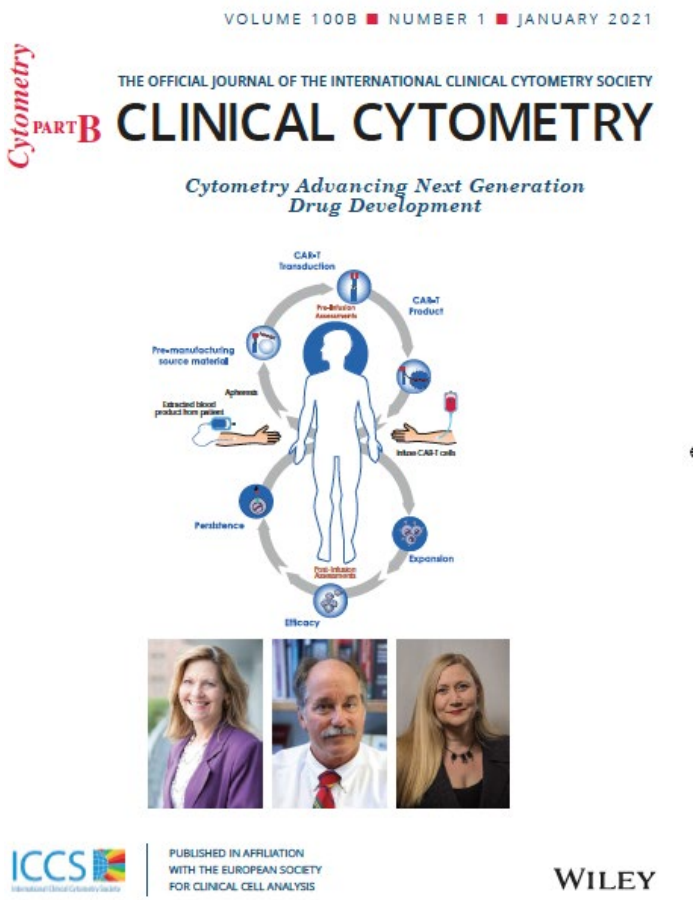
Relevant CLSI Validation Documents

Document #	Title
EP05-A3	Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline—Third Edition
EP06-A	Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach; Approved Guideline
EP09-A3	Measurement Procedure Comparison and Bias Estimation Using Patient Samples; Approved Guideline—Third Edition
EP10-A3-AMD	Preliminary Evaluation of Quantitative Clinical Laboratory Measurement Procedures; Approved Guideline—Third Edition
EP12-A2	User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline—Second Edition
EP15-A3	User Verification of Precision and Estimation of Bias; Approved Guideline—Third Edition
EP17-A2	Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures; Approved Guideline—Second Edition
EP21-Ed2	Estimation of Total Analytical Error for Clinical Laboratory Methods—Second Edition
EP23-A	Laboratory Quality Control Based on Risk Management; Approved Guideline
EP24-A2	Assessment of the Diagnostic Accuracy of Laboratory Tests Using Receiver Operating Characteristic Curves; Approved Guideline—Second Edition
EP25-A	Evaluation of Stability of In Vitro Diagnostic Reagents; Approved Guideline
EP26-A	User Evaluation of Between-Reagent Lot Variation; Approved Guideline
EP27-A	How to Construct and Interpret an Error Grid for Diagnostic Assays; Approved Guideline
EP28-A3c	Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guideline—Third Edition

Relevant CLSI Validation Documents, cont.

Document #	Title
EP30-A	Characterization and Qualification of Commutable Reference Materials for Laboratory Medicine; Approved Guideline
EP32-R	Metrological Traceability and Its Implementation; A Report
EP33, 1st ed.	Use of Delta Checks in the Clinical Laboratory
EP34, 1st ed.	Establishing and Verifying an Extended Measuring Interval Through Specimen Dilution and Spiking
EP35, 1st ed.	Equivalency of Specimen Types
EP39	Surrogate Sample Framework
EP40	Sample and Reagent Carryover
EP42	Laboratory-Developed Tests: A Workbook for Establishment and Implementation
EP43	User Verification of Precision Implementation Guide
EP44	User Verification of Bias Implementation Guide
EP45	Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures Implementation Guide
EP46	Risk Management Techniques to Identify and Control Laboratory Error Sources Implementation Guide
EP47	Evaluation of Total Analytical Error Implementation Guide
EP48	User Evaluation of Between-Reagent Lot Variation Implementation Guide
EP49	Precision Testing Quick Guide

Special Issue of *Cytometry Part B: Clinical Cytometry*
"Cytometry Advancing Next Generation Drug Development"



Recommendation Papers
Method transfer
Receptor Occupancy
Rare events

CAR-T Cell
Best practices
Monitoring CAR-T in clinical trials

Reviews
Immune monitoring for cancer immunotherapy
Biomarkers for antiviral therapies
SARS-CoV-2 immunopathology

Method Validation
Mass cytometry assay
Enrollment biomarker assay approved by NY State

Free Access https://bit.ly/3iDS5qb

RESOURCES

- **GenomeWeb Webinar: Quality Pillars for Building Measurement Assurance in Flow Cytometry 2018.** **GenomeWeb Flow Cytometry Quality Pillars**
- **ISAC/CYTOU Webinar: Validation the Key to Translatable Flow Cytometry a Three-part Webinar Series 2018**
  - **Instrument Qualification**
  - **Method Validation - Overview, Concepts**
  - **Method Validation - Planning and Excecution**
- Recommendations for the Validation of Flow Cytometric Testing During Drug Development: I Instruments. JIM, 363:104-119, 2011.
- Recommendations for the Validation of Flow Cytometric Testing During Drug Development: II Assays. JIM 363:120-134, 2011.
- Validation of Cell-Based Fluorescence Assays: Practice Guidelines from the International Council for Standardization of Haematology and International Clinical Cytometry Society. Cytometry Part B: Clinical Cytometry Special Issue volume 84B:2013
- Plant, Anne L., et al. "Improved reproducibility by assuring confidence in measurements in biomedical research." *Nature methods* 11.9 (2014): 895.
- Recommendations for the Evaluation of Specimen Stability for Flow Cytometric Testing During Drug Development. JIM, 418:1, 2015.
- Best practices in Performing Flow Cytometry in a regulated environment: feedback from experience within the EBF. Bioanalysis 9:1253, 2017.
- Flow cytometry method validation protocols. In Current Protocols in Cytometry, e53. doi: 10.1002/cpcy.53, 2018

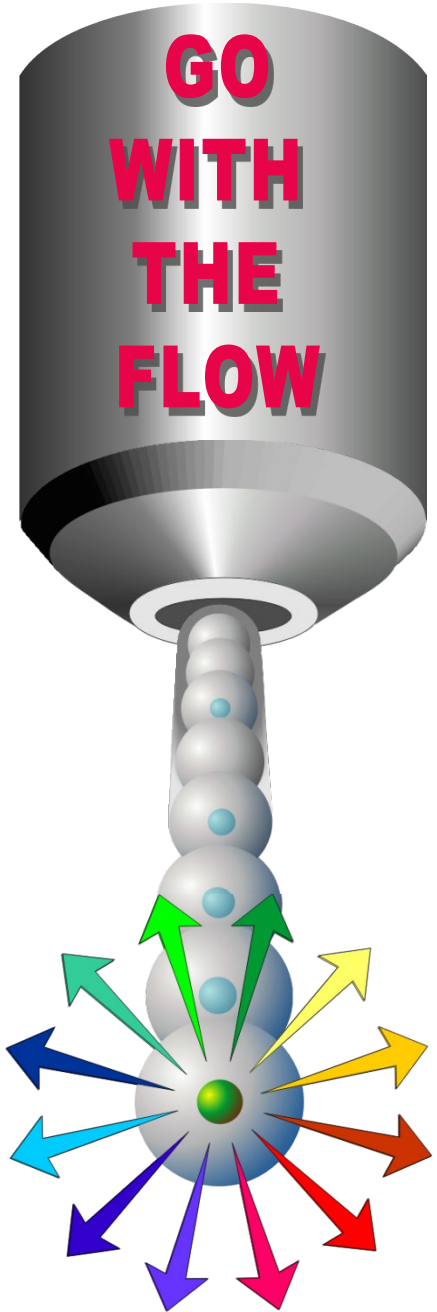


Figure courtesy of
Ira Schieren, Columbia University

Standards for Regenerative Medicine

Standards Coordinating Body

Dawn Henke
Senior Scientific Program Manager

Tuesday, February 16th, 2020

STANDARDS COORDINATING BODY
REGENERATIVE MEDICINE

The Need for Regenerative Medicine Standards

Regenerative medicine therapies present unique challenges related to product testing, scientific protocols, product quality and specifications, performance characteristics, and compliance criteria.

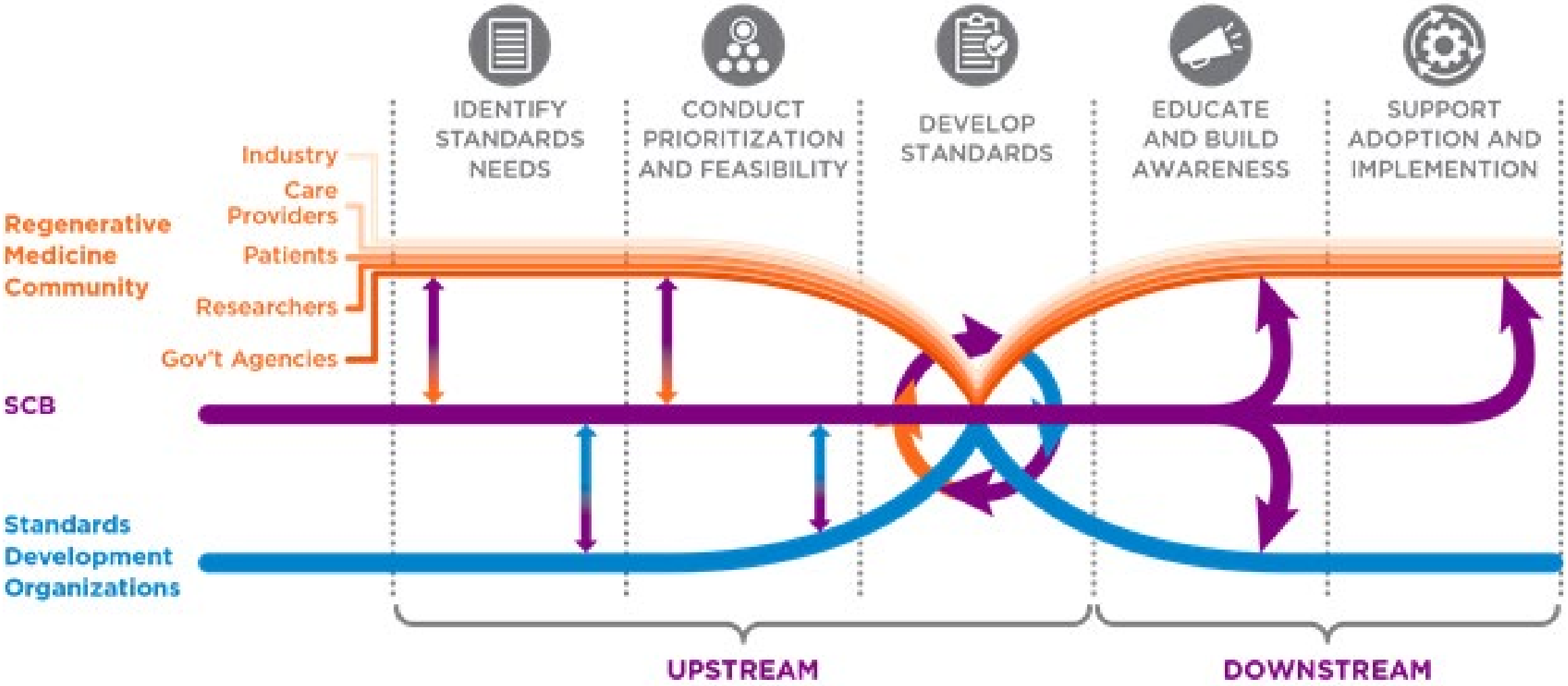
Need for Collaboration in Standards

- Openness
- Fairness
- Reflects the current state of the field
- Benefits the whole field
- Establish processes that can simplify logistics/pre competitive areas

Founding of SCB

- Established in 2016 and launched in January 2017, SCB is an **independent 501(c)(3)** organization
- Occupies unique niche within field with **no vested interests in specific scientific, commercial, clinical or policy approaches**
- SCB is **not an SDO**, but rather **coordinates** the standards development process
- Serves as **communication vehicle** among all stakeholders, including government agencies, critical to the development of standards

SCB's Role in Standards Development Process



Key SCB Education and Outreach Resources

Reports

The Regenerative Medicine Standards Landscape – **report available on SCB website**
Needed Standards in Regenerative Medicine – **report available on SCB website**

Webinars & workshops

Webinars on the **importance of standards**, the **current standards landscape**, and **standards development processes** (all available on the SCB website) - Workshops held with **NIST, BioFabUSA, ASTM International**, and **FDA**

Factsheets

Project factsheets on **standards in development**; factsheets on **ASTM and ISO standards development efforts** in regenerative medicine; factsheets on the **benefits of standards** in regenerative medicine, **defining standards terminology**, and **types of standards** (i.e., documentary and reference materials)

Newsletter

Quarterly newsletter that **updates on standards development efforts**, notes **upcoming events**, and lists **opportunities to get involved** in standards development

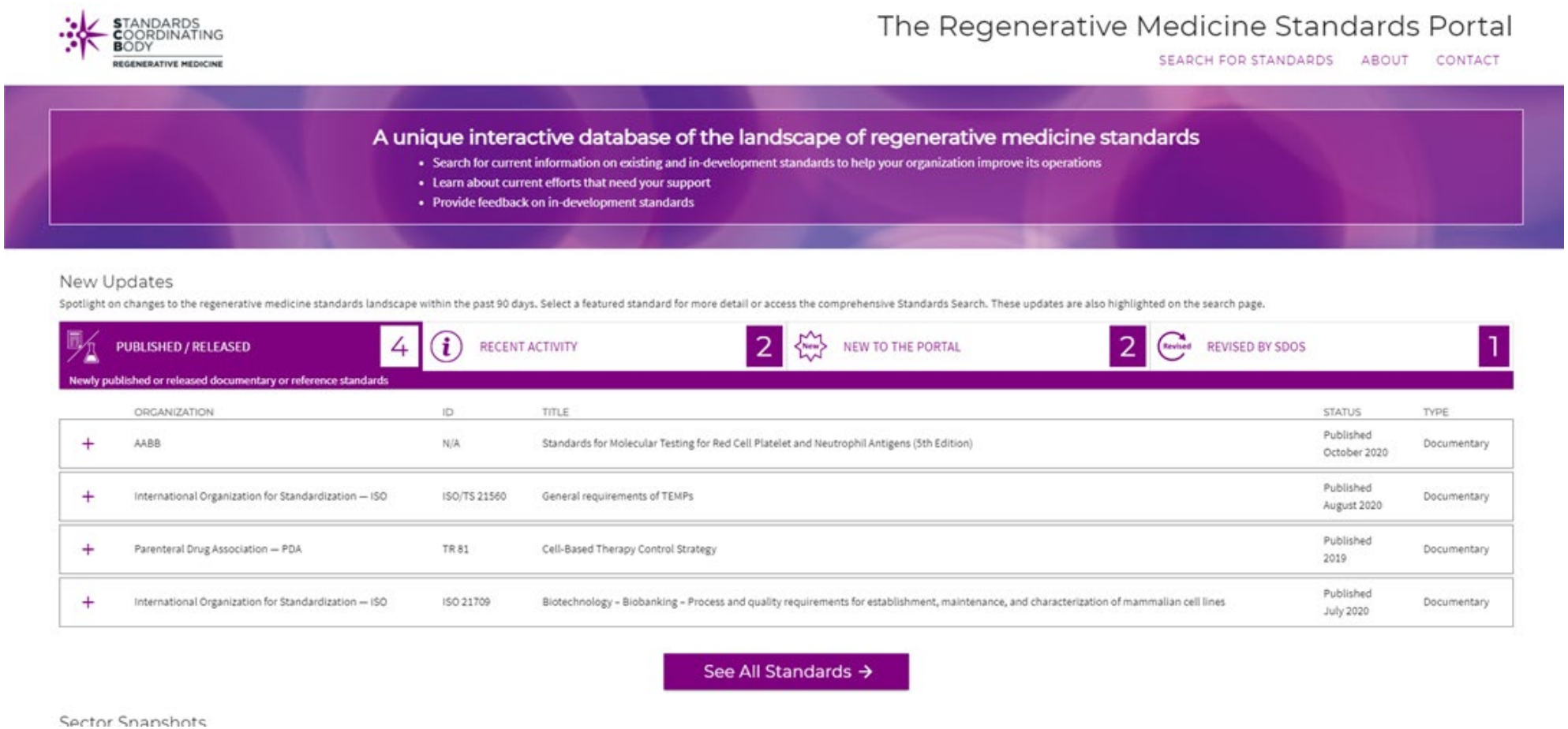
The Regenerative Medicine Standards Landscape

Develop and disseminate a landscape of existing standards (report available on the SCB website*) and potential needs in the regenerative medicine field.

- Overview of existing standards by sector
- Overview by application area (bioprocessing → clinical trials)
- Identify potential standards gaps or needs

*https://www.standardscoordinatingbody.org/publications

Regenerative Medicine Standards Portal



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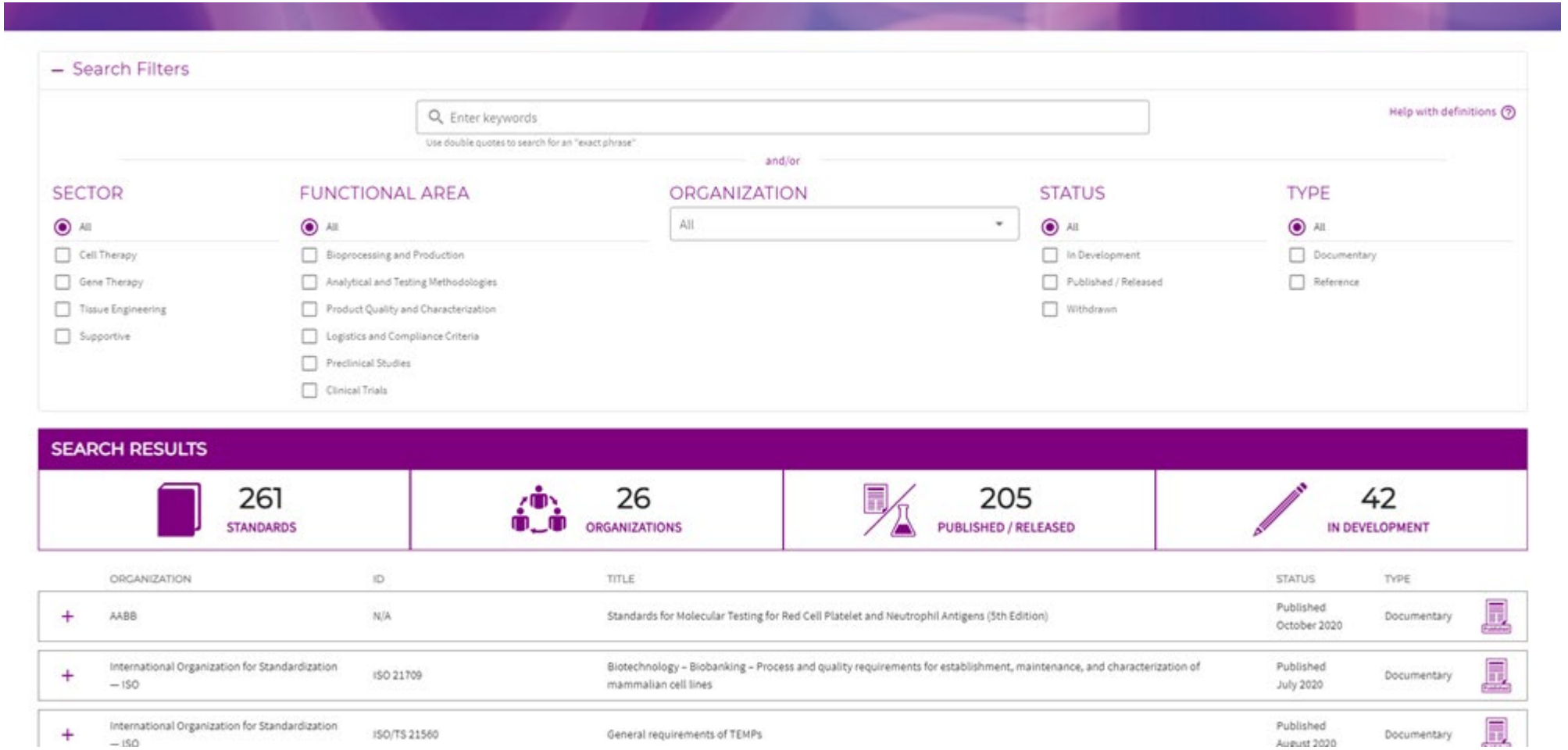


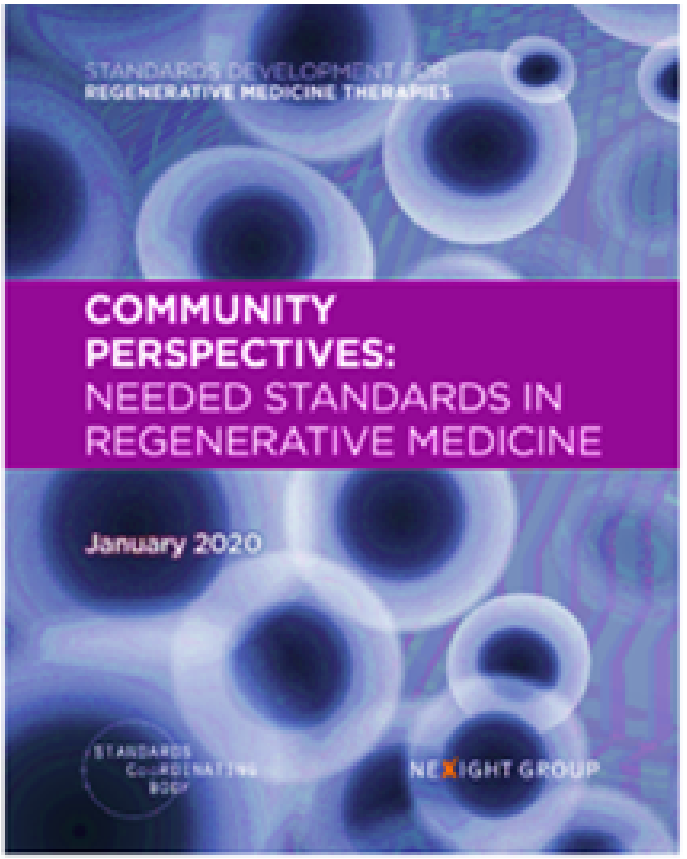
STANDARDS COORDINATING BODY
REGENERATIVE MEDICINE

Community Perspectives: Needed Standards in Regenerative Medicine

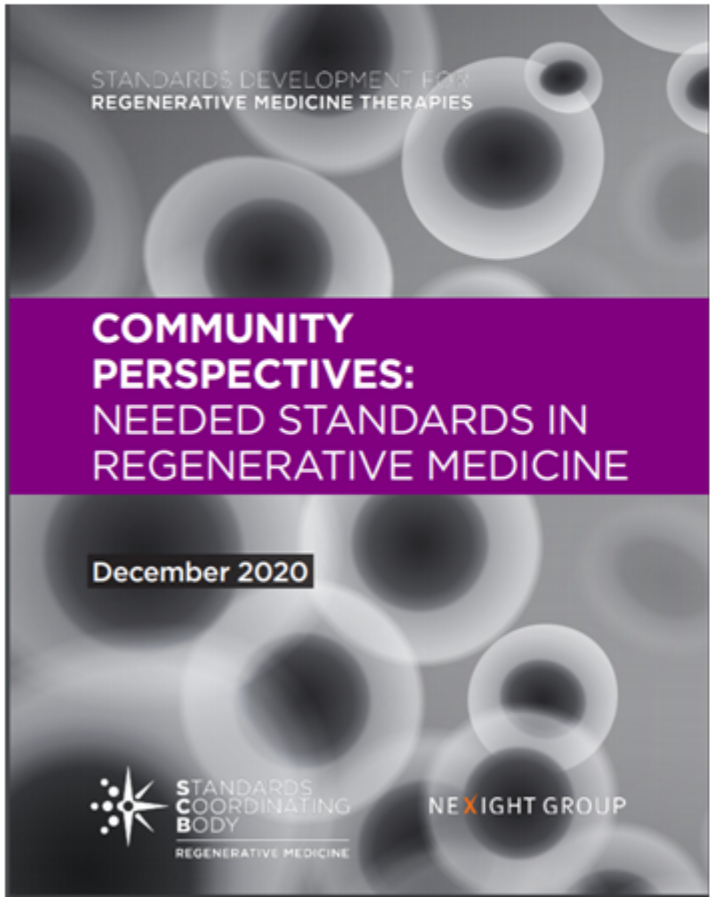
Outline standards needs identified by the community that could have the greatest benefit to the field of regenerative medicine (report available on the SCB website*)

- Overview of more than 30 needed standard areas identified and prioritized by the community
- Summaries of needed standards by sector (Cell Therapy, Gene Therapy, and Tissue Engineering)
- Overview by functional area (bioprocessing → clinical trials)

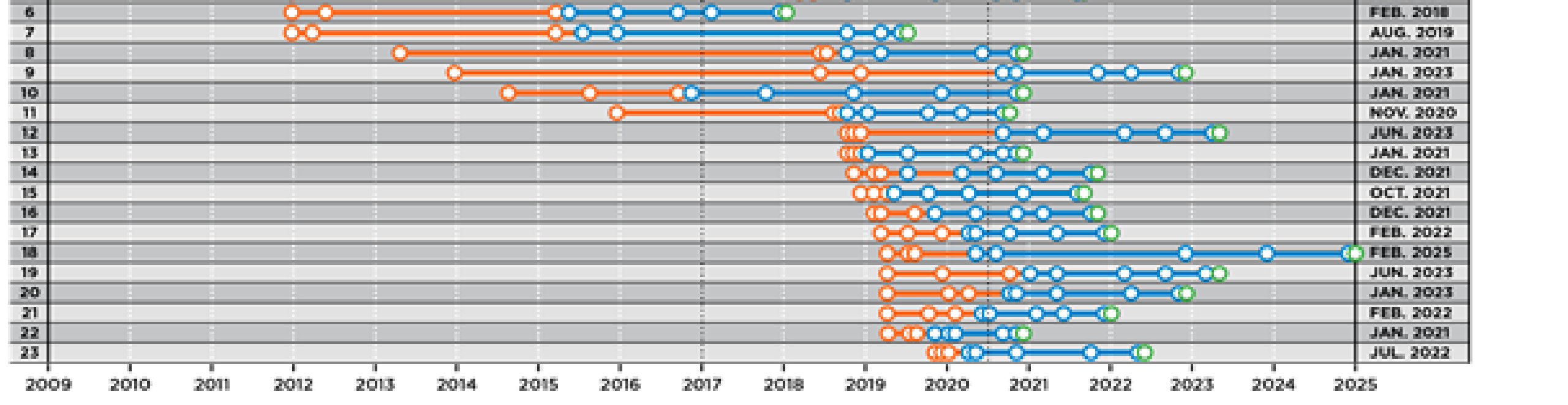
*https://www.standardscoordinatingbody.org/publications



Updated Needed Standards Report



- Updated in Dec 2020
- Involved surveys, interviews, sector calls
- Added 9 new needed standards areas
- Some standards reprioritized
- Report will be rolled into the new interactive portal
- Top prioritized areas and begin feasibility studies to determine next steps



Upstream | Development | Outreach

1. Identify Needs
2. Prioritize Standards
3. Assess Feasibility
4. Initiation
5. Initial Drafting
6. Review/ Comment
7. Final Voting
8. Finalization
9. Ongoing Outreach and Education

**Availability dates are estimates only. Development of a standard depends on SDO timelines, which can be time intensive and may vary significantly (particularly for reference materials).

1. Characterization of Human Cells for Therapeutic Use
2. Ancillary Materials Used in Cellular Therapy Production (3-Part TS)
3. Requirements for Cell Therapy Manufacturing Equipment
4. Rapid Microbial Testing Method Design and Validation Framework
5. Sampling Methods of Tissue Engineered Medical Products for Sterility Assurance
6. General Guidance on Cell Counting Part 1
7. General Guidance on Cell Counting Part 2
8. Characterization of Fiber-Based Scaffolds
9. Cell Collection Standards for Cell Therapies
10. Transportation Requirements of Cells for Therapeutic Use
11. Bioink Printability Test Method
12. Evaluating Pre-existing Immunity to Adeno-Associated Viruses
13. Cryopreservation of Cells (PDA-led project)
14. Bioprinter Hardware
15. Ancillary Materials used in Cellular Therapy Production (IS)
16. Bioprinter Software/Data Governance
17. ASME Thermal Medicine Tissue Properties
18. Base Requirements for Digital Platforms for Providers
19. Viral Vectors (Lenti/AAV) for Gene Therapy
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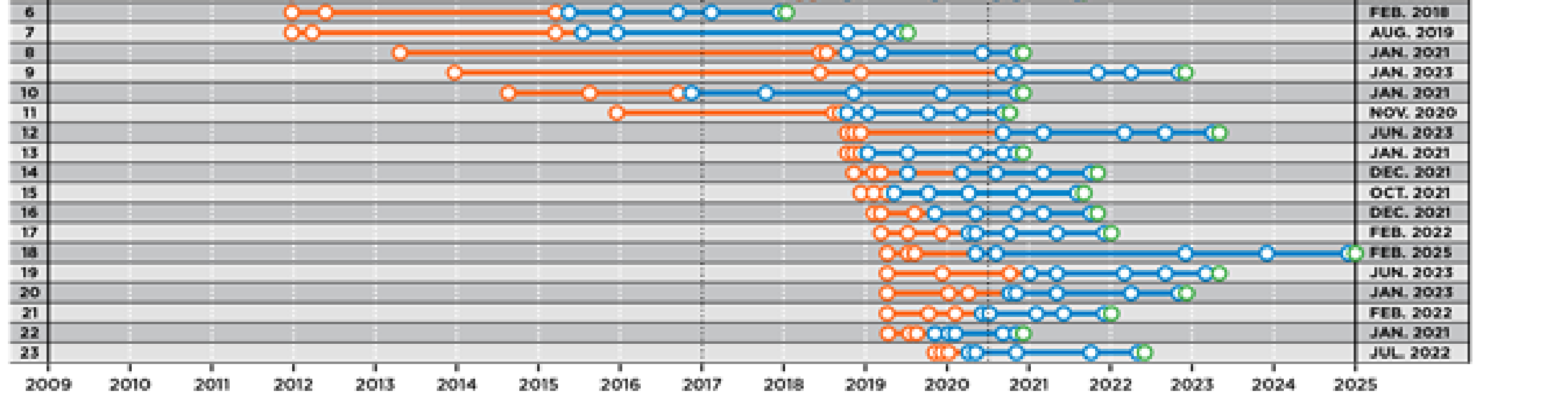
22. Base Labeling Requirements for Regenerative Medicine Products
23. Tissue Engineering Lexicon



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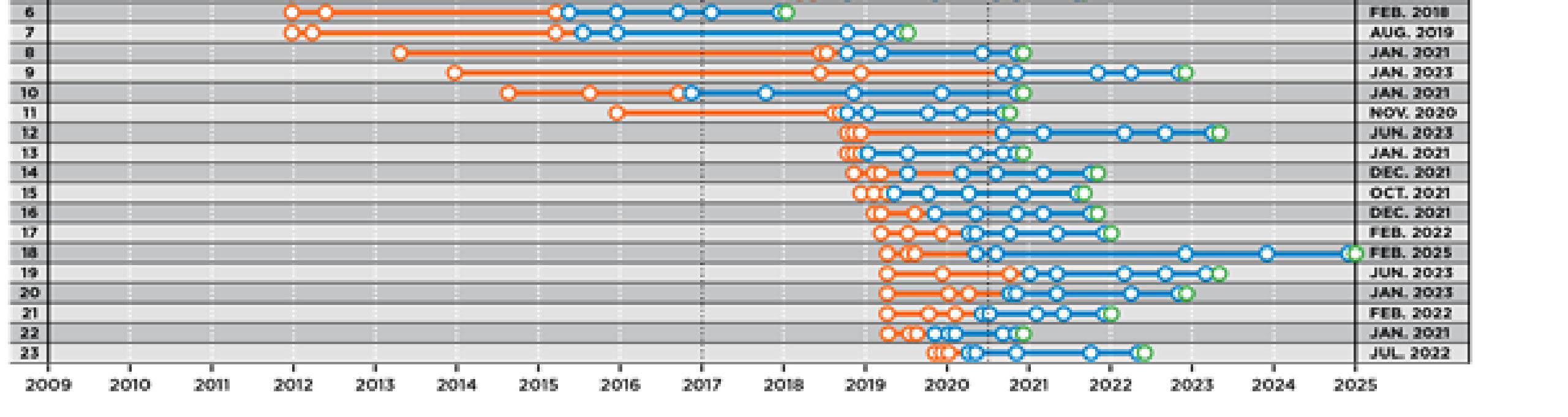

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New Projects:

- **Chain of Identity/Chain of Custody**
- **Standards for Lentivirus Vectors (feasibility**
- **Nucleic Acid Synthesis- General definitions and requirements for the production and quality control of synthesized gene fragments, gene, and genome**
- **General Requirements for cell line authentication**

Need for a Standards Development Forum

- Experts in the regenerative medicine community have identified areas of need that may benefit from the creation of new standards (Needed Standards Report)
- Every organization developing standards has its own areas of expertise and focus.
- Coordination among organizations developing standards is needed to address these identified standards needs.

SCB established the Standards Development Forum: A guided discussion between high-level decision-makers representing the major organizations developing standards.

Opportunities for New Joint Initiatives

1. Public Standards Development Resource
2. Open Ballot Update System
3. Biannual Meetings
4. Standards Education and Outreach

Standards for Flow Cytometry

- ISO 20391-1:2018 Cell counting — Part 1: General guidance on cell counting methods
- ISO 20391-2:2019 Cell counting — Part 2: Experimental design and statistical analysis to quantify counting method performance
- ISAC FCS 3.1 Flow Cytometry Standard Implementation guidance
- ISAC Minimum Information about a Flow Cytometry Experiment
- ISAC Mean Equivalent Soluble Fluorophores (MESF) and Equivalent Reference Fluorophore (ERF)
- USP <1027> Flow Cytometry
- USP <127> Flow Cytometric Enumeration of CD34+ Cells

FOR MORE INFORMATION VISIT

www.standardscoordinatingbody.org

OR CONTACT

admin@regenmedscb.org

National Institute of Standards and Technology
U.S. Department of Commerce

Flow Cytometry Standards Consortium Workshop

Flow Cytometry to Support Advanced Cell Manufacturing

Simon Lacey
Director, Translational and Correlative Studies Laboratory (TCSL)
Center for Cellular Immunotherapies,
University of Pennsylvania

February 16th 2021

Penn Medicine
Center for Cellular Immunotherapies

Flow Cytometry to Evaluate Engineered T cells

- **Engineered T cells for therapeutic use are becoming increasingly sophisticated:**
  - ***Introduction of engineered T cell receptors to direct specificity***
  - **Chimeric antigen receptor to target tumor or infectious disease antigens (CAR)**
  - **Chimeric AutoAntibody Receptors (CAAR), which target antibodies expressed on the surface of B cells.**
  - **Dominant-negative receptors such as dnTGF-βRII** **Kloss et al Mol Ther. 2018 Jul 5;26(7):1855-1866.**
  - **Switch Receptors**
  - ***CRISPR editing***
  - **TALEN editing**
  - **ZnFinger editing**
  - **Knock-in technologies**
  - **Engineering to secrete cytokines**
- **How can Flow be used to evaluate these cell products pre and post infusion?**

How can Flow be used to evaluate these cell products pre and post infusion?

Partial list:

- Detection of engineered cells in cell products by TCR or CAR or CAART marking
- Detection of engineered cells in post-infusion patient samples
- Surface marker phenotyping of engineered cells; differentiation, activation, exhaustion
- Evaluation of gene editing by loss of proteins encoded by edited genes
- Detection of decoy receptors on surface of engineered cells
- Detection of cytokine production by cells engineered to secrete cytokines (ICC assays)
- Examination of patient cellular immune responses to Cas9 or to engineered cell antigens using peptide libraries and flow ICC assays
- Flow-based cytotoxicity assays to measure killing function of engineered cells

CLINICAL TRIALS

CRISPR-engineered T cells in patients with refractory cancer

Edward A. Stadtmauer*†, Joseph A. Fraietta*, Megan M. Davis, Adam D. Cohen, Kristy L. Weber, Eric Lancaster, Patricia A. Mangan, Irina Kulikovskaya, Minnal Gupta, Fang Chen, Lifeng Tian, Vanessa E. Gonzalez, Jun Xu, In-young Jung, J. Joseph Melenhorst, Gabriela Plesa, Joanne Shea, Tina Matlawski, Amanda Cervini, Avery L. Gaymon, Stephanie Desjardins, Anne Lamontagne, January Salas-Mckee, Andrew Fesnak, Donald L. Siegel, Bruce L. Levine, Julie K. Jadlowsky, Regina M. Young, Anne Chew, Wei-Ting Hwang, Elizabeth O. Hexner, Beatriz M. Carreno, Christopher L. Nobles, Frederic D. Bushman, Kevin R. Parker, Yanyan Qi, Ansuman T. Satpathy, Howard Y. Chang, Yangbing Zhao, Simon F. Lacey*, Carl H. June*†

Science
Volume 367(6481):eaba7365
February 28, 2020

https://science.sciencemag.org/content/367/6481/eaba7365

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- Our previous studies had shown safety and promising efficacy of adoptive T cell transfer approaches using transgenic TCRs specific for the immunogenic NY-ESO-1 tumor antigen in patients with myeloma, melanoma, and sarcoma (Rapoport *et al.,* Nat. Med. 21, 914–921, 2015)

- Designed a first-in-human phase 1 human clinical trial to test the safety and feasibility of multiplex CRISPR/Cas9 genome editing for a synthetic biology cancer immunotherapy application.

- Autologous T cells engineered by lentiviral transduction to express an HLA-A2*0201–restricted TCR specific for the SLLMWITQC peptide in NY-ESO-1 and LAGE-1.

- CRISPR knock out of endogenous TRAC, TRBC, to increase exogenous TCR expression and reduce the potential for mixed heterodimer formation

- Limit the development of T cell exhaustion, which can be triggered by the checkpoint ligands PD-L1 and PD-L2 by deleting PDCD1.

NCT03399448. "Phase 1 trial of autologous T cells engineered to express NY-ESO-1 TCR and CRISPR gene edited to eliminate endogenous TCR and PD-1 (NYCE T Cells)"

- **June 21st 2016 Approval by Recombinant DNA Advisory Committee (RAC) at the NIH**

~17 months

- **IND approved Nov 17th 2017**

~14 months

- **First patient infused Jan 7th 2019**

~13 months

- **Publication Feb 28th 2020**

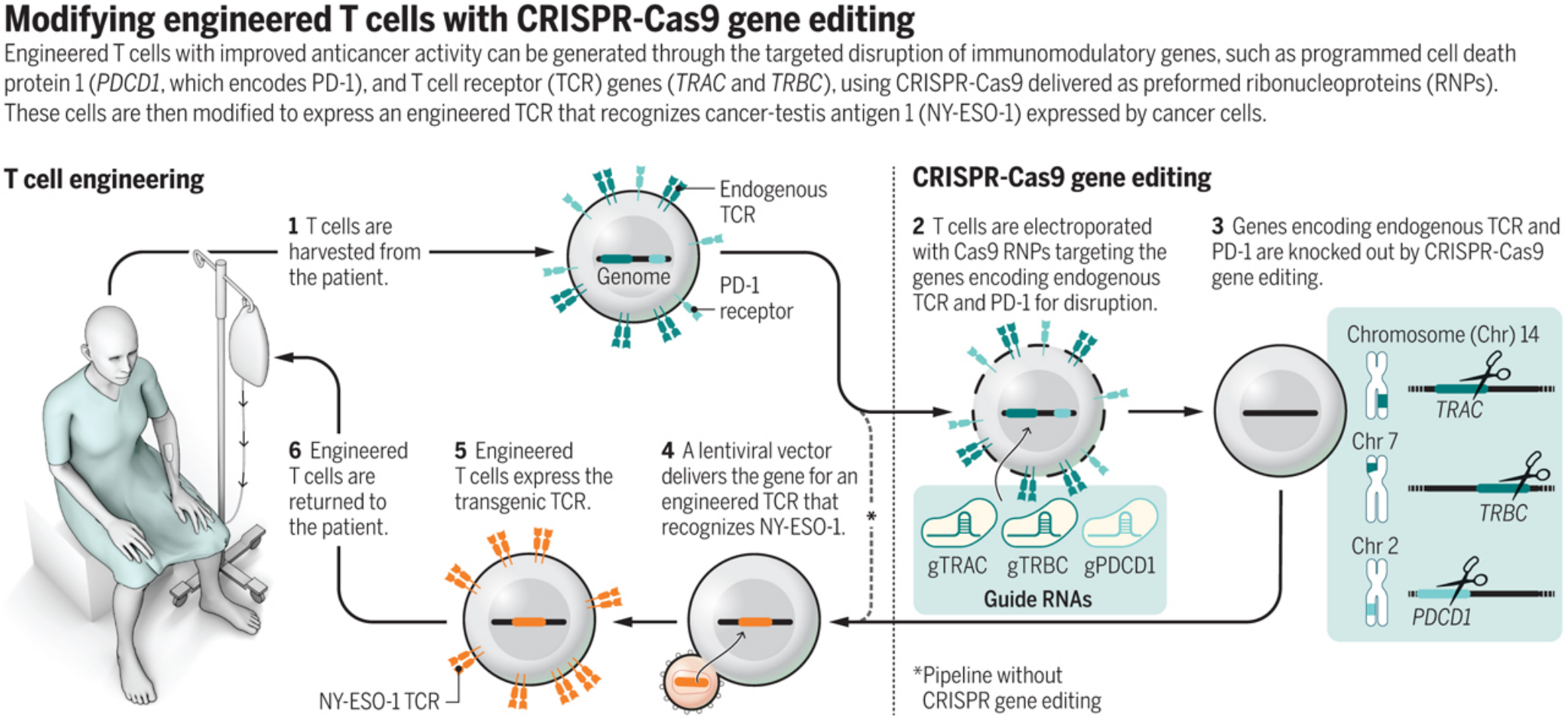
Lymphodepletion with cyclophosphamide and fludarabine and a single infusion of 1 × 10^8 manufactured CRISPR-Cas9–engineered T cells/kg

Table 1. Patient demographics and date of engineered T cell infusion. MM, multiple myeloma; BM, bone marrow; XRT, radiation therapy; ASCT, autologous hematopoietic stem cell transplant; ND, not done.

Subject ID (UPN) and infusion date	Sex and age	Diagnosis	Clinical sites	Prior therapy	Prior transplant or surgery	LAGE-1*, NY-ESO-1*, NY-ESO-1**
UPN35 7 January 2019	Female 66 years	Immunoglobulin G kappa MM 2008	BM, lytic bone lesions	Lenalidomide, pomalidomide, bortezomib, carfilzomib, daratumumab, panobinostat, etc. (eight lines of therapy; see supplementary materials)	Three ASCTs	Positive, positive, negative
UPN39 18 March 2019	Male 66 years	Myxoid and round cell liposarcoma 2012	Abdominal and pelvic masses	Doxorubicin, ifosfamide, XRT 60 gray, trabectedin, gemcitabine, taxol, XRT	Resection and debulking twice, left nephrectomy and partial sigmoid resection	ND, ND, positive
UPN07 5 August 2019	Female 62 years	Kappa light chain MM 2009	BM, lytic bone lesions	Lenalidomide, pomalidomide, bortezomib, carfilzomib, daratumumab, anti-CD38 immunoconjugate (six lines of therapy; see supplementary materials)	Two ASCTs	Positive, positive, negative

*qPCR **Immunohistochemistry

Manufacturing Schema



Jennifer R. Hamilton, and Jennifer A. Doudna Science 2020;367:976-977

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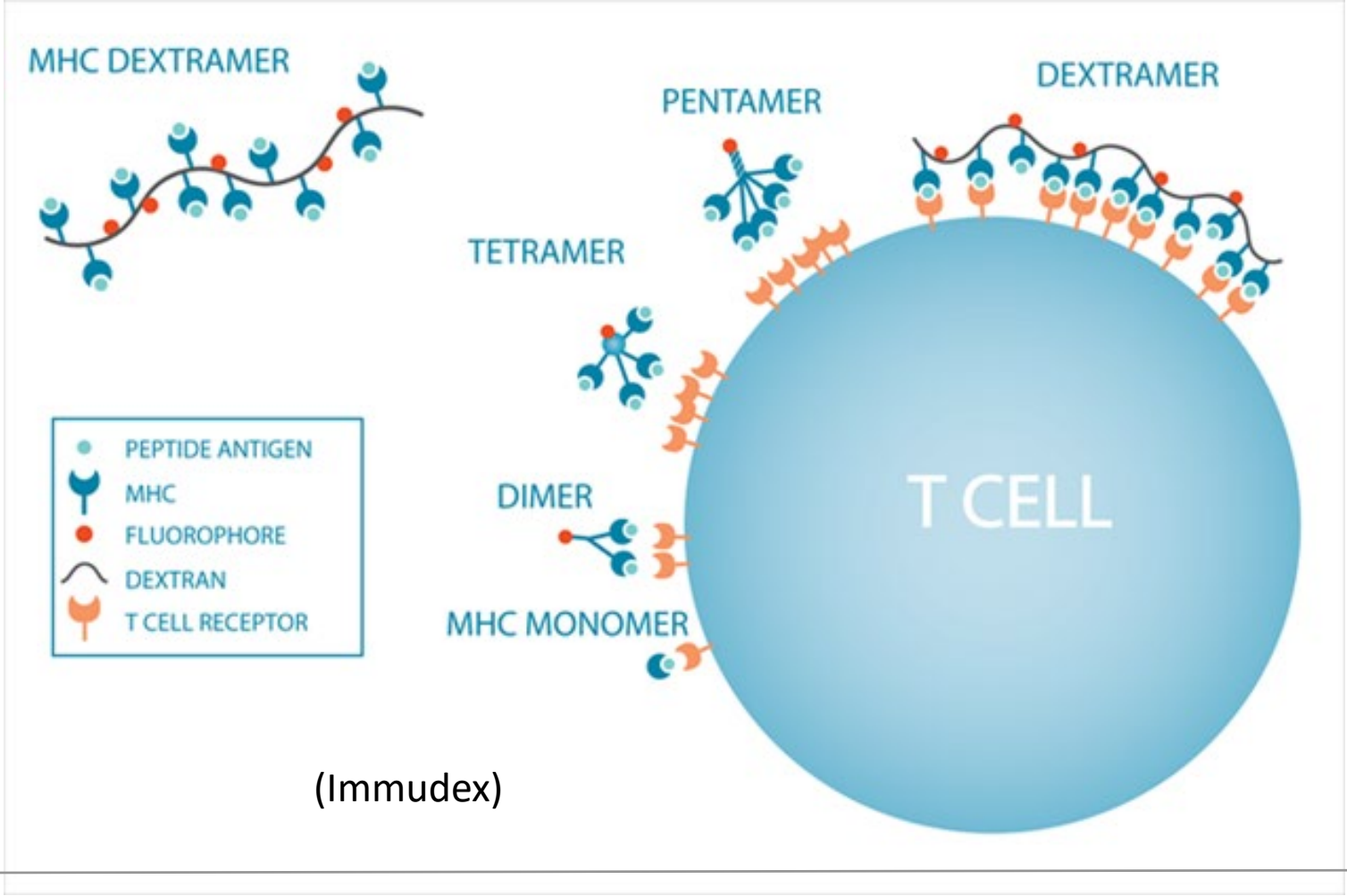
Center for Cellular Immunotherapies

Detection of Specific TCRs by Flow Cytometry

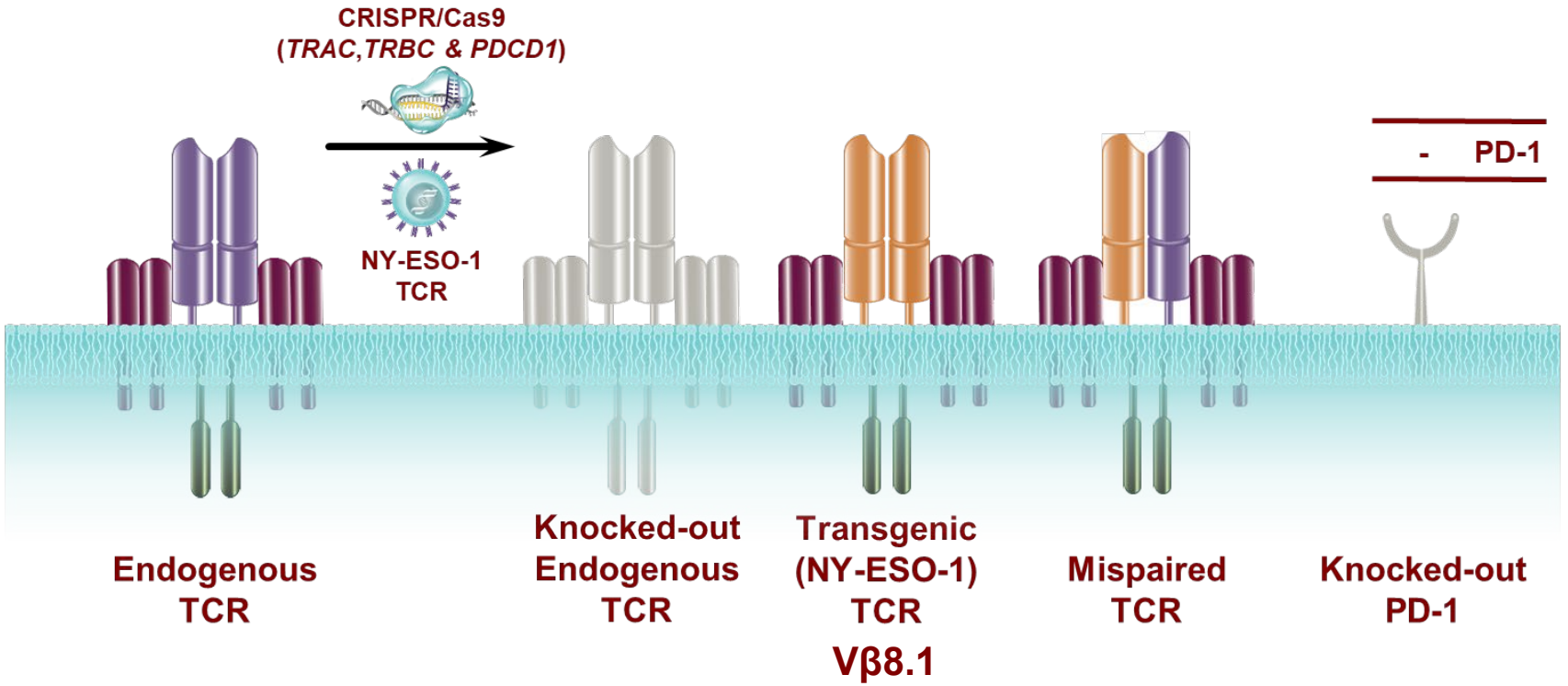


(Immudex)

- Can combine with antibodies in FACS to detect Vβ families.
- TCR diversity can be assessed using (e.g.) Beckman Beta Mark TCR Vβ repertoire kit

Flow Detection of NYESO Cells

Surface Markers for the Detection of TCR-transduced T cells				
CD3:	+	-	+	+
Dextramer:	-	-	+	-
TCR Vβ8.1:	-	-	+	+/-



Stadtmauer et al. Science 2020

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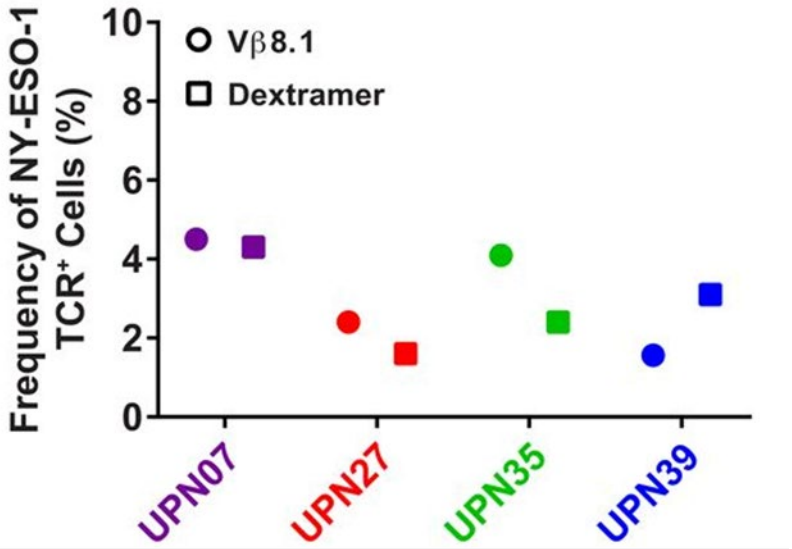
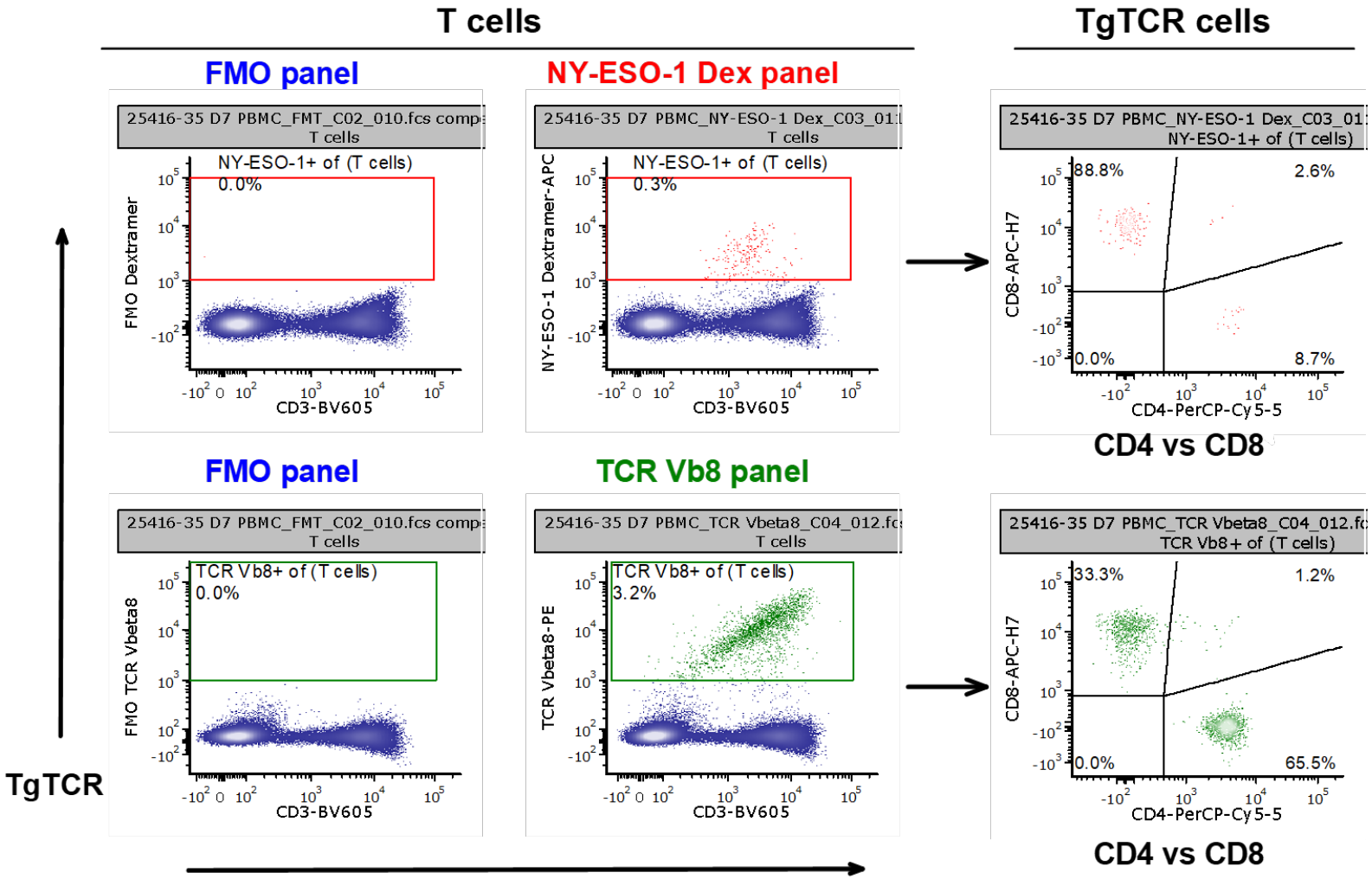
Flow Detection of TCR-Transduced Cells

Manufactured Cell Products



Patient 35 PBMC from 7 days post-infusion



Stadtmauer et al. Science 2020

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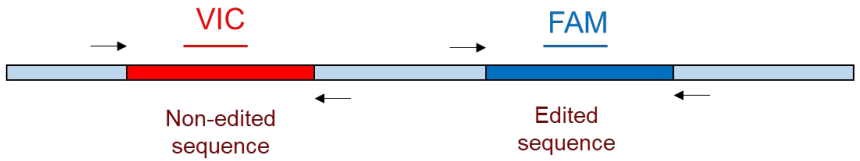
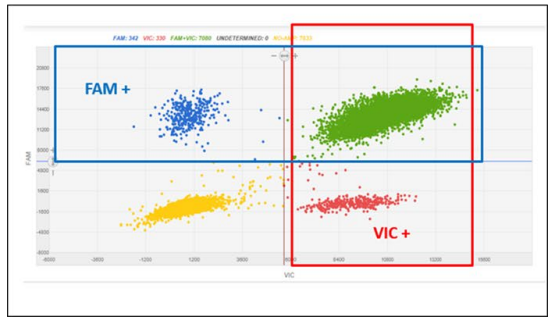
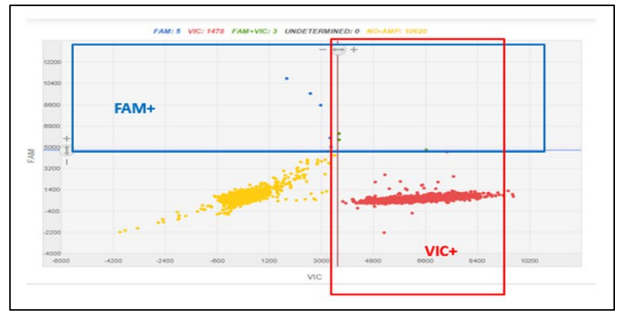
Assessment of Gene Disruption by Duplex Digital PCR



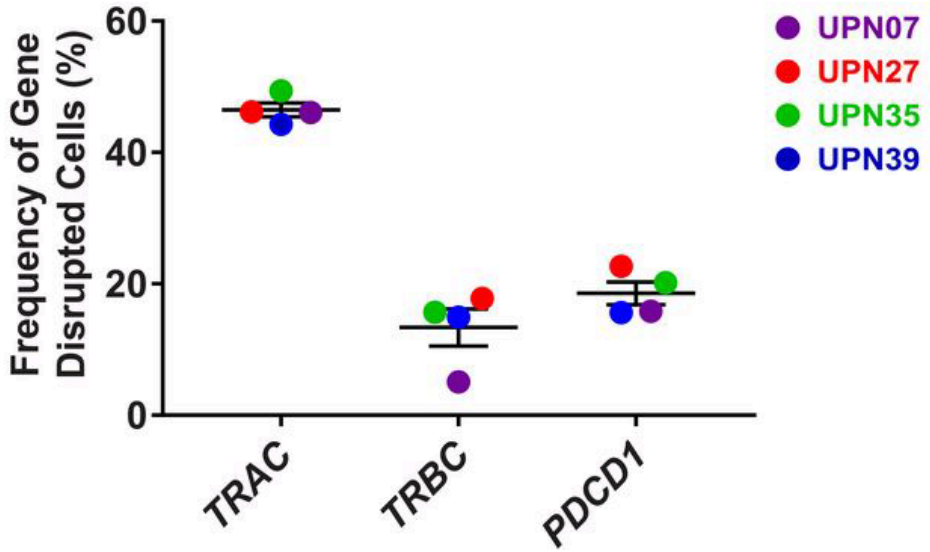


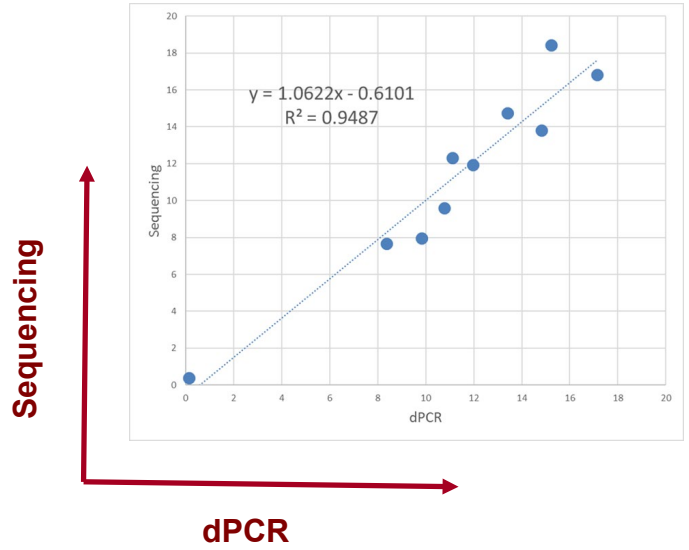
Non-edited template



Edited synthetic template

NYCE infusion products





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Disruption Efficiency = 100 – (FAM copies/µL/VIC copies/µL X 100)

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Assessment of PD-1 Gene Disruption by Flow

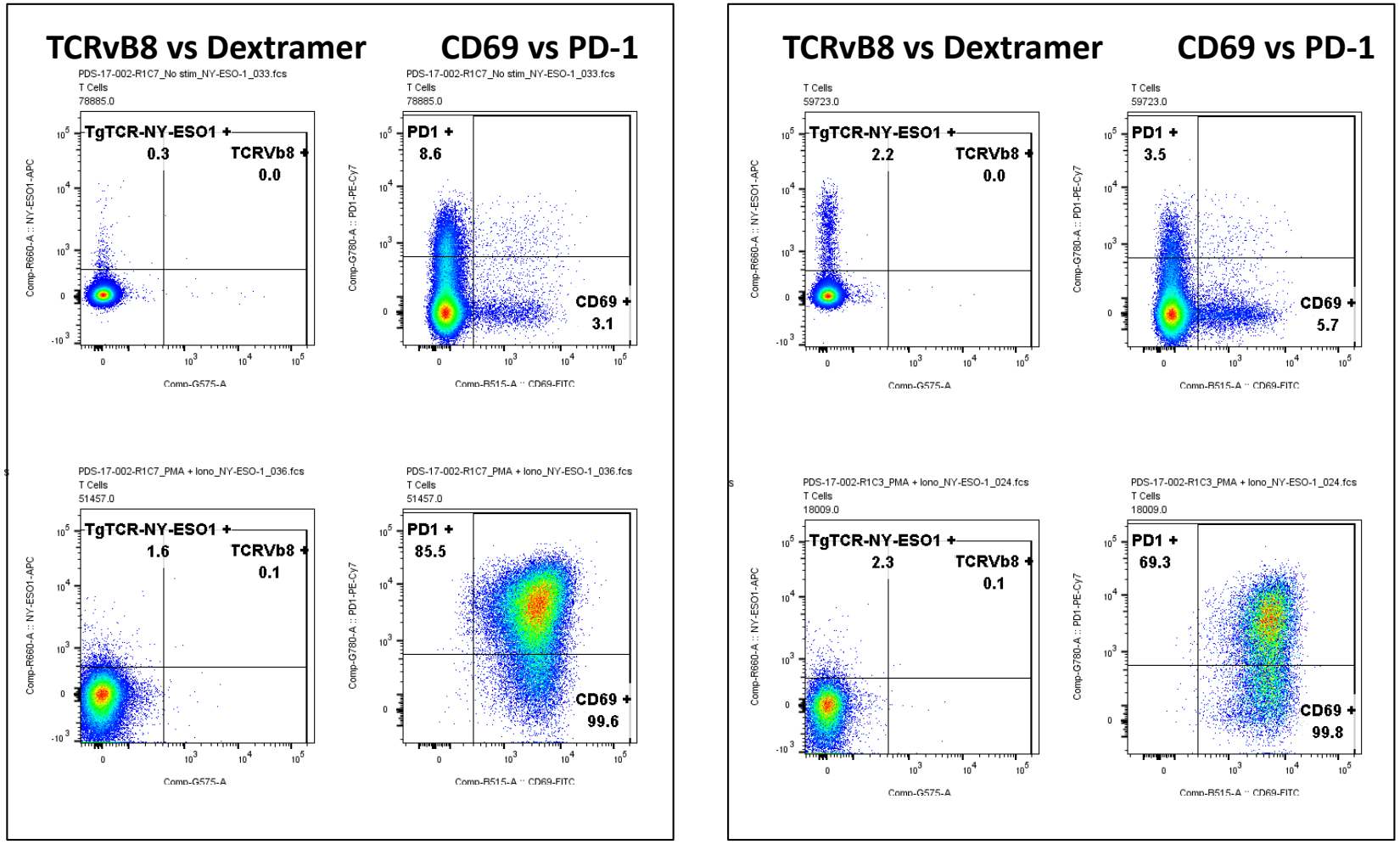
Non-edited product

PD-1 CRISPR edited product

No Stim

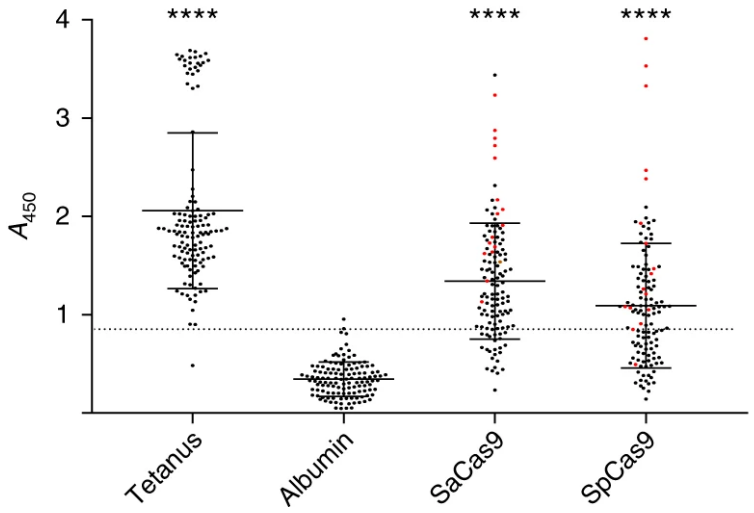
Gated on T cells (L-gate on CD4 vs CD8)

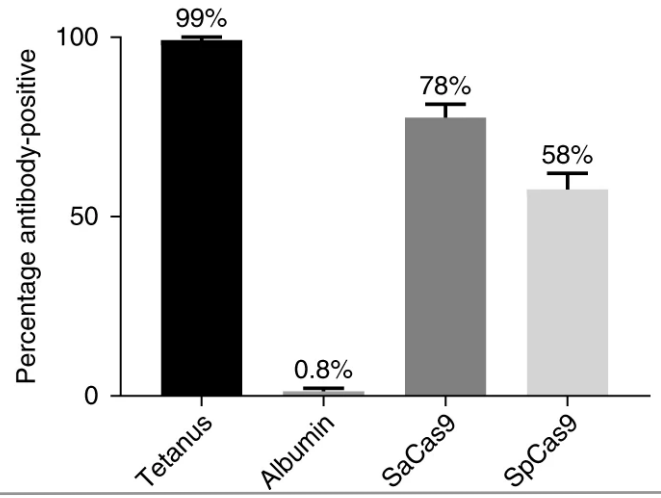
PMA+IONO 24hr



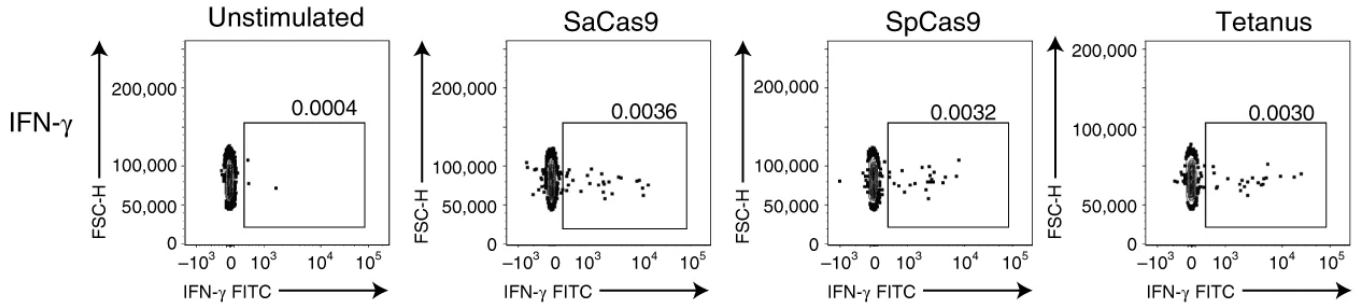
Immune Responses to Cas9 Are Widespread in Normal Donors

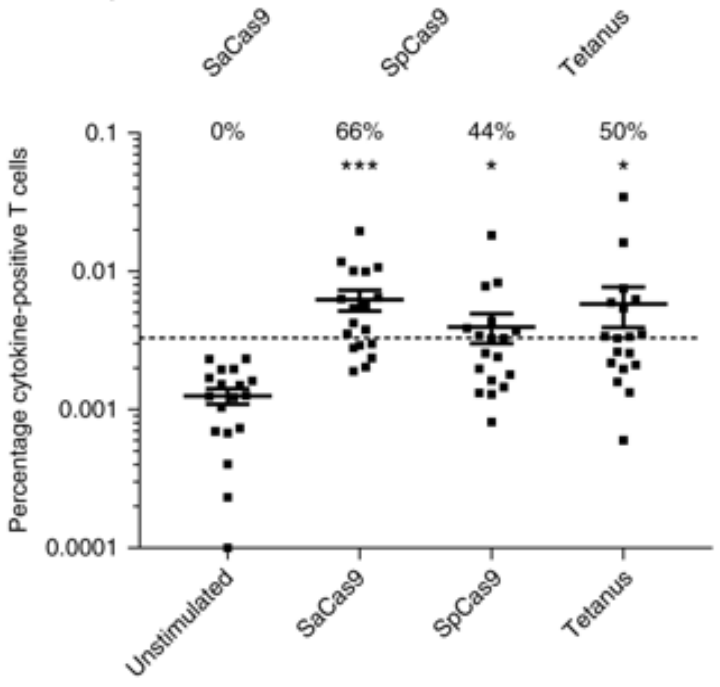
Humoral….





Cellular….





Charlesworth *et al. Nat Med* **25** 249-254, 2019

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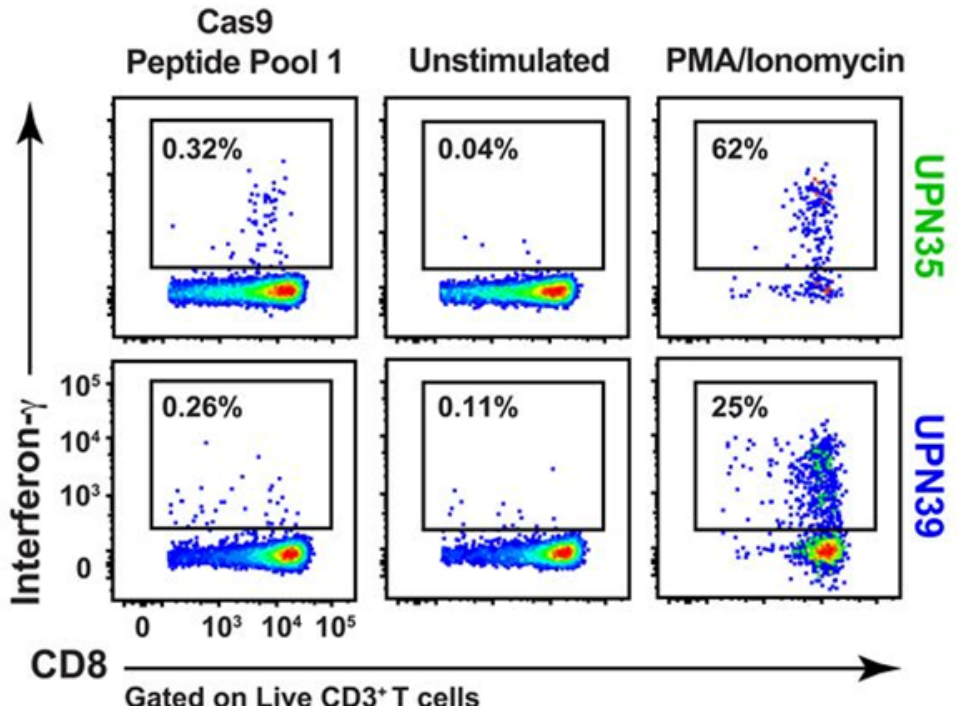
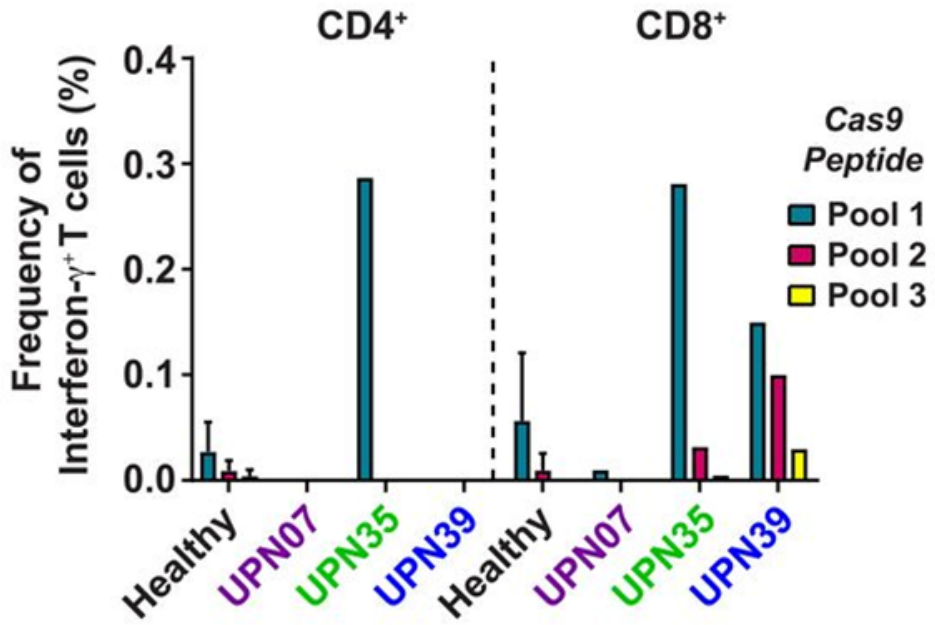
Cellular Immune Responses to Cas9





Healthy donor controls (n = 6). The background frequency of IFN-g–expressing T cells (unstimulated control group, DMSO alone) was subtracted.

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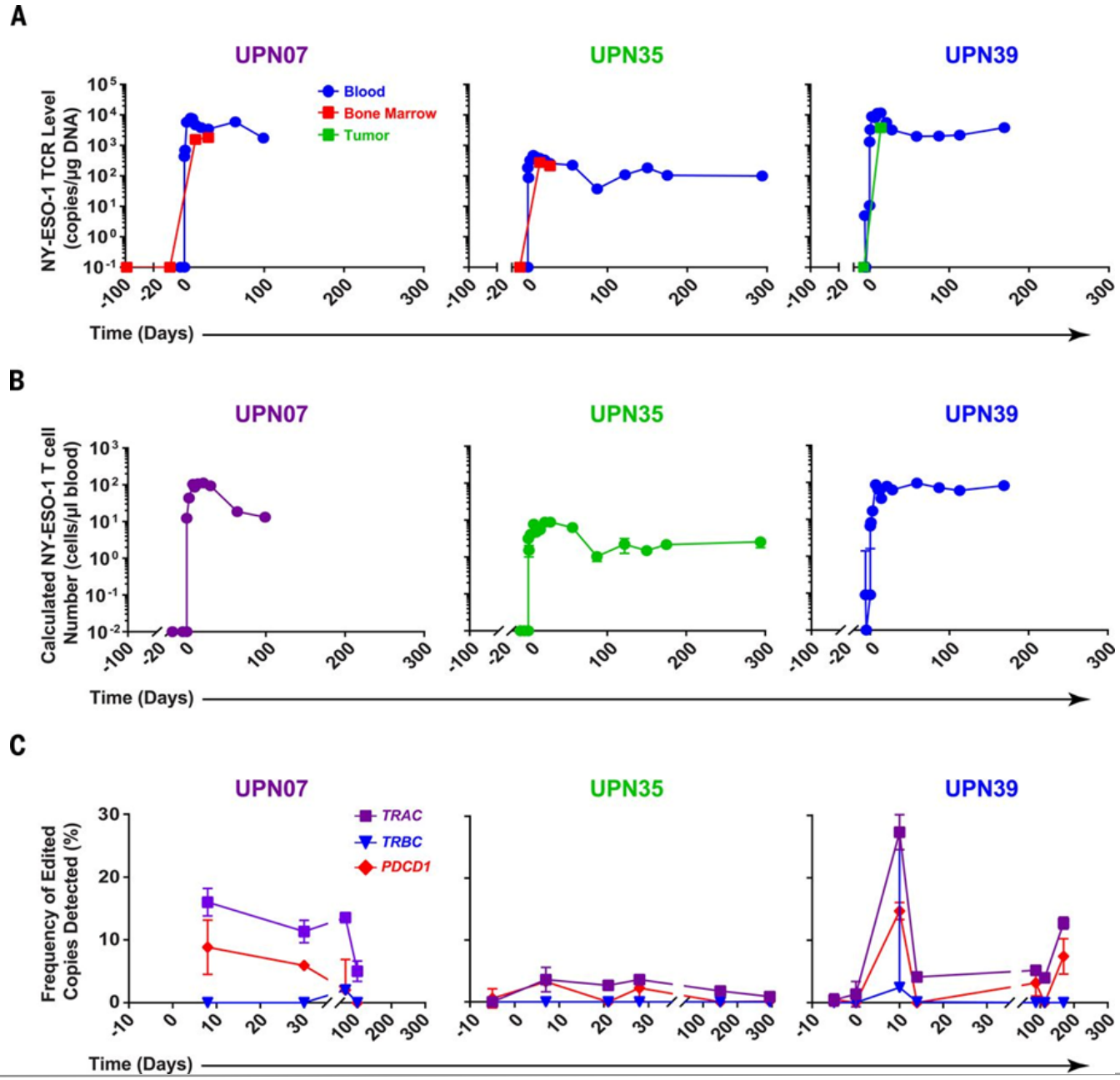
Sustained in vivo expansion and persistence of CRISPR-Cas9-engineered T cells in patients.

Decay half-lives of the transduced cells were 20.3, 121.8, and 293.5 days for UPN07, UPN35, and UPN39, respectively. The average decay half-life was 83.9 days.

Persistence higher than seen in our previous NY-ESO-1 TCR–engineered T cells trials where half-life was ~1 week.



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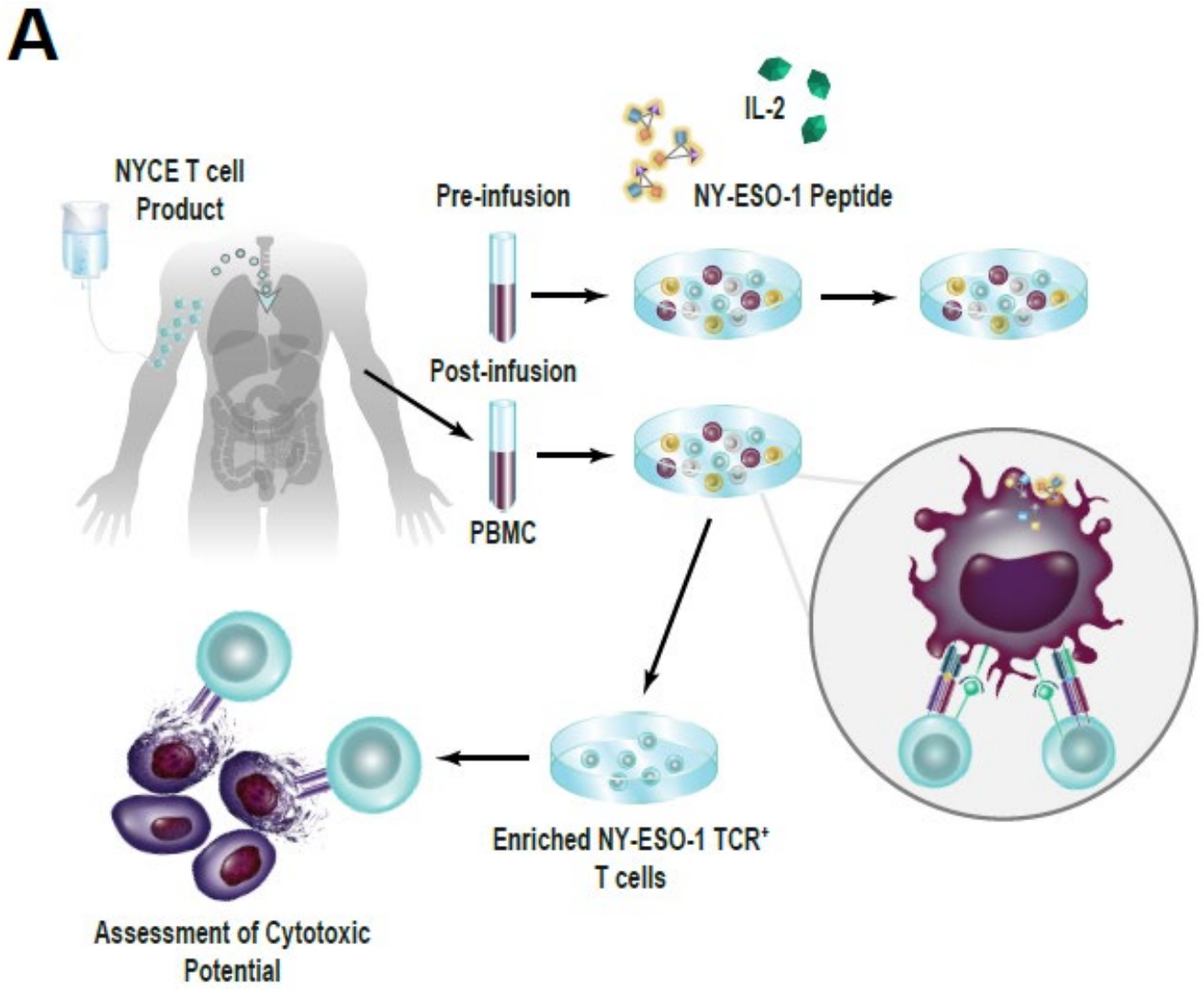
Are the NYCE cells functional after infusion? Schema for recovery and *in vitro* expansion with antigen



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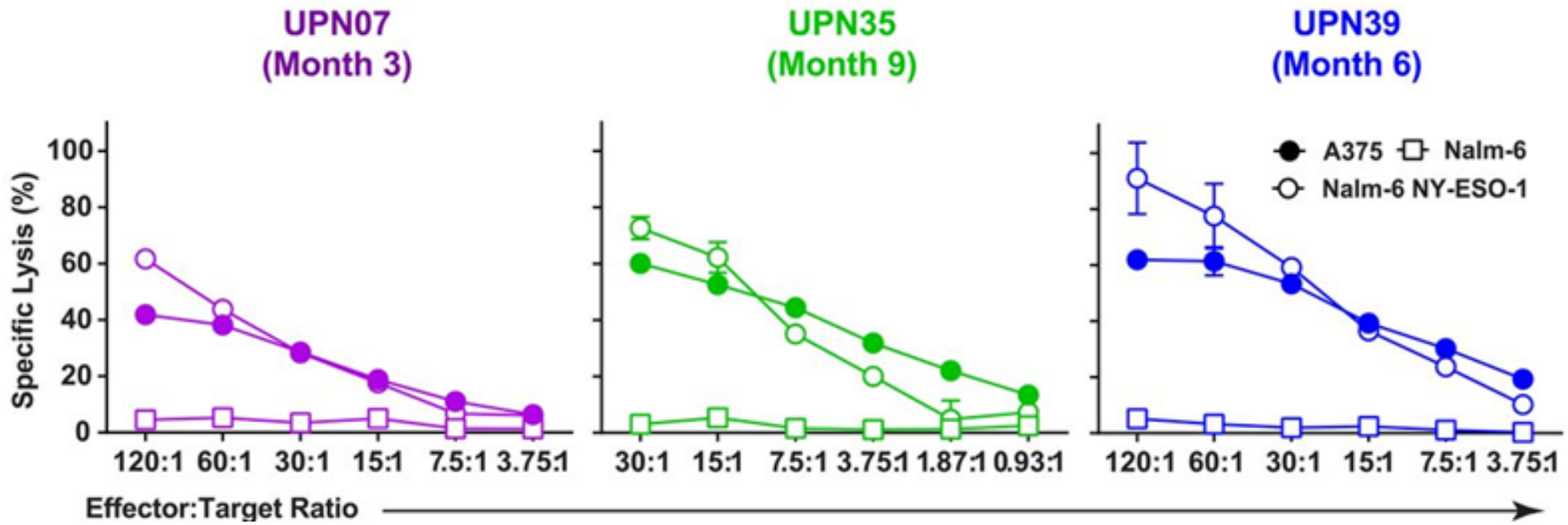
Are the NYCE cells Functional After Infusion?
NYCE cells recovered and expanded *in vitro* are cytotoxic to NY-ESO1 expressing tumor cells



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Summary:

- Flow cytometry is an essential tool for detection and evaluation of engineered T cells before and after infusion
- Maximize strength of flow methods by using them in conjunction with molecular assays to detect introduced sequences, gene editing and mRNA transcripts) and biochemical assays (Luminex, ELISA MSD etc.) that detect secreted proteins
- Be creative in adapting flow to the needs of the specific engineered product
- Include appropriate controls, validate assays, and attempt to generate quantitative as well as qualitative data

Acknowledgements

TCSL sample processing
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Jeff Finklestein
Tatiana Mikheeva
Chelsie Bartoszek
Brett Menchel

TCSL molecular assays
Irina Kulikovskaya
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Rachel Reynolds
Angela Kim
Todd Yoder

TCSL biomarker assays
Fang Chen
Natalka Koterba

TCSL Biostats & data mgt
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Mohsin Mahir

TCSL flow
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Harit Parakandi

PDL
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Jun Xu

Stanford (scRNAseq)
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Ansuman Satpathy
Kevin Parker

Lentiviral vector Integration site analysis and GUIDE-seq
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Chris Nobles

CVPF
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Megan Davis
Gabriela Plesa
Andrew Fesnak
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Al Garfall
Eric Lancaster
Kristy Weber

CCI & June lab
Carl June
Anne Chew
Bruce Levine
Regina Young
Jos Melenhorst
Beatriz Carreno
Julie Jadlowsky
Elizabeth Hexner
Wei-Ting Hwang
Yangbing Zhao

Questions?

PARKER INSTITUTE
for CANCER IMMUNOTHERAPY

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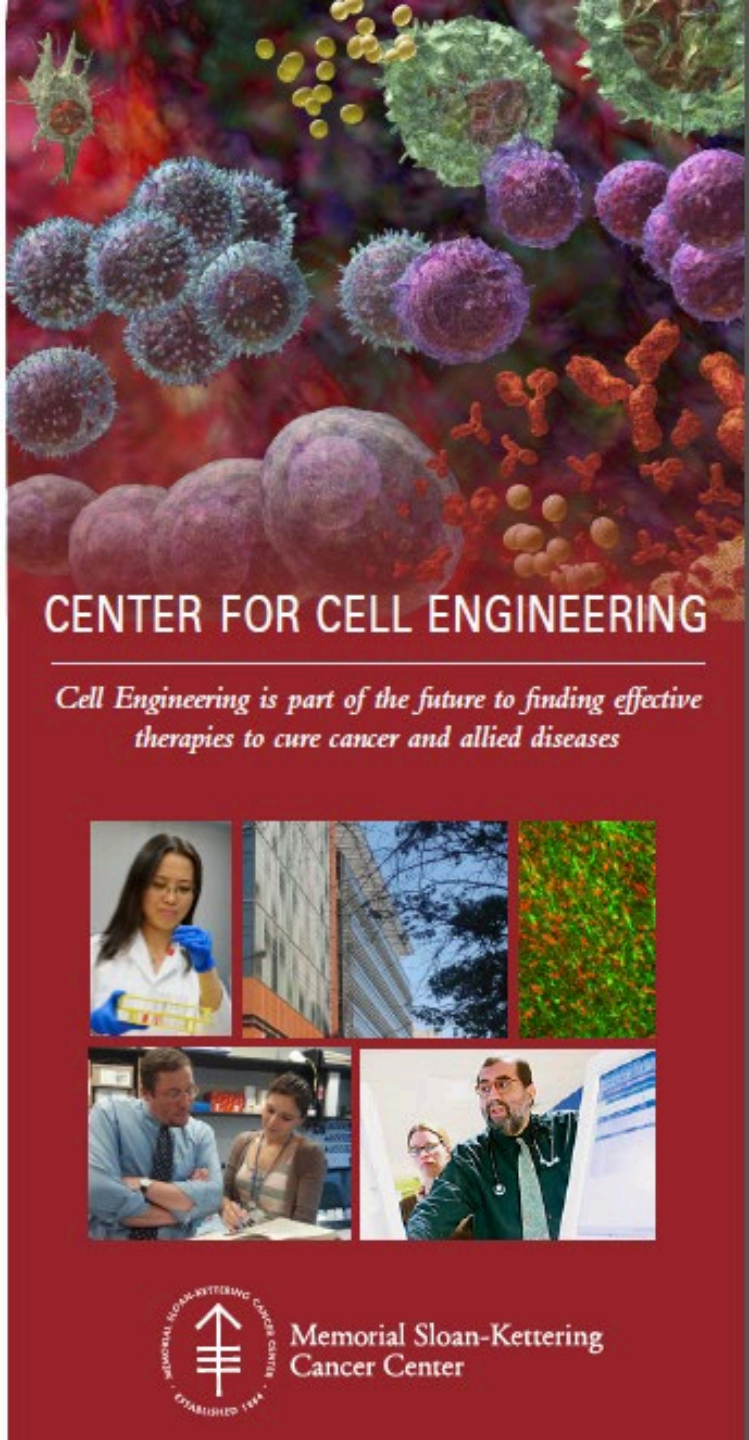
STAND UP TO CANCER®

Autologous and Allogeneic CAR T Cell Manufacturing

Xiuyan Wang, PhD

Memorial Sloan Kettering Cancer Center
New York, New York, USA



NIST Flow Cytometry Standards Consortium Workshop|NIST
Feb 16, 2021

Adoptive T Cell Therapy for Cancer

FDA News Release

FDA approval brings first gene therapy to the United States

CAR T-cell therapy approved to treat certain children and young adults with B-cell acute lymphoblastic leukemia

Kymriah – August 2017

FDA approves CAR-T cell therapy to treat adults with certain types of large B-cell lymphoma

Yescarta is the second gene therapy product approved in the U.S.

Yescarta – October 2017

FDA Approves First Cell-Based Gene Therapy For Adult Patients with Relapsed or Refractory MCL

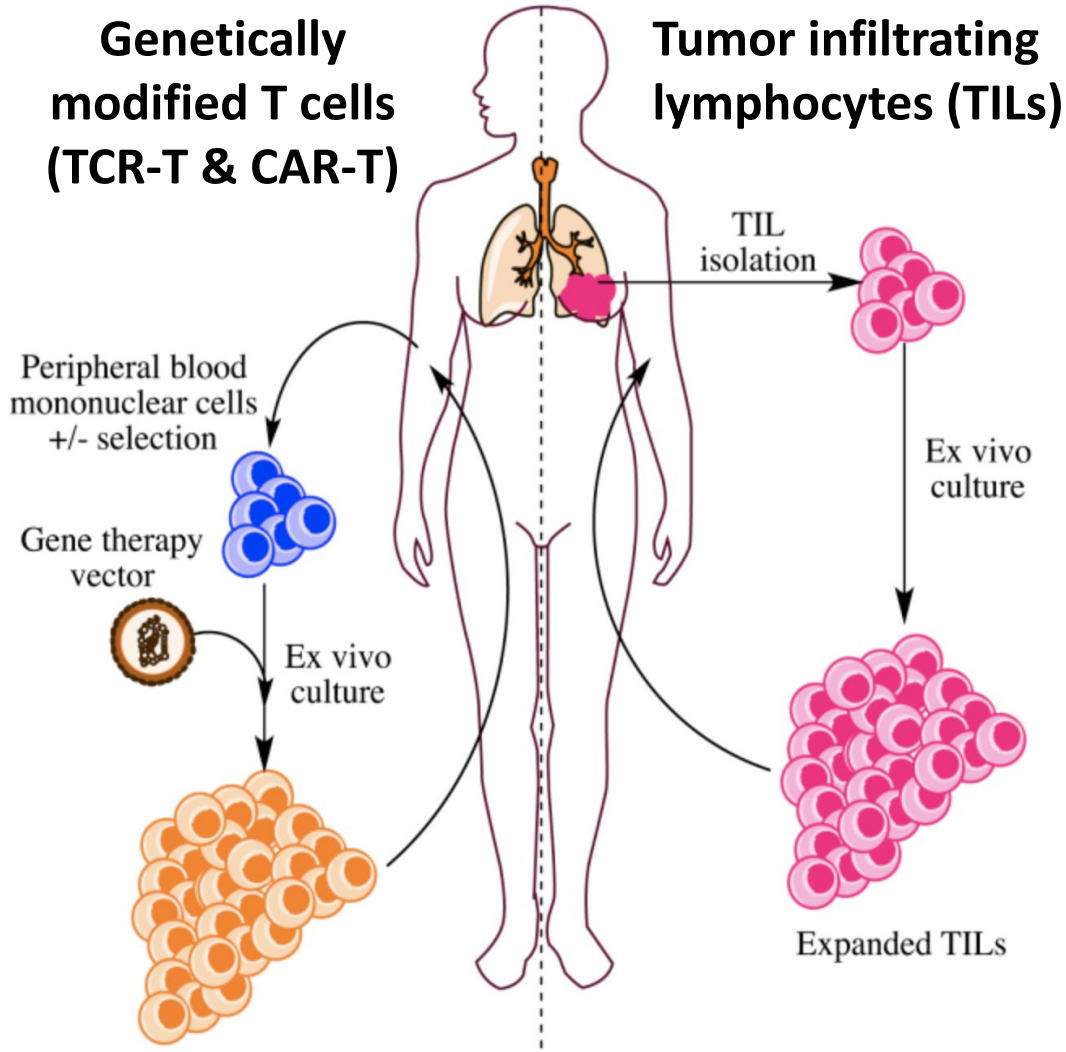
TeCartus the first cell-based gene therapy approved by the FDA for MCL

Tecartus – July 24, 2020

FDA Approves Lisocabtagene Maraleucel for Relapsed or Refractory Large B-cell lLymphoma

A new biologic for treating large B-cell lymphoma

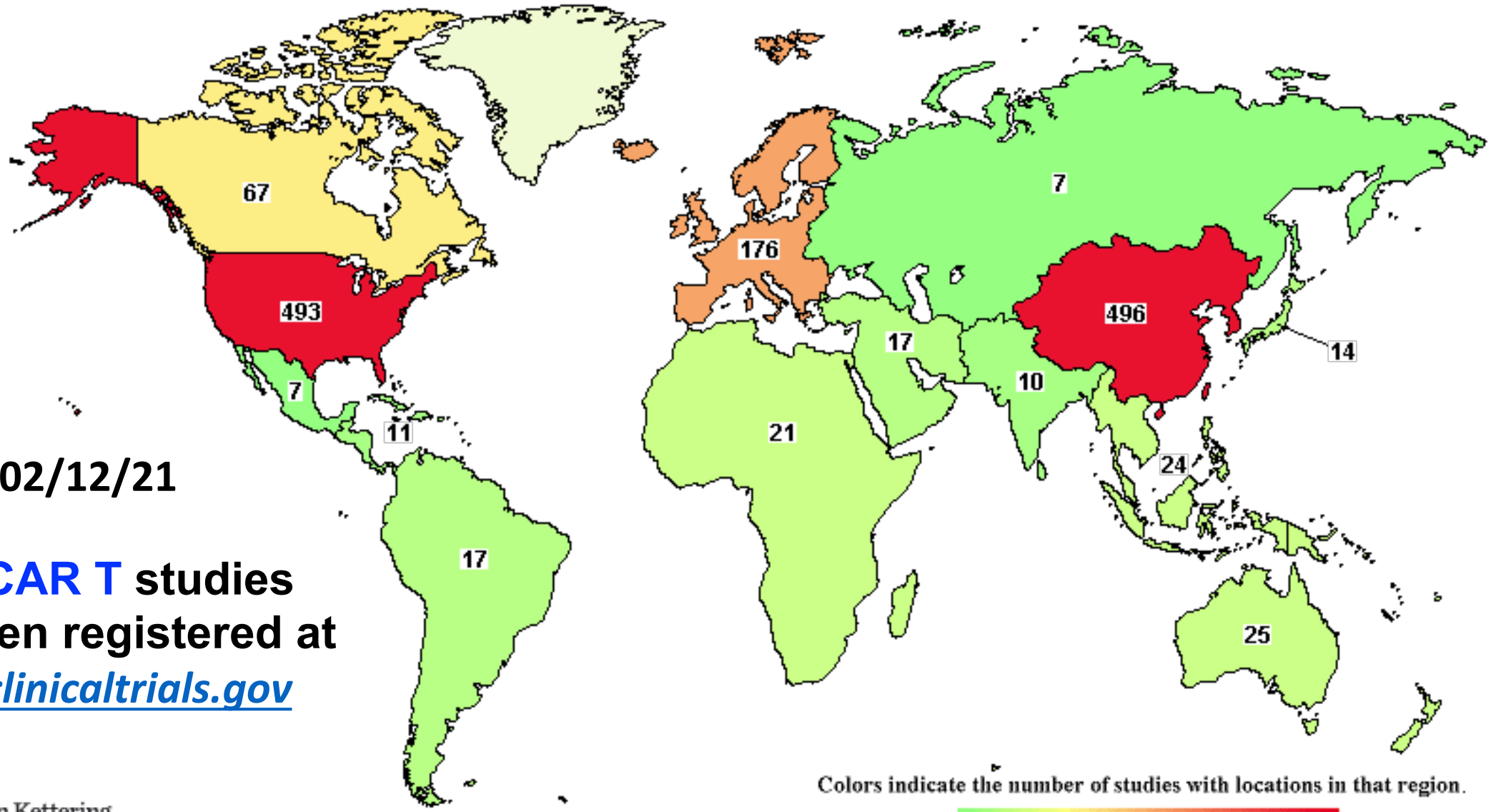
Breyanzi – Feb 5, 2021



Milone MC and Bhoj VG, *Mol Ther Methods Clin Dev 2018*

Memorial Sloan Kettering Cancer Center

Current CAR T Cell Clinical Trial



As of 02/12/21

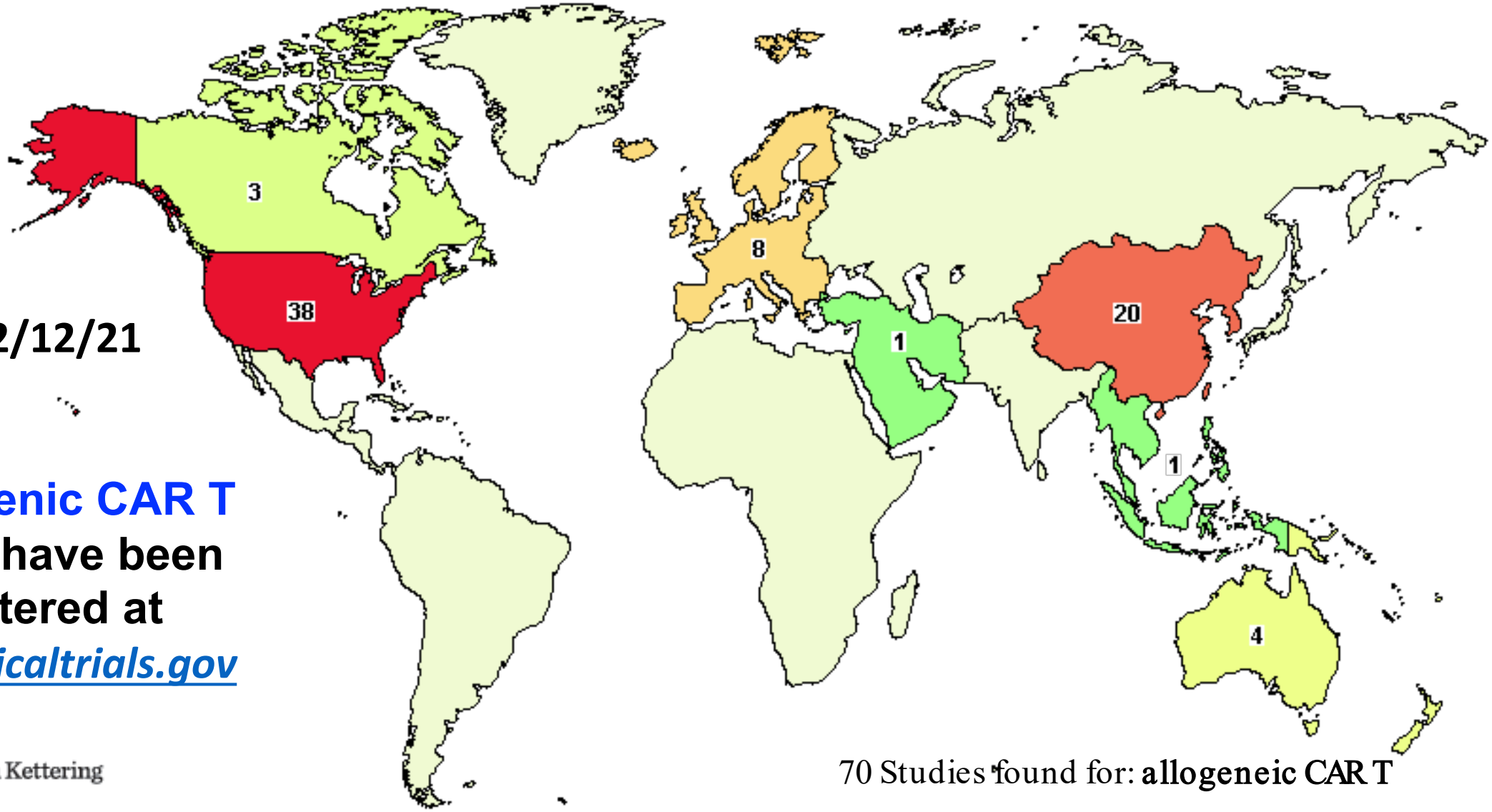
1294 CAR T studies have been registered at *www.clinicaltrials.gov*

Memorial Sloan Kettering Cancer Center

Current Allogeneic CAR T Cell Clinical Trial

As of 02/12/21

70 Allogenic CAR T studies have been registered at *www.clinicaltrials.gov*

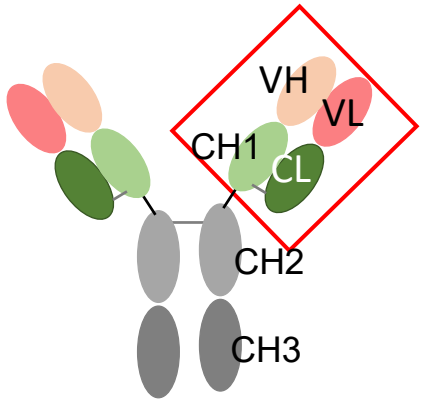


70 Studies found for: allogeneic CAR T

Memorial Sloan Kettering Cancer Center

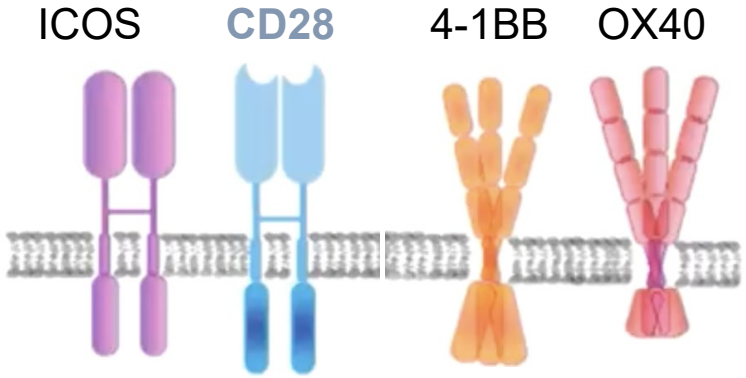
Chimeric Antigen Receptor (CAR)

Murine/human mAb

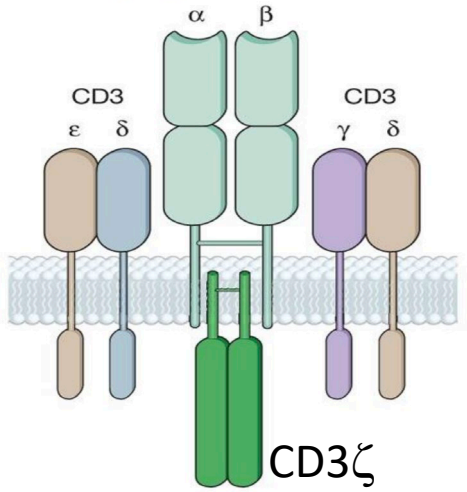


scFv

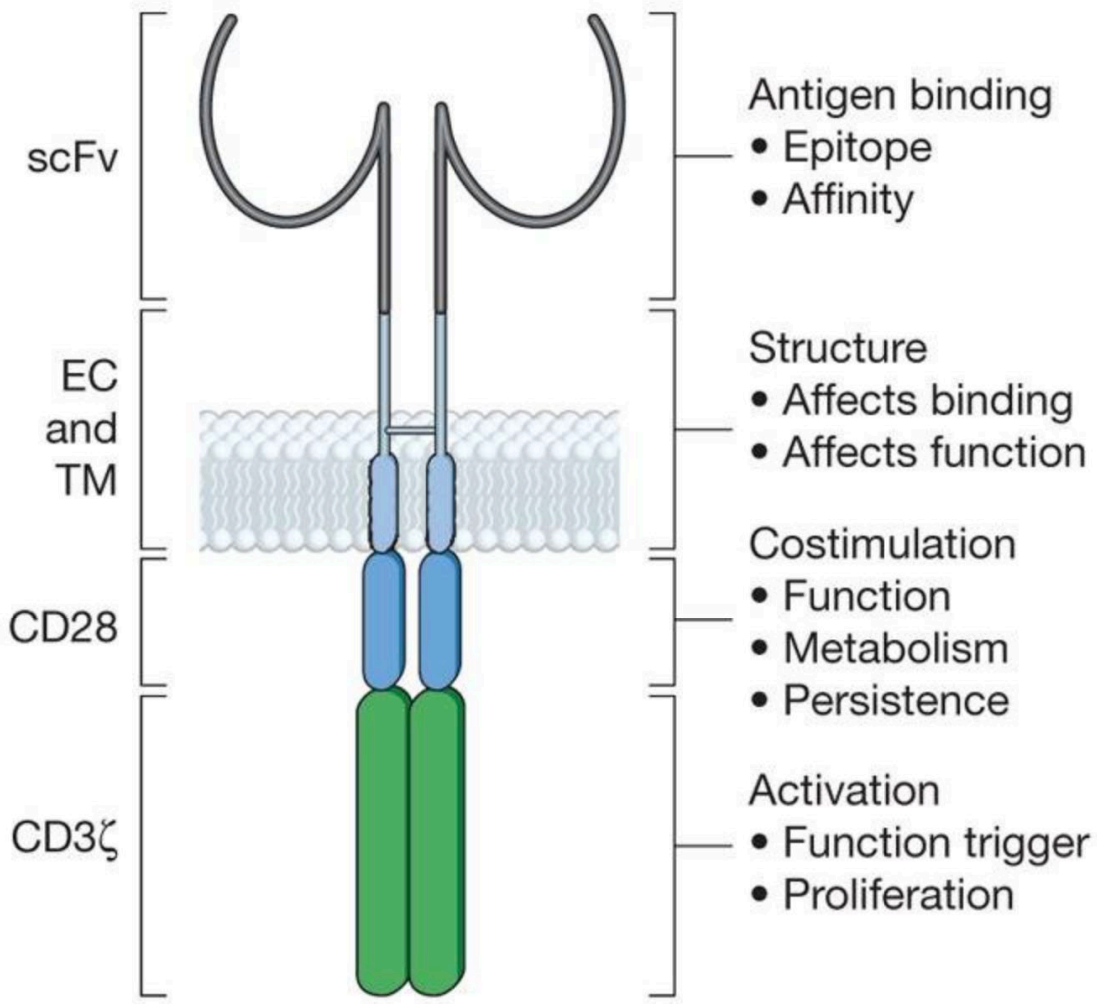
Co-stimulatory molecule



TCR/CD3 complex



FDA approved CAR design



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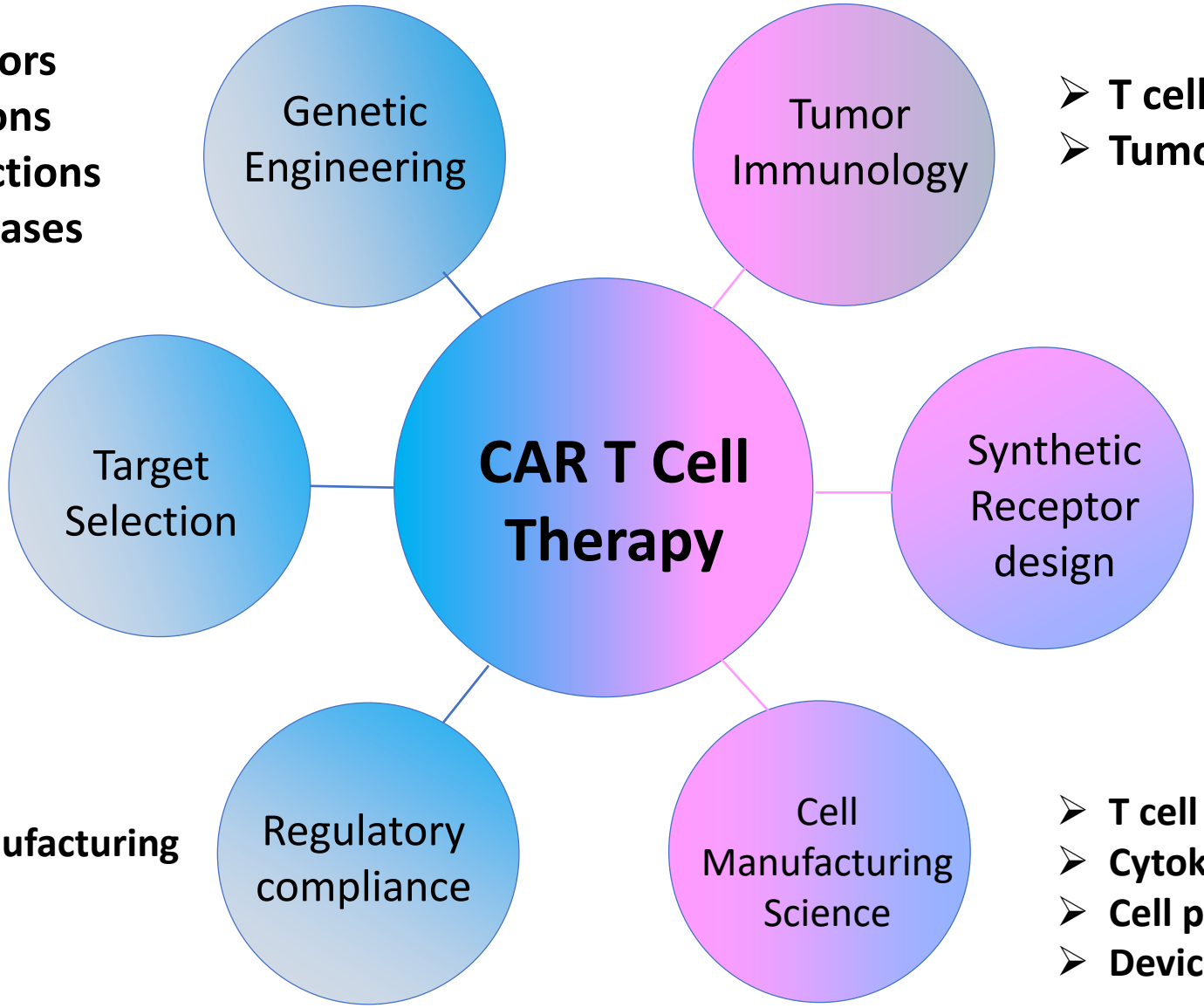
Assembling CARs for T Cell Therapy



- Retroviral vectors
- DNA transposons
- mRNA transfections
- Targeted nucleases

- T cell immunobiology
- Tumor microenvironment

- CD19 paradigm
- Target discovery (solid tumors)
- Combinatorial targeting

- T cell signaling
- Safety switches

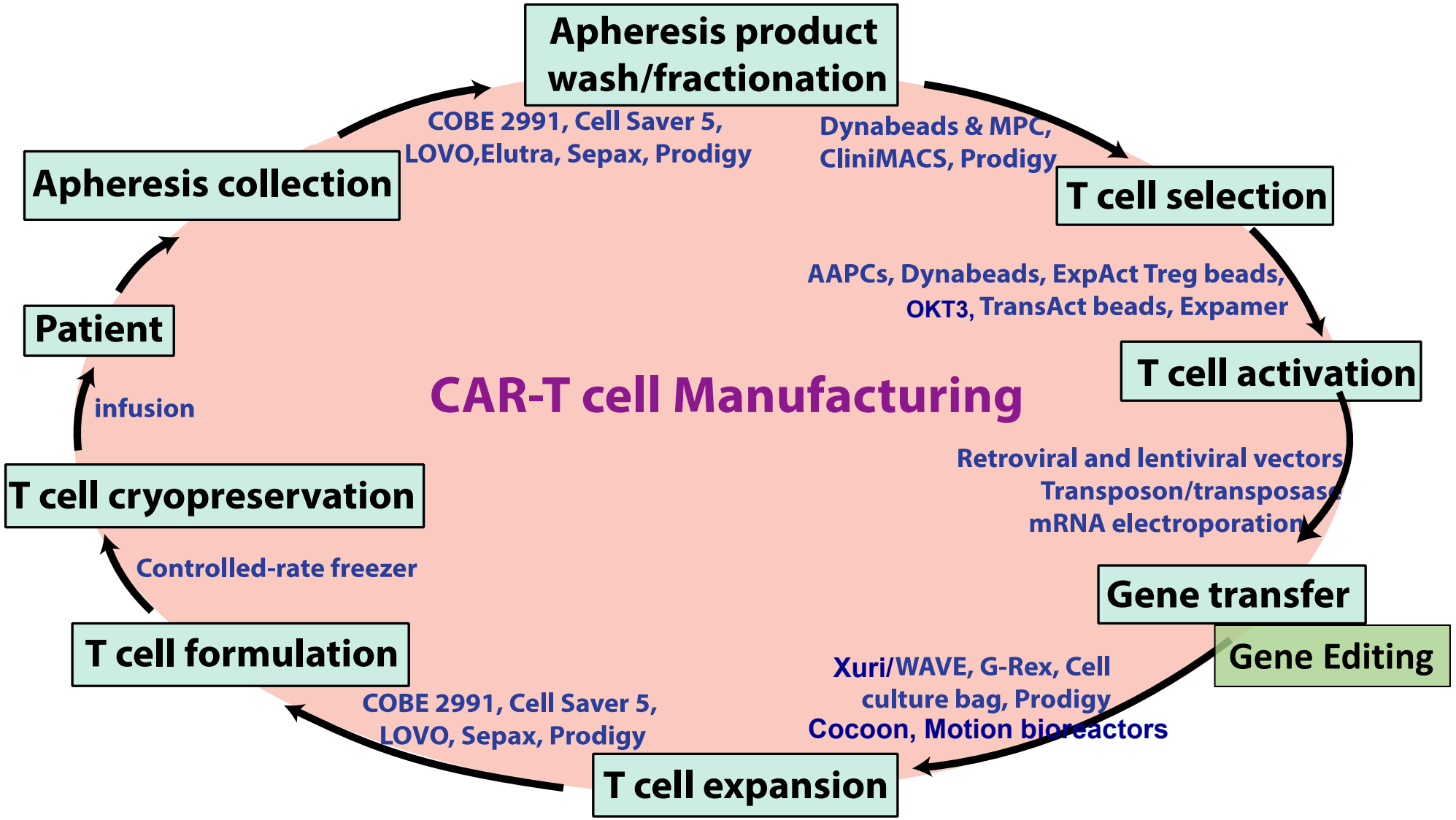
- cGMP manufacturing
- CFR210

- T cell activation
- Cytokines, small molecules
- Cell purification
- Devices

Memorial Sloan Kettering Cancer Center

Adapted from Riviere & Sadelain, Molecular Therapy, 2017

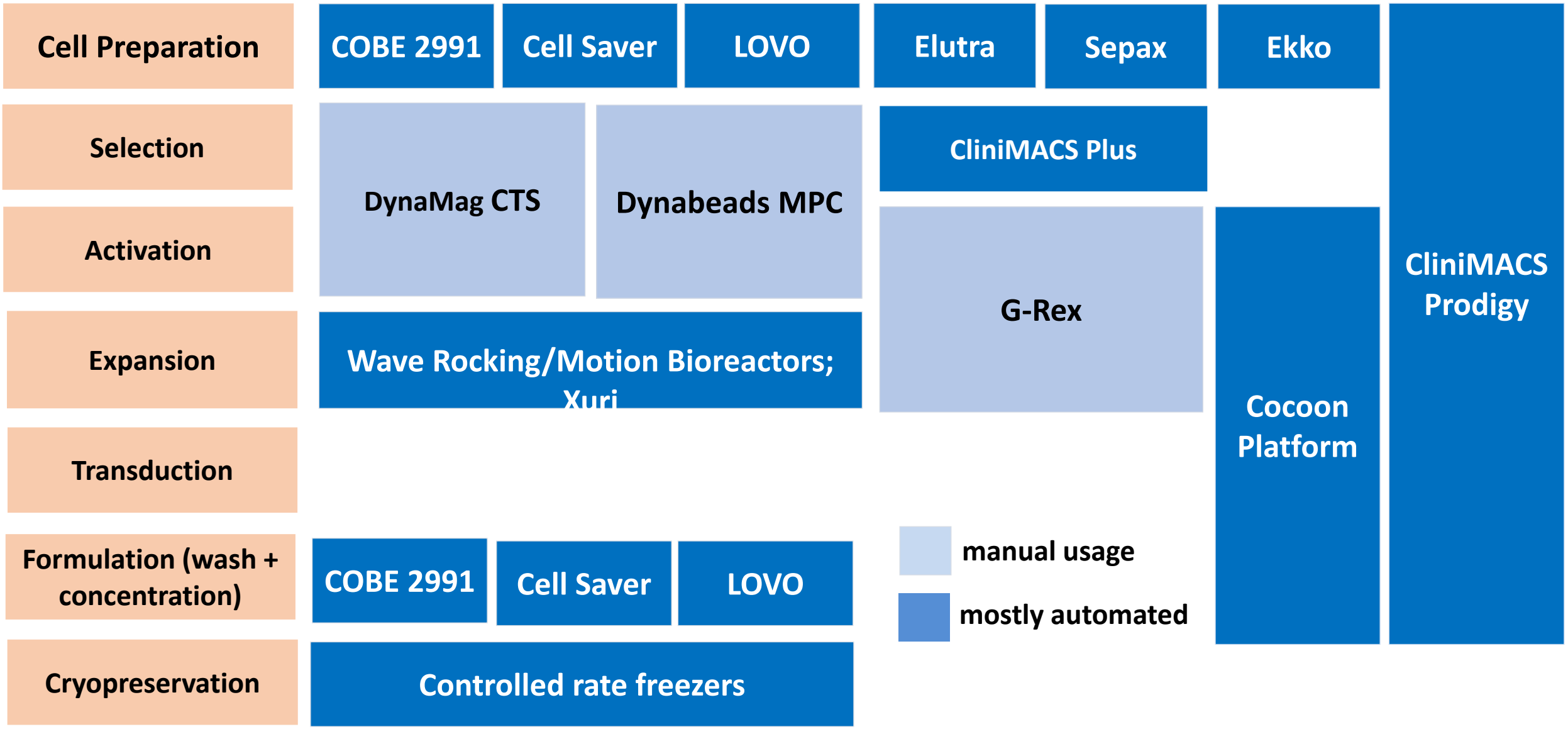
Major Steps in Autologous CAR-T Cell Manufacturing Process



Memorial Sloan Kettering Cancer Center

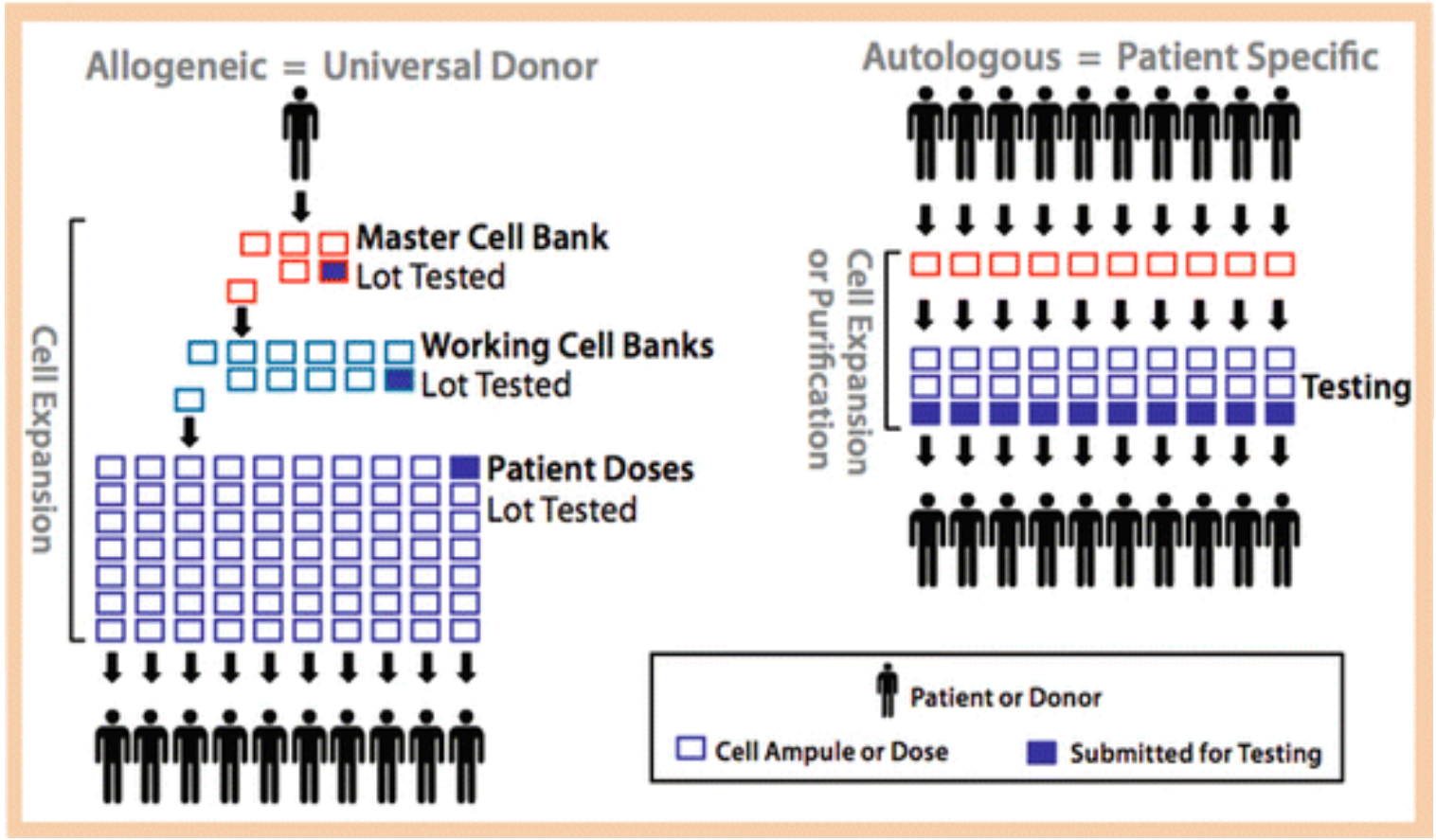
Adapted from Wang X. and Rivière I. *Molecular Therapy - Oncolytics, 2016*

Examples of Devices used for Clinical-Grade T Cell Manufacturing



Adapted from Kaiser et al Cancer Gene Ther. 2015 Mar; 22(2): 72–78.

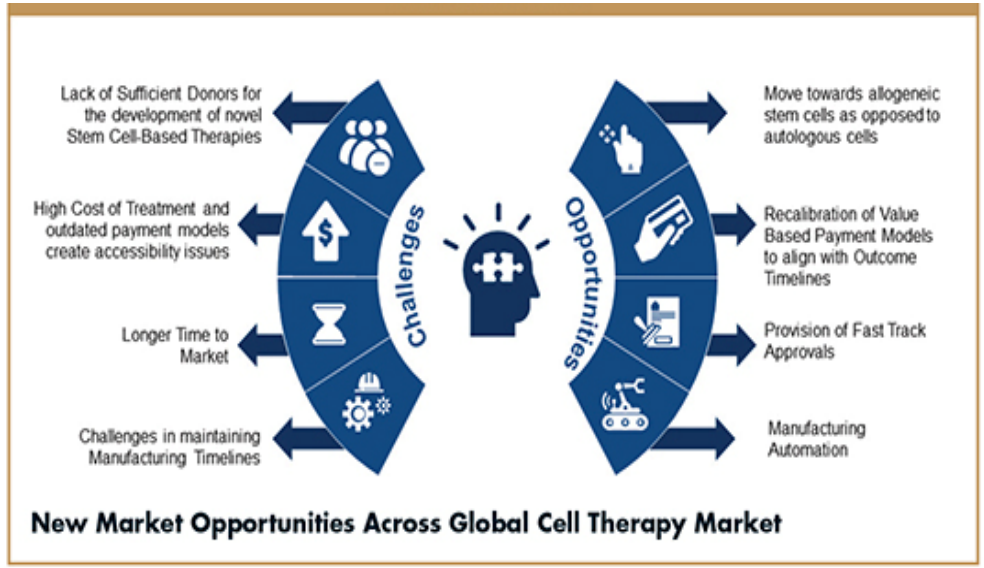
From Autologous to Allogenic Manufacturing



A.L. Van Deusen and M.E. McGary, Book Chapter, 2016, DOI 10.1007/978-1-4939-3228-3_7

Advantages of Allogenic Manufacturing

- **Cost effectiveness: large batch size**
- **Young/healthy donor tissue as starting material**
- **Availability: off-the-shelf**



Aarti Chitale., REGENERATIVE MEDICINE – Cell Therapy – The Quest for Finding the Cure, Nov/Dec 2018

Challenges of Allogenic Manufacturing

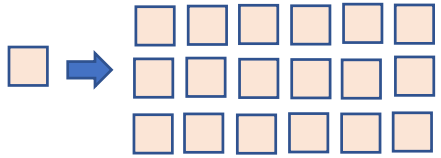
Starting Cells

*Criteria for choosing the best donor/source

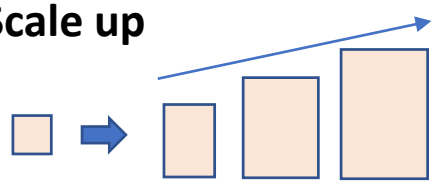
*Optimal passage# for MCB/WCB

Cell Expansion

Scale out



Scale up



Facility Design

End of Process

Large volume Harvesting

Formulation

Fill

Cryopreser-vation

Storage

Temperature Sensitive

Large quantity

Stability

Process Development Challenges

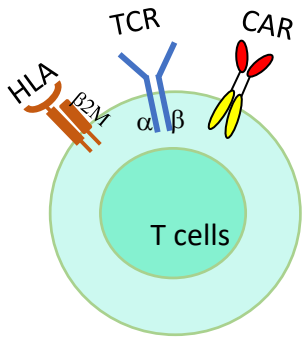
- Lack of expertise for large-scale production of cell-based products
- Strategy for a few liter scale to few hundred/thousand liters of culture
- Definition and maintenance of product critical quality attributes (CQAs)
- High cost of process development and conducting clinical trials

Strategies to Develop Allogeneic Cell Therapies with CAR

Two Main Issues to address for Allogeneic Cell Therapy: 1. Graft vs Host disease 2. Host clearance of infused cells

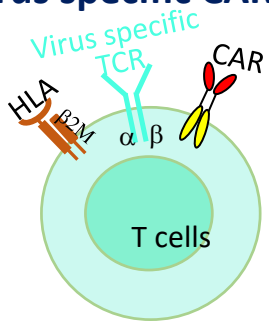
Stem Cell Donor-derived CAR T

for transplant relapsed patients

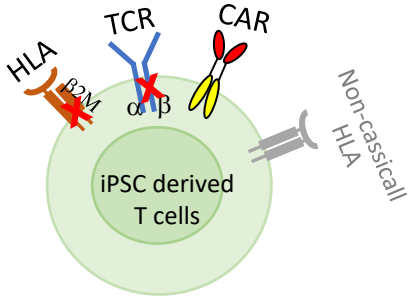


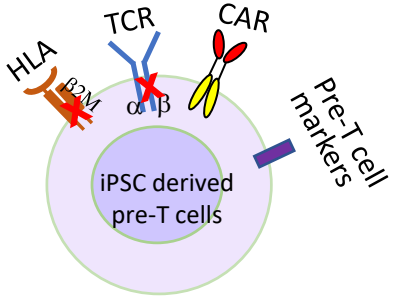
Non-Alloreactive T cells

Virus specific CAR T cells



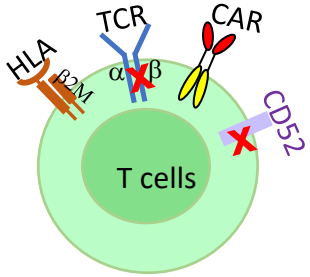
iPSC derived CAR T cells



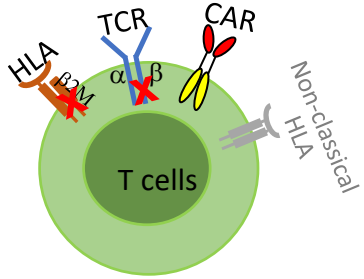


Gene Edited CAR T cells

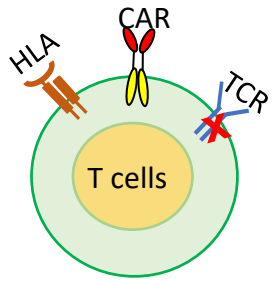
TCRα/CD52 edited



TCR/MHC edited

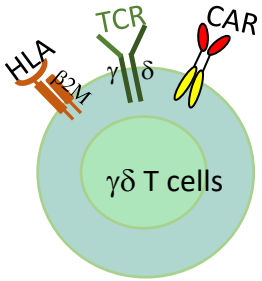


TRAC CAR

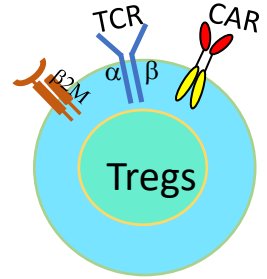


Alternative Effector Cells

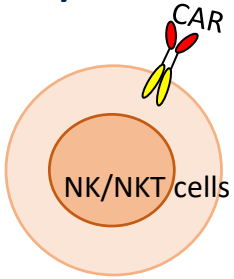
γδ T cells



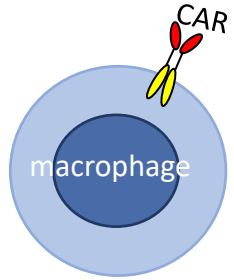
Treg cells



NKT/NK cells



Macrophage cells



Memorial Sloan Kettering Cancer Center

Critical Components and Characterization Requirement of Cell Products

Clinical Manufacturing Requirements

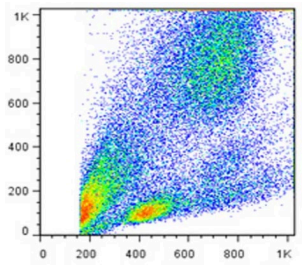
Safety

- **Microbiological safety**
  - Demonstrate product is sterile and absence of mycoplasma
- **Virological safety**
  - Demonstrate product is free of infectious agents and RCV
- **Genomic stability**
  - Demonstrate product is absence of genotoxicity and tumorigenicity

Purity

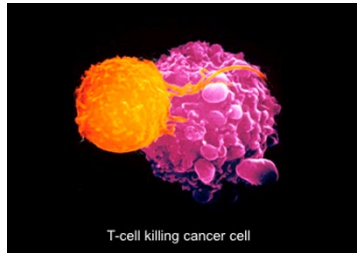
- Demonstrate product is free of endotoxin and pyrogen
- Determine levels of impurities introduced during process such as antibiotics, cytokines, small molecules
- Determine the levels of impurities arising from raw materials such as FBS, feeder cells or matrix

Identity



- Demonstrate phenotypic expression of surface markers
- Demonstrate differentiation stage
- Demonstrate absence of immunogenicity. *etc*

Potency



- The most difficult feature to characterize
- Measure the biological function of a product
- Ideally the testing results can correlate with clinical outcomes

In-Process Monitoring for Production

Quality by Design

Characterization of the Starting Cell Population

Starting Cell Characterization/ Attributes	Read out
Quantity	Cell count
Health	Viability
Cell Composition	FACS of lineage markers
Cell Differentiation stages	FACS for specific markers
Tumor burden if applicable	FACS for tumor markers

In-process sampling and monitoring

In-process decision points

Product characterization

Final CAR T Cell Product Lot Release Testing

Test Attribute	Testing for Autologous Therapy	Testing for Allogenic Therapy	Testing method
Safety	Gram Stain / Sterility	Gram Stain / Sterility	Gram Stain
	Sterility	Sterility	USP<71> or BacT/Alert
	Mycoplasma	Mycoplasma	MycoAlert, MycoPCR or USP
	Endotoxin level	Endotoxin level	UPS or kinetic chromogenic LAL
	Residual beads if applicable	Residual beads if applicable	Visual count under Microscope
	Cytokine independent proliferation	Cytokine independent proliferation	*In vitro* culture
	Replication competent virus (RCR, RCL, RCA)	Replication competent virus (RCR, RCL, RCA)	Indicator cell assay, PERT or PCR
Purity	% CD3+ T cells	% CD3+ T cells	Flow Cytometry
	Residual tumor burden if applicable	Residual tumor burden if applicable	Flow Cytometry
	Residual ancillary reagents (such as cytokines)	Residual ancillary reagents (such as cytokines)	USP or ELISA
		Residual feeder cells if used	qPCR
		Residual parental cell line	qPCR
Identity	% CAR+ CD3+	% CAR+ CD3+	Flow Cytometry
	Copy# of transgene	Copy# of transgene	qPCR
		additional markers (such as TCR knock down)	Flow Cytometry
		additional differentiation stage marker (such as CD7)	Flow Cytometry
Potency	Cytotoxicity	Cytotoxicity	Cytotoxicity T Lymphocyte Assay
Dose	Cell count& Viability	Cell count& Viability	Automatic Cell Counter

Center for Cell Engineering

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P. Adusumilli MD
J. Mansilla-Soto, Ph.D
J. Eyquem, Ph.D (UCSF)
J. Feucht, MD (Tubingen Univ.)

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M. Geyer MD
Medical Teams, Nurses, RSA
E. Smith MD (Dana Farber)

Cytotherapy Lab
Blood Donor Room

Biovec Pharma

M. Caruso
K. Ghani

Our Patients!

Funding Sources

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Thank you!

Gaps and Needs in Flow Cytometry for Anti-Tumor T cell Therapies

11 Feb 2021

Melissa Myint, PhD
Director of Translational Research

MANA THERAPEUTICS

Company Introduction

MANA Therapeutics

Educating Immune Cells. Eliminating cancer.

Our mission is to develop unique “off-the-shelf” cellular therapies
that improve outcomes for cancer patients.

MANA was founded in November 2018, based on research and human proof-of-concept clinical trials conducted by Dr. Catherine Bollard, her team at Children’s National Hospital in Washington, D.C., and colleagues at Johns Hopkins Medical Center.

In Māori culture, MANA is the force that drives an individual’s leadership, character, and influence. It is strengthened by helping and caring for others. It is this sentiment that inspires and drives our evolution as we grow as a team and company.

Company Leadership:
Extensive Experience in Healthcare and Cell Therapy

Leadership

Martin B. Silverstein, M.D.
President and Chief Executive Officer

Madhusudan V. Peshwa, Ph.D.
Chief Technology Officer

Arthur (Andy) Hurwitz, Ph.D.
Senior Vice President,
Head of Research

Barb Geiger, B.S.N., R.N.
Senior Vice President,
Clinical Operations

Michael Kuo
Vice President,
Quality and Supply Chain

Tunç Toker
Vice President,
Program Management

Founders

Catherine Bollard, M.D., MBChB
Children's National Hospital
George Washington University School of
Medicine and Health Sciences

Marc Cohen
Executive Chairman
Cobro Ventures, Co-Founder

Conrad Russell Y. Cruz, M.D., Ph.D.
Children's National Hospital
George Washington University School of
Medicine and Health Sciences

Patrick Hanley, Ph.D.
Children's National Hospital
George Washington University School of
Medicine and Health Sciences

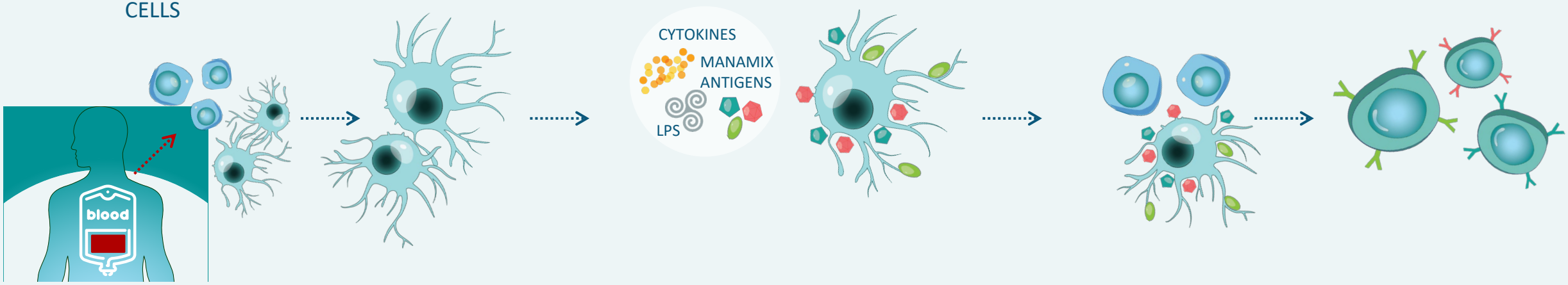
The MANA EDIFY™ Platform

Our EDIFY Platform allows education of T Cells to recognize multiple tumor antigens without genetic modification

1 ISOLATE T CELLS & MONOCYTES FROM APHERESIS PRODUCTS AND CULTURE MONOCYTES TO MATURE DENDRITIC CELLS

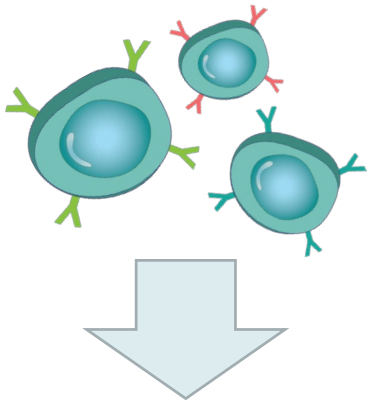
2 LOAD MATURE DENDRITIC CELLS WITH TUMOR-ASSOCIATED ANTIGEN PEPTIDES

3 STIMULATE TAA-SPECIFIC NAÏVE T CELLS; EXPAND OBTAINED MEMORY T CELLS INTO MANA T CELL PRODUCTS FOR DIRECT TUMOR KILLING IN PATIENTS



Flow cytometry is critical for characterizing our final drug products before release

Typical Flow Cytometry Assay Development for Anti-Tumor T cell Products



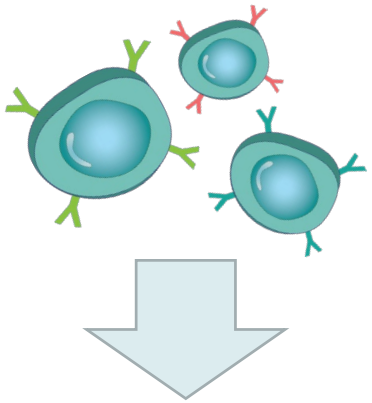
Flow Cytometry to Characterize Drug Product for Target Release Criteria (e.g. Purity, Identity, etc)

Research Method Transfer to Analytical Development

Analytical Method Development for Manufacturing Process

Method Implementation at CDMOs

Common Challenges in Flow Cytometry Assay Development



Flow Cytometry to Characterize Drug Product for Target Release Criteria (e.g. Purity, Identity, etc)

Methods developed with immune cells derived from **healthy** (non-disease state) donors

Autologous Products: Methods implemented on **disease state** (patient) immune cells

Research Method Transfer to Analytical Development

Analytical Method Development for Manufacturing Process

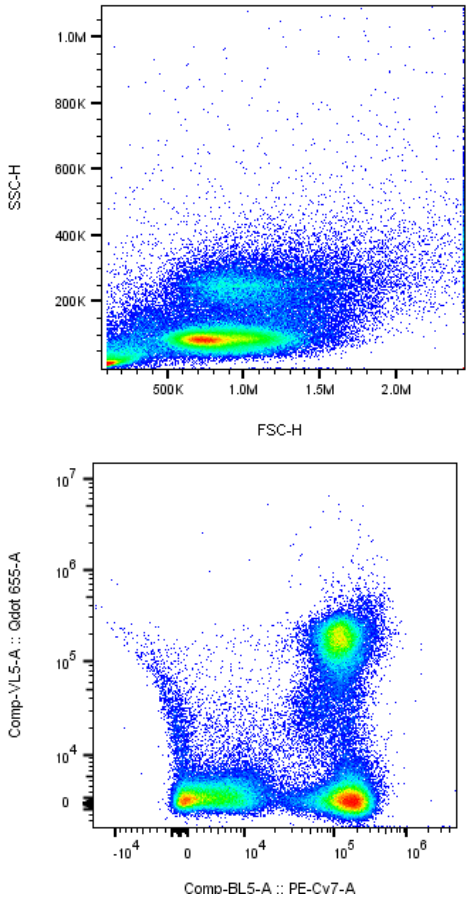
Method Implementation at CDMOs

Methods developed with **in-house flow cytometer**

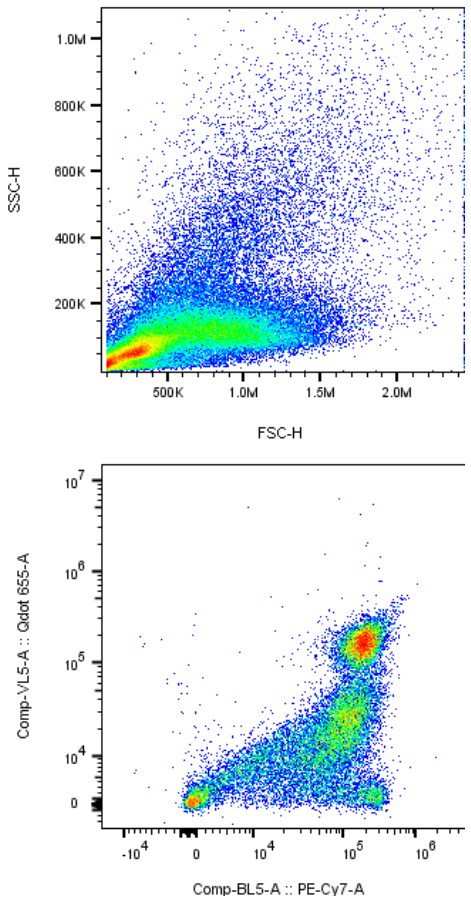
Methods developed with flow cytometer at CDMO – **Make/model not typically identical to in-house unit**

Major Challenge: Samples for Method Development vs Final Implementation

Surrogate Samples for Early Method Development



Actual Samples for Method Implementation



- Surrogate samples may not behave identically to actual samples used in final clinical implementation
  - *Common Example*: Healthy donor material vs patient, disease state, material
- More representative samples not typically available for early method development
  - Access to specific disease state specimens is limited (but growing!)
- Challenges arises when actual samples yield “abnormal” flow profiles. Unclear how operators should handle these deviations

Major Challenge: Intrinsic Differences in Equipment Setup

- Instrument settings can impact data visualization for a given sample
  - *Example*: PMT voltage settings directly impact the separation between a positive and negative population
- Setting specifications are not universal, even between instruments of identical make/model
- Challenges are exacerbated by the wide variety of available instrument models and unique setups within a model

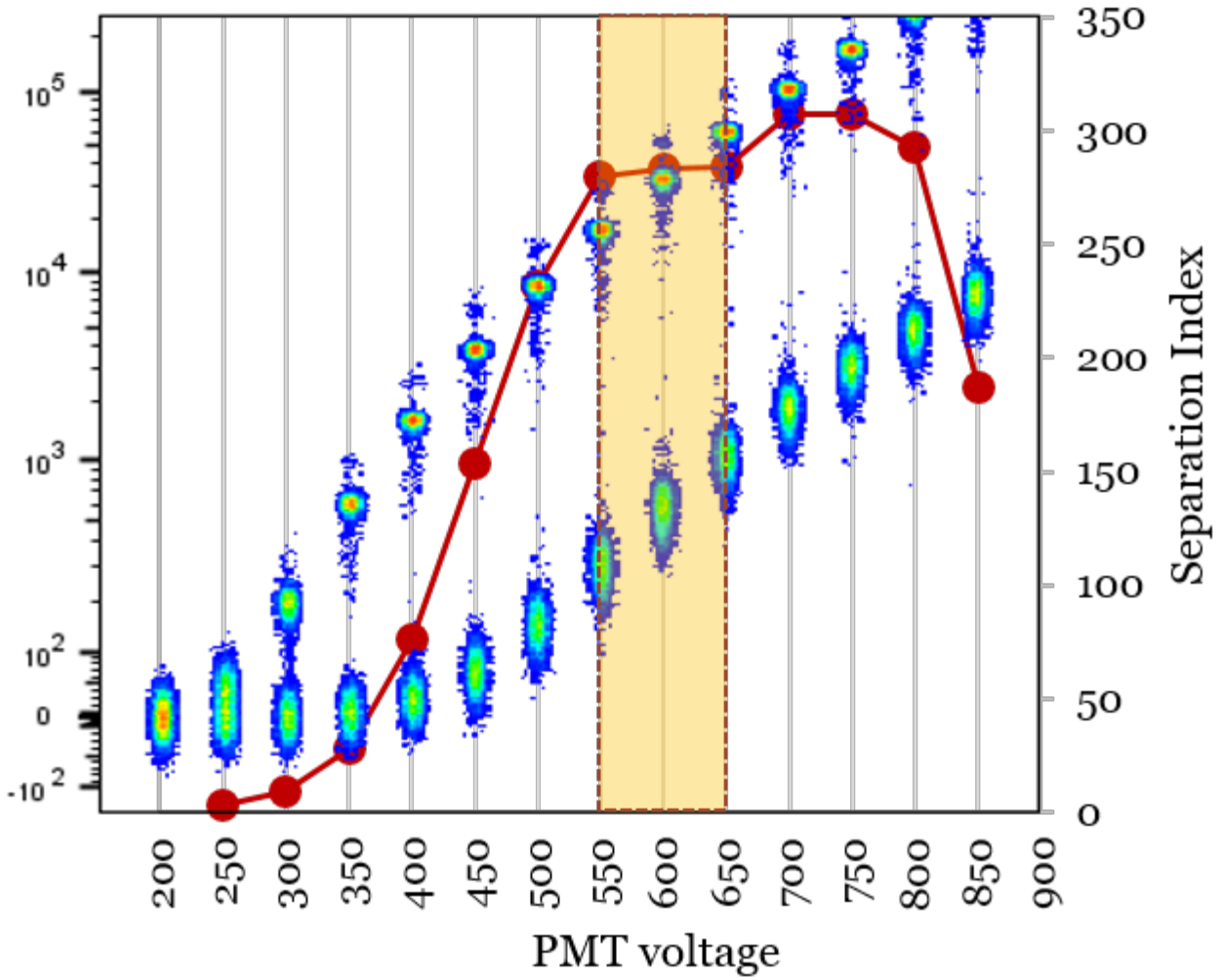


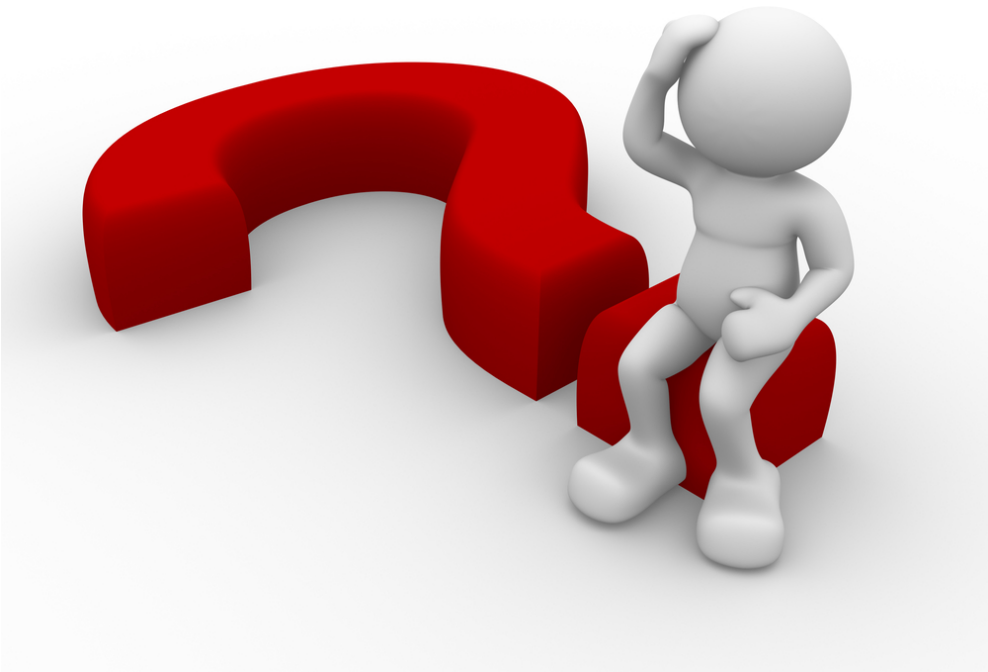
Image Source: https://www.uth.edu/imm/service-centers/flow-cytometry/pmt-voltage-optimization

Other Common Challenges

- No clear standards for sample prep:
  - Washing steps?
  - Staining at room temp or on ice?
  - Fixing method?

- No clear standards for negative controls:
  - Unstained vs FMOs vs isotype controls?

- Universal standards may be difficult to set because every cell type will be different!
  - T cells
  - Antigen-presenting cells
  - CD34+ cells
  - Naïve vs activated vs otherwise differentiated etc



(Realistic) Wishlist for Establishing Industry Flow Cyt Standards

- Absolute universal protocol is not entirely realistic
  - Every cell type is different
  - Every flow cytometer is different

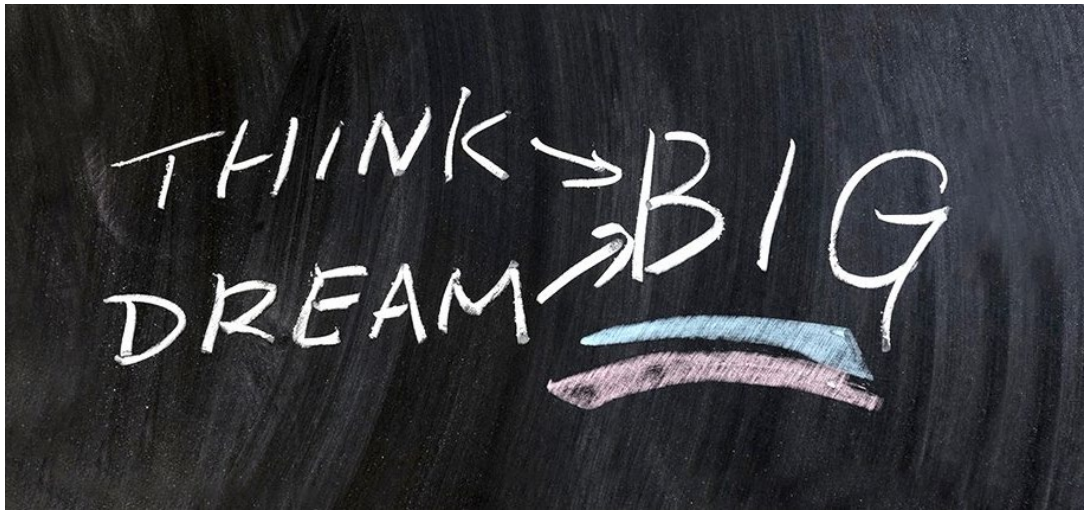
- Instead, let's consider establishing standard set of "Best Practices"
  - Regular instrument performance checks
  - Guidelines for voltage settings and target separation indexes
  - Guidelines for sample prep
  - Panel optimization and antibody titrations
  - Sample requirements for method validation
    - Specifications for positive and negative controls
  - Including flexible language in final SOPs that allow operators to consult with designated subject matter experts if results are abnormal

(BLUE SKY!) Wishlist for Establishing Industry Flow Cyt Standards

If we could snap our fingers and make anything come true….

Universal standard samples for method development:

- Standard cell lines for target applications
  - Establish standards for BOTH “clean” and “dirty” sample types
- Beads (or alternative “off-the-shelf” material) that can mimic cells in their ability to bind antibodies for flow analysis



Universal standards that enable quantification:

- **Ultimate Goal**:
  For a given target, be able to quantify number of molecules detected/expressed based on the measured MFI by flow.

Thanks for your attention!

Questions?

Special acknowledgements to:
Madhusudan V. Peshwa (MANA)
Michael Kuo (MANA)
Lindsay Kelly (MaxCyte)

IVD Quantitative Cellular Diagnostics in FCM: Barriers and opportunities in Clinical Practice

Bruce H Davis, MD

Bruce H Davis MD, Inc
Member, Flow Cytometry Standards Consortium

Talk Outline and Conflicts

- Components of a successful clinical IVD quantitative cell based assay
- Current challenges in delivering fully automated IVD quantitative cellular assay and influence of LDT regulation
- Current Assays needed at semi-quantitative interpretative level for leukemia/lymphoma/myeloma/MDS interpretation and MRD require multiple markers (colors). Only fluorescence calibrators are normal internal cells (if present).
- Quantitative Multiplexed Antigen Measurements by Fluorescence Cytometry (QMAM-FC)
- Anticipate niche needs with focused clinical use represent opportunity for defined automated IVD kits (examples provided)

- BH Davis holds patents for CD64 cellular measurements and related royalty bearing monoclonal antibodies

IVD Assays for Flow Cytometry: Components of cell-based diagnostics

Components of IVD Cellular Assays	Development Stage (1-5)	Important Issues	Outstanding Issues
Reagent specificity	5	Production QC & Clones Important, In-house production or quality OEM	Multiple vendors divide market, Clone selection important
Stable Formats at Rm Temp	4	Validation studies must be ISO and FDA, Real time data in final IVD format	Stability QC Data needed
Assay Controls - 2 levels needed, Design can be internal or external	3	Validation studies must be ISO and FDA, Real time data in final IVD format	Internal QC assays limited, stabilization of ext. material limited
Assay Controls - Compensation settings and calibrators	3	Validation studies must be ISO and FDA, Real time data in final IVD format	compensation consensus limited, JCTLM calibration procedures not established
Data analysis software	2	Manual gating requires prof cert, Automated gating software needed	Need AI Software validated, (e.g. Verity Gemstone)
Reporting Software	3	Autovalidation not routine, Interpretive nature of L/L reporting	integrate controls and EQA
Traceability and Standards	1	Lack of stable standards, reference methods	Disjoint between ISO requirements and JCTLM approvals

Status of Cellular Diagnostics: Automation, Calibration and Traceability

1. IVD regulations in EU require both device validation and traceability for all IVD assays to a reference standard or methodology
2. Current principals for quantitative antigen measurements using bead calibration, along with biologic calibration to healthy reference range, using software for analysis can be many cell antigen measurements – requires authoritative calibration service
   - Traceability is intuitive and feasible for chemistry analytes, but no consensus exists on cellular measurements
   - Such reference methods/materials must be blessed by Joint Committee on Traceability in Laboratory Medicine (JCTLM)
3. The proposed NIST flow cytometry standards consortium service will provide:
   - accurate quantitative measurements by flow cytometry using NIST-certified FITC, PE and APC beads with MESF unit value assignment
   - ability for ERF assignments for other fluorochromes
   - meets current untested thinking on traceability with efficient antibody – fluorochrome labeling.

Current Cellular Diagnostics in Laboratory Hematology - I

- **Blood cell counts (absolute and relative %)**
  - **Leukocytes – immunologic responses, infections, cancers, toxic effects**
  - **RBCs – trauma, toxic effects, genetic disorders**
  - **Platelets – bleeding disorders**
- **Morphologic features with microscopy**
  - **often disjoint between care giver and diagnostician (pre-test bias, if its the the same person)**
  - **Experiential dependent expertise with large inter-observer variations; issues of accuracy and terminology**
  - **Geographic differences in medical dogma and teachings, differences in staining techniques and artifacts**
- **Immunologic lymphocyte subset quantitation for selected infectious disorders (non-specific, becoming more obsolete?)**
- **Hematologic malignancy taxometry - (leukemia and lymphoma immuno-phenotyping)**
- **Activation status of PMNs and monocytes, myeloid suppressor precursor cells in infection/sepsis**
- **Activation marker quantitation: mHLA-Dr, mCD169 and nCD64 – no IVD reagents available, still at LDT stage globally**
- **Functional assays are time-intensive and difficult to standardize - limited availability for rare genetic conditions**
  - **PMN functions of oxidative burst, phagocytosis**
  - **Lymphocyte mitogenic assays**

Current Cellular Diagnostics in Laboratory Hematology - II

- **Selected *in vitro* tissue typing (HLA-Dr), phenotype activities for athletics (blood doping), transplant medicine, disease risk analysis (HLA-B27), PNH (PIG proteins) and other membrane defect syndromes**

- **HematoFlow, ClearLab or EuroFlow panels and related automated leukocyte differentials – cost effective workflow?**

- **Circulating Tumor Cells (CTCs), Cell Free DNA, lineage microparticles – still not part of routine practice protocols**

- **Minimal Residual Disease (MRD): currently defining clinical applications and standardization of solutions, but expensive and still under technical and regulatory development - applications in lymphoma, ALL, AML, myeloma, more?**

- **Chimeric receptor analysis (e.g. Car-T cell) – Companion Diagnostics and production QC : applications and standardization of solutions still under development and construct dependent – value of quantitative cytometry not clear (what is acceptable QC for Car-T cell manufacturing and treatments?)**

Cellular blood assays with unrealized IVD potential

Cell Target	Cellular Assay	Clinical Utility	Estimated ROC	IVD Status
RBC	Immature Reticulocyte Fraction (IRF)	Anemia Assessment	AUC = 0.80 - 0.90	global markets
	Reticulocyte Hemoglobin Content - CHR	Anemia Assessment	AUC = 0.70 - 0.80	global markets
	RBC RDW	Virtually Every Disese claimed	AUC = 0.55-0.60	global markets
	HbF Content - F Cells	Sickle Cell Therapeutic Monitor	?	RUO
	HbS Content	Sickle Cell Therapeutic Monitor	?	RUO
	HbA1C Content	Better Diabetes Monitor	?	RUO
	Microparticles CD235a+	Disease monitor of SSC & hemolytic diseases	?	RUO
Platelets	Large Platelet Fraction or "immature platelet fraction (IPF)"	Thrombocytopenia - transfusion guide	AUC = 0.75 - 0.80	global markets or pending
	Reticulated Platelets (RNA content)	Thrombocytopenia - transfusion guide	AUC = 0.80-0.85	RUO
	MPV	Virtually Every Disese claimed	AUC = 0.55-0.60	RUO
	microparticles - CD41/42/61+	Disease monitor - ITP, HUS, DIC	?	RUO
Leukocytes	PMN CD64 Expression	Infection detection and monitoring	AUC = 0.85 - 0.90	CE IVD, but off market
	Monocyte HLA-Dr Expression	Sepsis monitoring	AUC = 0.80 - 0.85	CE-IVD, but limited use
	Car-T Cells and other chimeric lymphs	Therapeutic and Manufacturing monitor	?	Clinical trials RUO status?
	Microparticles - CD45+ or subset+	Therapeutic and Manufacturing monitor	?	RUO
	Myeloid Functional Assays	rare diseases - CGD, MPO	AUC = 0.95 - 0.98	CE-IVD, but limited use
	Leukocyte Immuno-differential	Work efficiency?	?	RUO
	Apoptosis - annexin V	Therapeutic monitor?	?	RUO
Others	Minimal Residual Disease (MRD)	Leukemia monitoring & therapeutic decisions	AUC = 0.90 - 0.95	CE-IVD, but limited use
	Circulating Tumor Cells (CTC)	Disease prognosis and diagnosis	AUC = 0.90 - 0.95	CE-IVD, but limited use

Cellular blood assays with practical IVD potential

Cell Target	Cellular Assay	Clinical Utility	Estimated ROC	IVD Status
RBC	Immature Reticulocyte Fraction (IRF)	Anemia Assessment	AUC = 0.80 - 0.90	global markets
	Reticulocyte Hemoglobin Content - CHR	Anemia Assessment	AUC = 0.70 - 0.80	global markets
	RBC RDW	Virtually Every Disese claimed	AUC = 0.55-0.60	global markets
	HbF Content - F Cells	Sickle Cell Therapeutic Monitor	?	RUO
	HbS Content	Sickle Cell Therapeutic Monitor	?	RUO
	HbA1C Content	Better Diabetes Monitor	?	RUO
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	Reticulated Platelets (RNA content)	Thrombocytopenia - transfusion guide	AUC = 0.80-0.85	RUO
	MPV	Virtually Every Disese claimed	AUC = 0.55-0.60	RUO
	microparticles - CD41/42/61+	Disease monitor - ITP, HUS, DIC	?	RUO
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Standardization, Calibration, traceability

Nonspecific, no traceability

Standardization, Calibration, or traceability

NETs??

Little utility
IVD too costly

Little utility
Standardization

Status of Cellular Diagnostics: Calibration and Traceability

1. IVD Regulations in EU require traceability, which while intuitive and feasible for chemistry analytes, no consensus exists on cellular measurements. Reference methods/materials blessed by JCTLM
2. Existing principals for quantitative antigen measurements for leukocyte CD64 measurements using bead calibration and automated software analysis can be applied to other cell measurements
3. Provides ability for accurate quantitative multiplexed measurements by flow cytometry using NIST-certified FITC beads with MESF unit value assignment or ERF, meeting existing thinking on traceability.

ERF Assignment of Beads with SRM® 1934 (or equivalent)		
Fluorochrome Bead Label	Laser Excitation (nm)	SRM® 1934 material
Fluorescein (FITC)	488	Fluorescein
Phycoerythrin (PE)	488	Fluorescein
Brilliant Blue 515 (BB515)	488	Fluorescein
peridinin-chlorophyll-protein (PerCP)	488	Nile Red
PerCP-Cy5.5	488	Nile Red
PE-Cy7	488	Nile Red
Allophycocyanin (APC)	633	APC
APC-R700	633	APC
APC-H7	633	APC
APC-Cy7	633	APC
Violet 450	405	Coumarin 30
BV421	405	Coumarin 30
V500	405	Coumarin 30
BV510	405	Coumarin 30
BV605	405	Coumarin 30

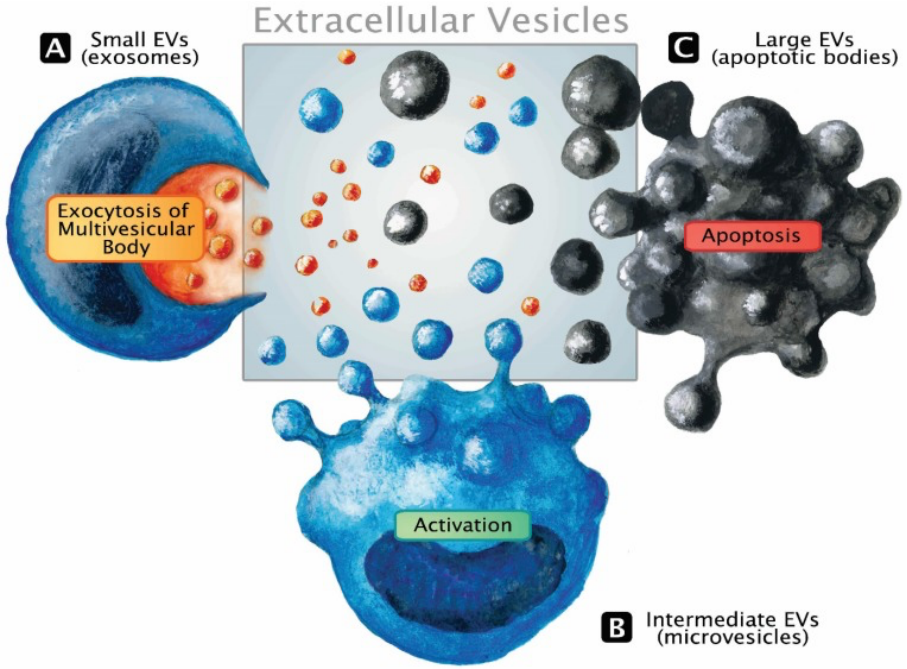
Niche Cell Diagnostics Example #1: Cell Activation of Innate Immune Response in infection/sepsis

Intended Medical Uses:

1. R/O Sepsis Screen – vastly superior to procalcitonin
2. Diagnostic to determine if viral or bacterial infection
3. Prognostic indicator of sepsis patients – mHLA-Dr levels
4. Therapeutic monitor for infection (bacterial or viral)
5. Disease monitor for Covid-19 and all severe infections
6. Determinate as to D/C of antibiotics or therapeutic change indicator
7. Only specific monitor of neutrophils and monocytes functional status
8. Early detection of infections in post-operative and immuno-suppressed states

		LeukoCD-Innate Panel (6 tube assay)						
CD Target	**Purpose**	**FITC**	**PE**	**ECD**	**PerCP**	**APC**	**Draq 7**	**Bead Population #**
CD64 (22&32.2 clones)	nCD64	1						1
CD169	mCD169	2						2
HLA-Dr	mHLA-Dr	3						3
CD35 (C3b/C4a receptor)	nCD35	4						4
CD88 (C5a receptor)	nCD88	5						5
MPO (myeloperoxidase)	gating - NETs	6						6
CD45	gating				1,2,3,4,5,6			
CD163	gating		1,2,3,4,5,6					
CD15	gating					1,2,3,4,5,6		
Draq 7	gating - live/dead - NETs						1,2,3,4,5,6	
CD16 + CD3 + CD19	gating			1,2,3,4,5				

Extracellular Vesicles and NETs as biomarkers of Infection



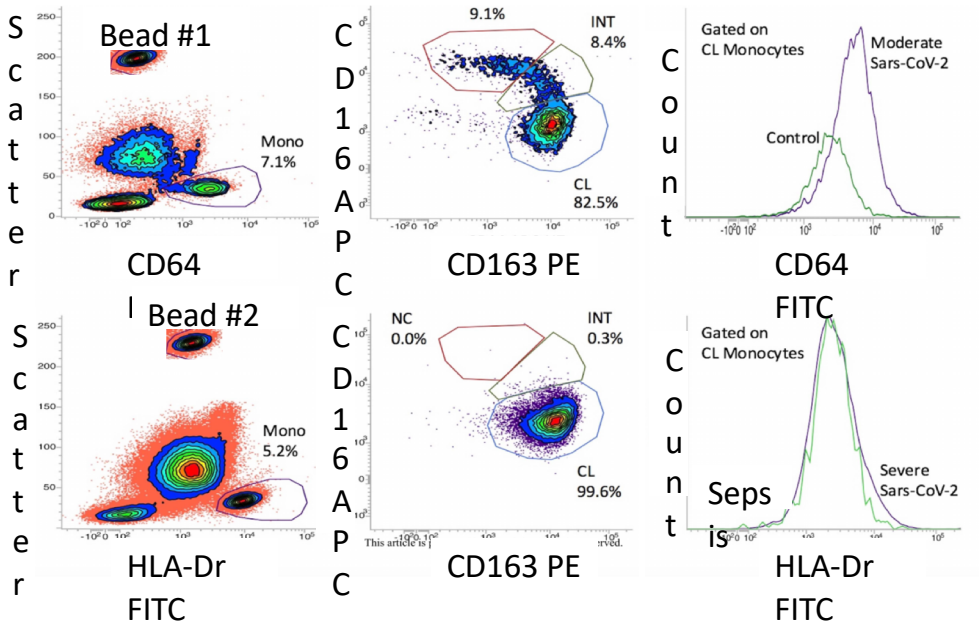
PMN derived neutrophil extracellular traps (NETs): chromatin (DNA), vesicles (elastase, MPO, lactoferrin), PMN membranes –

1. Masuda S, Shimizu S, Matsuo J, et al. Measurement of NET formation in vitro and in vivo by flow cytometry. Cytometry A. 2017;91(8):822-829. doi:10.1002/cyto.a.23169
2. Gavillet M, Martinod K, Renella R, et al. Flow cytometric assay for direct quantification of neutrophil extracellular traps in blood samples. Am J Hematol. 2015;90(12):1155-1158. doi:10.1002/ajh.24185
3. Rada B. Neutrophil Extracellular Traps. Methods Mol Biol. 2019;1982:517-528. doi:10.1007/978-1-4939-9424-3_31
4. Zuo Y, Yalavarthi S, Shi H, et al. Neutrophil extracellular traps in COVID-19. JCI Insight. 2020;5(11):e138999. Published 2020 Jun 4. doi:10.1172/jci.insight.138999;
5. Carmona-Rivera C, Kaplan MJ. Induction and quantification of NETosis. Curr Protoc Immunol. (2016) 115:14.41.1–14. 10.1002/cpim.16 doi:10.1007/978-1-4939-9424-3_31;
6. Denning NL, Aziz M, Gurien SD, Wang P. DAMPs and NETs in Sepsis. Front Immunol. 2019;10:2536. Published 2019 Oct 30. doi:10.3389/fimmu.2019.02536

Example of Quantitative Multiplexed Antigen Measurements by Fluorescence Cytometry (QMAM-FC): Leuko-Innate Screen by *multiplexed single-cell-resolved gene expression profiling*

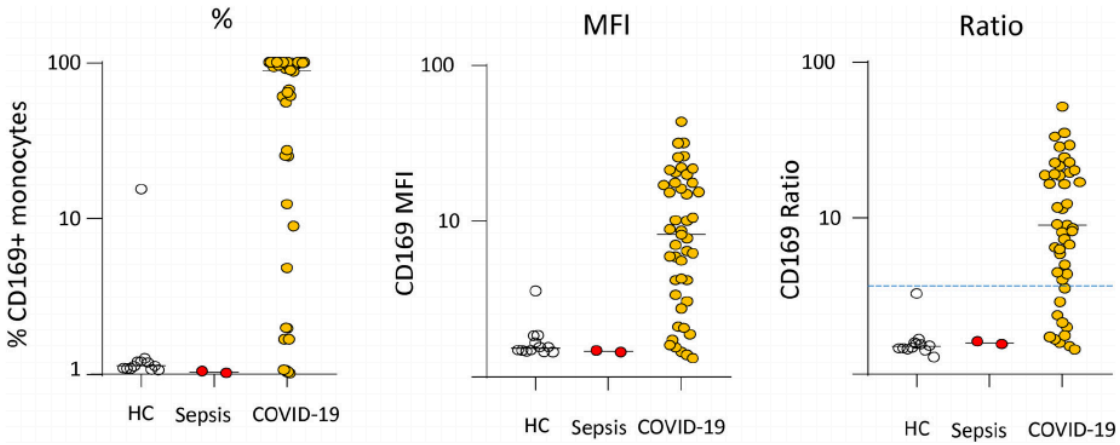
Patient Number

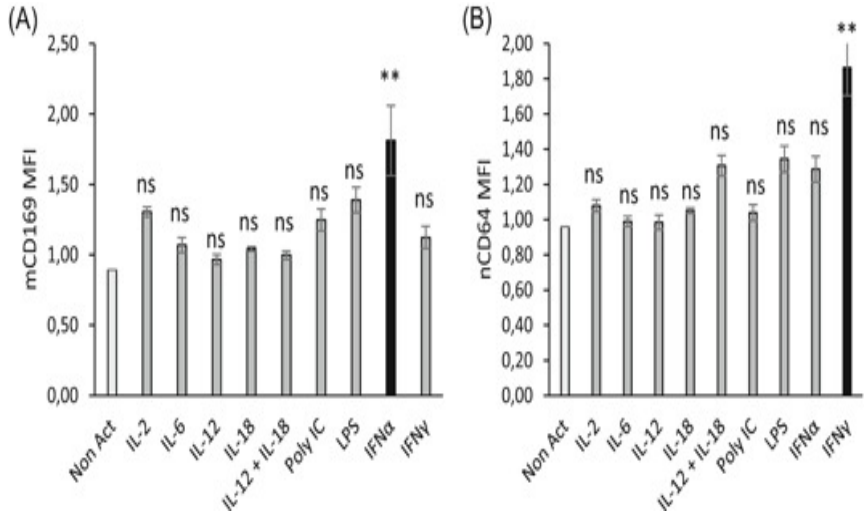
Assay Profile Subunits	1	2	3	4	5	6	7	8	9	10	11	12
Leukocyte CD64	A											
Leukocyte HLA-Dr	B											
Leukocyte CD169	C											
Leukocyte CD35 (C3aR)	D											
Leukocyte CD88 (C5aR)	E											
Neutrophil Extracellular Traps	F											
PMN:Lymph ratio; eos count	G											
Controls	H											



Same approach used for Leukocyte CD64 measurements (Leuko64, CE IVD for infection screening) with time-resolved assay concatenation of unique reference beads calibration to allow for multiplexed fluorescence molecules/cell measurements (limited only by ability to resolve bead populations). When data is analyzed using patented Gemstone software, the assays will be of high precision (CV $\leq$5%), sensitivity (<1,000 molecules per cell analyte) and linearity (4-6 logs), allowing true multiplexed quantitative cytometry compliant with current and incoming EU CE IVD guidelines.

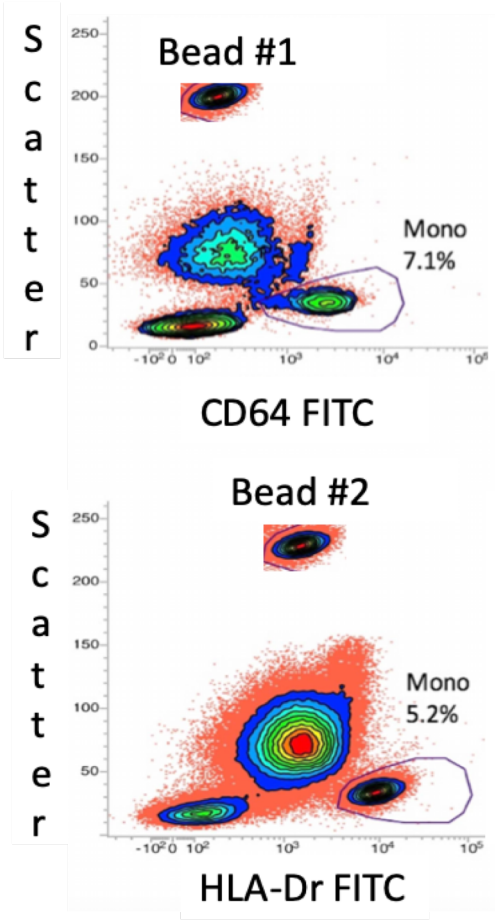
Monocyte CD169 in Covid-19 Infection: marker of viral infection

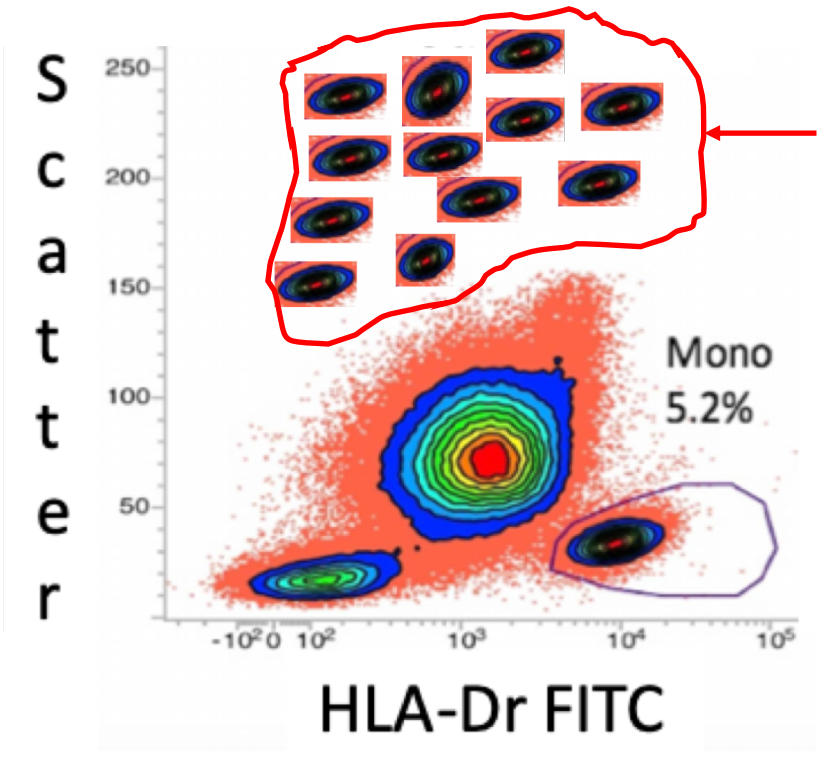




1. Bourgoin P, Biéchelé G, Ait Belkacem I, Morange PE, Malergue F. Role of the interferons in CD64 and CD169 expressions in whole blood: Relevance in the balance between viral- or bacterial-oriented immune responses. Immun Inflamm Dis. 2020 Mar;8(1):106-123. doi: 10.1002/iid3.289. Epub 2020 Feb 7. PMID: 32031762; PMCID: PMC7016842.
2. Ortillon M, Coudereau R, Cour M, Rimmelé T, Godignon M, Gossez M, Yonis H, Argaud L, Lukaszewicz AC, Venet F, Monneret G. Monocyte CD169 expression in COVID-19 patients upon intensive care unit admission. Cytometry A. 2021 Feb 5. doi: 10.1002/cyto.a.24315. Epub ahead of print. PMID: 33547747.
3. Bedin AS, Makinson A, Picot MC, Mennechet F, Malergue F, Pisoni A, Nyiramigisha E, Montagnier L, Bollore K, Debiesse S, Morquin D, Bourgoin P, Veyrenche N, Renault C, Foulongne V, Bret C, Bourdin A, Le Moing V, Van de Perre P, Tuaillon E. Monocyte CD169 expression as a biomarker in the early diagnosis of COVID-19. J Infect Dis. 2020 Nov 18:jiaa724. doi: 10.1093/infdis/jiaa724. Epub ahead of print. PMID: 33206973; PMCID: PMC7717347.

Quantitative Multiplexed Antigen Measurements by Fluorescence Cytometry (QMAM-FC): Advance in Cellular Diagnostics

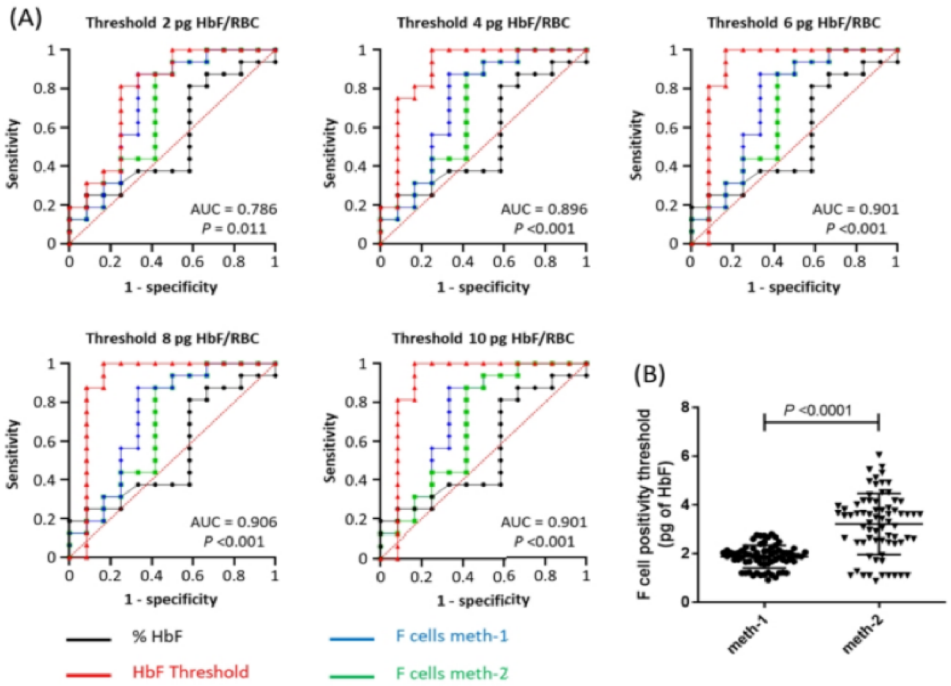




Assay calibration beads unique to each assay allows for unichromic multiplexing by flow cytometry; each assay from separate well or tube has one time-resolved portion of a list mode file each with a unique reference bead.

As with multiplexed immunoassays, scores of quantitative cellular assays can be performed, but using FITC, PE and APC and other such superior traceable calibrator fluorochromes.

Niche Cell Diagnostics Example #2: RBC HbF content for Fetomaternal hemorrhage and Sickle Cell Disease

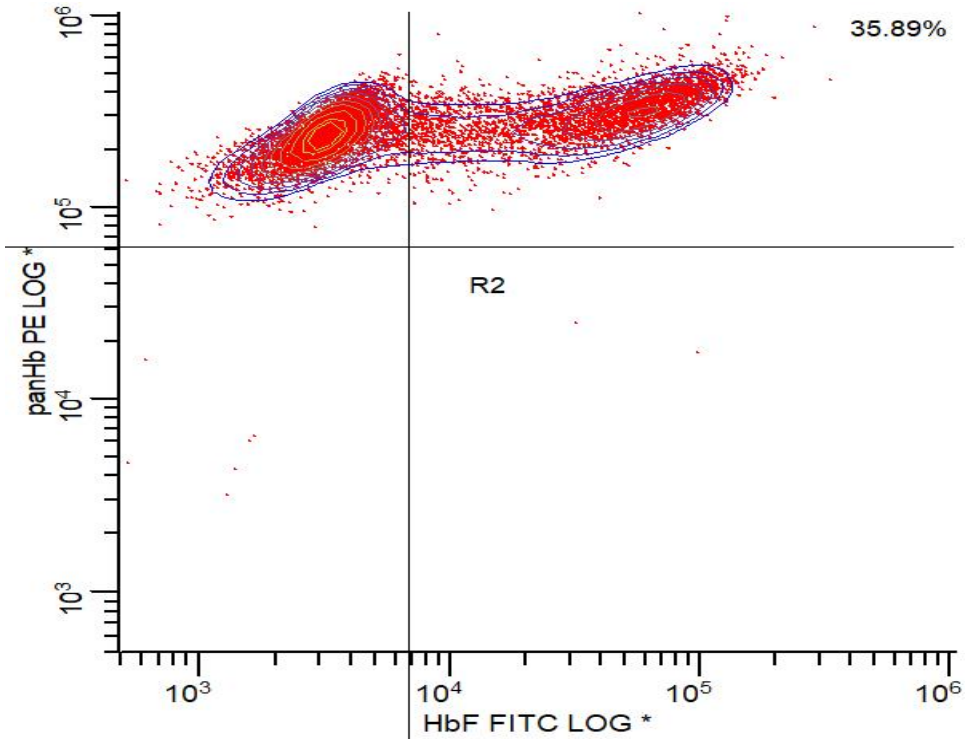


F Cell Count with optimized HbF threshold + Fetal RBC count

- Complement CRISPR therapies designed to increase F cells to reduce tendency for RBC to shape change in Sickle Cell Disease and bypass retarded RBC production in Thalassemia
- Potential for companion diagnostic roll with the emerging Rx and on-going therapeutic monitoring for dose adjustments
- Automated assay with software would generate market acceptance
- Assay design might be enhanced with the use of calibration bead population to define HbF thresholds and built-in QC

1. Frangoul H et al. CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia. N Engl J Med. 2021 Jan 21;384(3):252-260. doi: 10.1056/NEJMoa2031054. Epub 2020 Dec 5. PMID: 33283989.
2. Esrick EB et al. Post-Transcriptional Genetic Silencing of *BCL11A* to Treat Sickle Cell Disease. N Engl J Med. 2021 Jan 21;384(3):205-215. doi: 10.1056/NEJMoa2029392. Epub 2020 Dec 5. PMID: 33283990.

F Cell Enumeration for Sickle Cell Disease



Bivariate plot of RBCs of total hemoglobin (anti panHb PE, IQ Products, Groningen, NL) vs. HbF (anti HbF1, FITC, IQ Products, Groningen, NL) from an individual with hetero-cellular hereditary persistence of HbF.
Such an approach could be used to rapidly assess that ~36% fraction of RBCs having a defined threshold level of HbF per cell

Chen JC, Bigelow N, Davis BH. Proposed flow cytometric reference method for the determination of erythroid F-cell counts. *Cytometry*. 2000;42(4):239-246.

Conclusions and Premonitions

- Components of a successful clinical IVD quantitative cell based assay need further development in calibration and regulatory understanding
- IVD Cellular assays need to address dearth of traceability and standards and establish a dialogue with JCTLM to define process of certification
- Fully automated IVD quantitative cellular assays will be influenced by LDT regulation (subsidizes poor quality?) and industry response to clinical need
- Current Assays at semi-quantitative interpretative level for leukemia/lymphoma/myeloma/MDS interpretation and MRD require multiple markers (colors). Professional interpretation an uncontrolled variable. Only fluorescence calibrators are normal internal cells (if present).
- Quantitative Multiplexed Antigen Measurements by Fluorescence Cytometry (QMAM-FC) or more robust gating approaches with spectral analysis or mass cytometry allows IVD opportunities through NIST consortium services
- Anticipate niche clinical needs with focused clinical use represents an opportunity for defined automated IVD kits – automation key to success to avoid manual gating