British Pharmacopoeia

Advanced Therapy Medicinal Products Guidance

Application of Flow Cytometry

Medicines & Healthcare products Regulatory Agency

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Contents

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1. ABBREVIATIONS

7AAD	7-Aminoactinomycin D
ABC	Antibody Binding Capacity
APC	Allophycocyanin
ATMP	Advanced Therapy Medicinal Product
BMR	Batch Manufacturing Record
BP	British Pharmacopoeia
CAR	Chimeric Antigen Receptor
CAT	Committee for Advanced Therapies
CDx	Cluster of Differentiation x
CE	European conformity
CGT	Cell and Gene Therapy
CMO	Contract Manufacturing Organisation
CQA	Critical Quality Attribute
CRO	Contract Research Organisation
DAPI	4′,6-diamidino-2-phenylindole
DC	Dendritic Cell
DMSO	Dimethyl sulfoxide
DQ	Design Qualification
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
EU	European Union
FC	Flow Cytometry
FDA	Food and Drug Administration
FIO	For Information Only
FMO	Fluorescence Minus One
FS	Functional Specification
FSC	Forward Scatter
FSC-A	Forward Scatter-Area
FSC-H	Forward Scatter-Height

FSE	Field Service Engineer
GXP	Good X Practice (where X refers to manufacturing, clinical, distribution etc)
GVHD	Graft Versus Host Disease
HSC	Haematopoietic Stem Cell
ICH	The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IOQ	Installation/Operational Qualification
IMP	Investigational Medicinal Product
iPSC	induced Pluripotent Stem Cell
IQ	Installation Qualification
IVD	*In Vitro* Diagnostics
LDT	Laboratory Developed Test
LoD	Detection Limit
LoQ	Quantitation Limit
LPS	Lipopolysaccharide
MdFI	Median Fluorescence Intensity
MFI	Mean Fluorescence Intensity
MEPE	Molecules of Equivalent Phycoerythrin
MESF	Molecules of Equivalent Soluble Fluorochrome
MOI	Multiplicity of Infection
MSC	Mesenchymal Stem Cell
NGS	Next Generation Sequencing
NIBSC	The National Institute for Biological Standards and Control
NK	Natural Killer
OQ	Operational Qualification
PE	Phycoerythrin
PI	Propidium iodide
PQ	Performance Qualification
Ph. Eur.	European Pharmacopoeia
PBS	Phosphate Buffered Saline
PMT	Photomultiplier Tube
QC	Quality Control
QMS	Quality Management System

RA	Risk Assessment
RBC	Red Blood Cell
RPE	Retinal Pigment Epithelial
RSD	Relative Standard Deviation
SD	Standard Deviation
SSEA	Stage-Specific Embryonic Antigen
SIT	Sample Injection Tube
SMA1	Spinal Muscular Atrophy Type 1
SOP	Standard Operating Procedure
SSC	Side Scatter
UK	United Kingdom
UKCA	UK Conformity Assessed
UPS	Uninterruptible Power Supply
URS	User Requirements Specification
USA	United States of America
USP	United States Pharmacopeia
VMP	Validation Master Plan

2. INTRODUCTION

The cell and gene therapy communities comprise a diverse set of stakeholders interested in the application of flow cytometry: research scientists, therapy developers, clinical institutions, vendors, service providers and regulators.

As an analytical technique, flow cytometry can be used to characterise the physical and chemical attributes of a population of cells or particles qualitatively or quantitatively. The fundamental principles of the technique are well documented in the literature[1'2] and have been summarised in pharmacopoeial texts (Ph. Eur. 2.7.24, USP <1027>). Efforts to establish best practice for performing flow cytometry in a regulated environment have been made.[3'4]

Flow cytometry is a widely used but technically complex tool; the practical application of which is recognised to be diverse and often challenging to standardise. Standardisation is critical in supporting robust data generation, enabling data comparability between users and instruments, and when applied to development of ATMPs, ensuring reproducible product quality, safety, and efficacy throughout the entire product lifecycle.

The guidance provided within this framework is structured in the form of four "themes" (Table 1). Each theme consists of a series of technically focused chapters and case studies illustrating best practice for flow cytometry application; the adoption of which is intended to promote standardisation

[1] *Kalodimou, V. E., & AABB. (2013). Basic principles in flow cytometry. Bethesda, Md: AABB Press.*
[2] *Cossarizza, Aet al. Guidelines for the use of flow cytometry and cell sorting in immunological studies. Eur. J. Immunol. 2017. 47: 1584–1797.*
[3] *der Strate BV et al. Forum. Bioanalysis. 2017; 9(16):1253-1264.*
[4] *CLSI. Validation of Assays Performed by Flow Cytometry, 1st ed. CLSI document H62. Wayne, PA: Clinical Laboratory Standards Institute; 2021.*

of the technique within the cell and gene therapy community. Several aspects of the guidelines apply to all flow cytometry users and should be given consideration regardless of the context in which the assay is being developed; their purpose is primarily to facilitate the generation of reproducible high-quality data (Themes 1 and 2). If the assay under development is intended for use as part of a human medicine development programme, then it is important to recognise that when used for control of manufacture, it will need to conform to appropriate regulatory guidelines.

It is not the intention of this guidance to define analytes representative of common cell populations. Perspectives on critical phenotypic attributes that characterise specific cell populations will be product specific and can be highly dynamic as fundamental knowledge develops. Therefore, it is recommended to review pharmacopoeia monographs, published institutional position statements, and/or the general literature should be consulted and reviewed to support identification of representative analytes for populations of interest. A selection of recommended references for commonly analysed cell types are detailed in Table 2.

Table 1: The four themes of the Framework for Standardising the Application of Flow Cytometry within the Cell and Gene Therapy Community.

Themes	**Relevant CGT Community Stakeholders**	**Objective**
Theme 1 - Overarching points for consideration when initiating the application of flow cytometry	All Users	This theme is focused on supporting the user to clearly define the purpose of the assay being developed; the equipment and environment in which it will be implemented; how data acquired will be used; and if applicable, existing regulations and guidelines that may shape the development of the assay.
Theme 2 - Best practice guidelines when considering assay configuration and the development of sample workflows	All Users	This theme is focused on supporting the user by demonstrating the value of building a strong understanding of how assay performance may be impacted by: the quality attributes of the material being analysed, sample processing, flow cytometer configuration, panel design, staining protocol, data acquisition, and data analysis strategies.
Theme 3 - Best practice guidelines for users operating within GMP environments	Therapy developers, CMOs, CROs, Clinical Institutions	This theme is focused on supporting the user by: summarising the regulatory requirements and procedures to which the assay must conform; illustrating challenges and approaches to mitigating the risk associated with fulfilling assay validation strategies (including multi-site comparability); providing examples of good data reporting practices; and demonstration of the importance of performance trending and review.
Theme 4 - Monograph Development	Therapy developers, Regulators	Presents a vision for the field, where through the standardisation of practice and an improved understanding of cell and gene therapies, agreement can be reached supporting the development of flow cytometry monographs in relation to the performance of specific assays.

Table 2: References to support analyte definition for commonly analysed cell types.

Cell Type	References
General	Characterization of human cells for clinical applications - Guide (PAS 93:2011)
MSC	Dominici M *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy (2006) 8 (4): 315-317
	Bourin *et al.* Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics (IFATS) and Science and the International Society for Cellular Therapy (ISCT). Cytotherapy (2013) 15(6): 641–648
	Mendicino M *et al.* MSC-based product characterisation for clinical trials: An FDA perspective. Cell Stem Cell (2014) 14(2): 141-145
iPSC	Sullivan S *et al.* Quality control guidelines for clinical-grade human induced pluripotent stem cell lines. Regen.Med. (2018) 13(7): 859–866
T-Cell	Janetzki *et al.* "MIATA"—Minimal Information about T Cell Assays. Immunity. 2009 October 16; 31(4): 527–528.
	Hollyman *et al.* Manufacturing validation of biologically functional T cells targeted to CD19 antigen for autologous adoptive cell therapy. J Immunother. (2009) 32(2): 169–180
	Singh H *et al.* Manufacture of T Cells Using the Sleeping Beauty System to Enforce Expression of a CD19-specific Chimeric Antigen Receptor. Cancer Gene Ther. (2015) 22(2): 95-100
	Mousset CM e*t al.* Comprehensive Phenotyping of T Cells Using Flow Cytometry. Cytometry A. (2019) Jun; 95(6):647-654
HSC	USP <127> Flow cytometric enumeration of CD34+ cells
NK	Koehl U *et al.* Advances in clinical NK cell studies: Donor selection, manufacturing and quality control. Oncoimmunology (2016) 5(4): e1115178
RPE	Plaza Reyes A, *et al.* Identification of cell surface markers and establishment of monolayer differentiation to retinal pigment epithelial cells [published correction appears in Nat Commun]. (2020) 11(1):1609
DC	Kalantari T, Kamali-Sarvestani E, Ciric B, *et al.* Generation of immunogenic and tolerogenic clinical-grade dendritic cells. Immunologic Research. (2011) 51(2-3): 153-160
CAR-T	Sarikonda G, Mathieu M, Natalia M, et al. Best practices for the development, analytical validation and clinical implementation of flow cytometric methods for chimeric antigen receptor T cell analyses. Cytometry B Clin Cytom (2020) Dec 29. doi: 10.1002/cyto.b.21985. Epub ahead of print. PMID:

3. POINTS FOR CONSIDERATION WHEN DEVELOPING A FLOW CYTOMETRIC ASSAY

Flow cytometry is used as an analytical technique throughout a diverse range of scientific fields and settings (See Section 3.1). The effort spent on method development and validation should reflect the phase of drug development and the intended use of data; with the objective of ensuring the assay is

"fit-for-purpose". At the beginning of any flow cytometry-based assay development programme, it is important that the purpose of the assay (See Section 3.2) is clearly defined in combination with the intended environment of application. This will shape the subsequent steps of the assay development and validation programme (See Figure 1).

In the context of ATMP development, the existing framework of guidelines and standards should also be consulted to understand the regulatory expectations (See Section 3.3) from the outset of the development programme. It will be necessary to consult different regulatory guidelines and standards depending upon the phase of development, and geographical location.

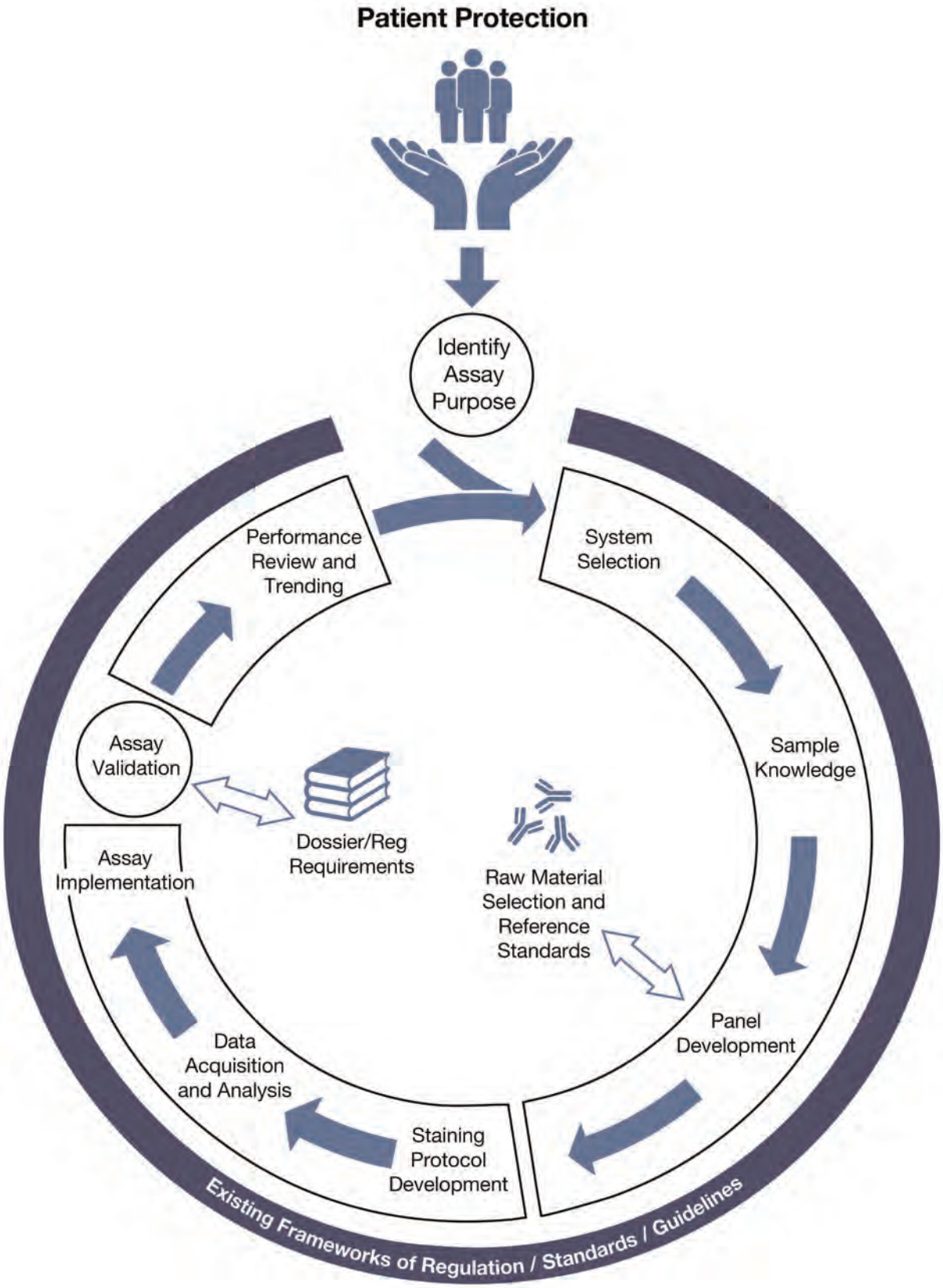


Figure 1: Key stages in the development of a flow cytometry based analytical assay.

3.1 Environmental settings of assay development and application

Flow cytometry assays may be deployed in a range of scientific settings or environments. It is important to recognise at the outset of an assay development programme who the intended end user is, and the regulatory status in which the assay will be operated; these factors may influence the assay configuration and workflows. For example, the assay may be developed for an end-point application within:

- An academic research setting.
- An ATMP development programme (including GMP manufacture).
  - Within the sponsor's own facilities.
  - Within outsourced contract vendors (e.g. CROs & CMOs).
- Clinical settings such as pathology laboratories for the analysis of patient samples.
- Clinical trials setting.
- Pre-clinical.

Its application may be at a single site or across multiple sites (within the same geographical region or across different geographical regions). This has the potential to impact factors such as, but not limited to:

- Availability of different equipment types. See Section 4 for points to consider when selecting flow cytometry equipment to support assay development (Note: consideration should also be given to the ancillary equipment to support the assay set-up and workflows).
- Staff experience and training levels.
- Sample workflows - for example considerations such as the impact of regional variations in temperature and humidity on assay performance, or the sample age at point of analysis. See Section 5 for further points to consider in setting up sample workflows.
- The requirement for assay validation - See Section 8 for further points to consider when validating flow cytometry assays.
- Software used to support data acquisition, interpretation, and analysis.
- Availability of reagents.

3.2 Identifying the assay purpose

Typically, the purpose of a flow cytometry-based assay is to establish at least one of three attributes of a sample population: identity, purity, and/or potency (Table 3). Flow cytometry can also be used to establish cell count and viability, See Section 5. When considered in the context of the development of human medicines, these attributes may be directly related to the product's safety and therefore it is essential that the assay is robust and reproducible. It is important to recognise that multiple assays may be needed to establish each attribute, and that flow cytometry could be just one tool in an analytical strategy.

At the outset of the research or development programme the key analytes representing the identity, purity and/or potency attributes of the cells may be unknown. This may be due to factors such as:

- Variation in material composition and quality (e.g. for autologous starting materials or clinical patient samples).
- Differences observed between healthy and diseased phenotypes.
- Rarity of cell populations.
- The impact of sample degradation or growth conditions on cell characteristics.

At the beginning of any assay development programme researchers and developers should strengthen their knowledge around the samples to be analysed (See Section 5). It will be common to utilise assay panel designs (See Section 6) that support analysis of a broader range of analytes. However, as research or development progresses, critical quality attributes (CQAs) should be identified and assay

panels may be restricted to those critical analytes only. The advantage of restricting panels to critical analytes is that this drives more rapid assay turn-around times, simplifies data acquisition and analysis (See Section 7) and supports the use of simpler equipment within controlled GMP environments. Whilst in the context of QC release assays for clinical / commercial products – reducing the number of analytes where possible facilitates successful validation of the assays and ultimately release of these products. This does not mean that additional analysis for information only (FIO) cannot be performed using broader panels for research purposes. However the data generated should be segregated from that of the assay and treated separately.

Understanding the assay's purpose may help set expectations on the required level of assay performance. For example, in the analysis of samples across multiple sites (manufacturing or clinical), it is essential that comparability is maintained, and it should be recognised that the greater the number of sites expected to perform the assay, the more challenging it may be to ensure consistency in assay performance and comparability.

Table 3: Sample population attributes typically associated with the development of flow cytometry-based assays for cell or gene therapies.

Attribute	**Definition**
Identity	The objective of identity assays is to recognise a specific population of interest, when compared to other populations (or in the context of therapy development, other products). Cell Therapies: In the case of cell-based products, the assay should confirm the identity of the cellular components. Depending on the cell population and origin, cells will be characterised in terms of phenotypic and/or genotypic profiles. These markers may be based on gene expression, antigen presentation, biochemical activity, response to exogenous stimuli, capability to produce biologically active or otherwise measurable molecules. Gene Therapies: In the case of vector or nucleic acid-based therapies, flow cytometry can be utilised to confirm expression of the genetic insert product upon cell culture transduction/transfection (respectively).
Purity	The objective of purity/impurity assays is to confirm the correct "composition" of the product and to determine the percentage of the total population comprising the target of interest. Cell Therapies: Where a specific cell type is required for the indication, the unwanted cells (which may be defined as product related impurities) should ideally be defined and their amount in the final product should be controlled by appropriate acceptance criteria. For example, when differentiating cells from a pluripotent starting material, the population of interest could contain other cells that are of different lineages and/or differentiation stage or that may be unrelated to the intended population. In cases where the desired biological activity and efficacy of the product requires a complex mixture of cells, the cell mixture needs to be characterised and its composition controlled by appropriate in process controls and release testing. For example, for T-cell based products, it is common to restrict the percentage of remaining NK and B-cells to ensure optimal activity and reduce other risks such as EBV transmission.
Potency	The objective of a potency assay is to provide a quantitative measure of biological activity based on an attribute of the product, which is linked to its relevant biological properties, associated as closely as possible to the likely mechanism of action of the product. Biological activity describes, therefore, the specific ability or capacity of a product to achieve a defined biological effect.[5] In the context of ATMP development, developing a potency assay early in development can give added value, but is not a pre-requisite for Phase I clinical trials. A potency assay will be required for a licensed product and it is advisable to have a potency assay in place for the pivotal clinical trials. Cell Therapies: If the mechanism of action of the cells can be clearly related to specific antigens (i.e., tumour-specific antigens, tumour-associated antigens), the potency assay may be based on quantification of these antigens by suitable methods, such as flow cytometry analysis.[6] Since cell viability is an important parameter for product integrity and directly correlated to the biologic activity, the ratio between non-viable and viable cells should be determined and appropriate specifications should be set.

[5] *ICH Topic Q6B, Step 4 Note for Guidance on Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products. CPMP/ICH/365/96*

[6] *EMA/CHMP/BWP/271475/2006 rev.1 Guideline on potency testing of cell-based immunotherapy medicinal products for the treatment of cancer.*

3.3 Existing guidelines and standards related to flow cytometry assay development for ATMPs

The purpose of this section is to provide an overview of the existing guidelines and standards concerning the use of flow cytometry analysis in ATMP development and manufacturing. Regulatory authority requirements and expectations for the development of ATMPs are constantly evolving as technologies advance and knowledge is expanded. Thus, this section is not an exhaustive list of all guidance and ATMP developers should familiarise themselves with regulatory guidelines which apply to their respective context (for example country, facility setting, and product type). Guidelines can be divided into those associated with defining quality attributes for specific product types and starting materials (Table 4) and those associated with ensuring that products (and assays) are consistently produced and controlled according to the appropriate quality standards (Table 5).

Table 4: Guidance documents defining expectations relating to product characterisation for ATMPs.

Agency	**Reference**	**Title**
EMA	EMEA/CHMP/410869/2006	Guideline on Human Cell-Based Medicinal Products
EMA	CAT/CHMP/GTWP/671639/2008	Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells
EMA	EMA/CAT/80183/2014	Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products
EMA	EMA/CHMP/BWP/271475/2006 rev.1	Guideline on potency testing of cell-based immunotherapy medicinal products for the treatment of cancer
EMA	EMA/CAT/852602/2018*	Draft guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials*
Ph. Eur.	Chapter 5.14	Gene transfer medicinal products for human use
FDA	2008-D-0205	Guidance for Industry: Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)
USP	Chapter <1046>	Cellular and Tissue-Based Products guidelines.
ISO	ISO 20391-1:2018	Biotechnology - Cell counting - Part 1

**Guidance in draft.*

Table 5. Guidance document defining standardized procedures to ensure product quality standards.

Agency	Reference	Title
EMA	EudraLex Volume 4 Part IV	GMP requirements for Advanced Therapy Medicinal Products
ICH	Q2(R1)	Validation of analytical procedures: text and methodology
ICH	Q6B	Test Procedures and Acceptance Criteria for Biotechnological/Biological Products

In the context of ATMP development, flow cytometry analysis is used as an analytical tool for in-process control and product characterisation. As such, flow cytometry analysis should be conducted in accordance with GMP requirements when in a regulatory environment. Biomarker selection for a given flow cytometry panel should be performed during process development/validation, it is product-specific and should be aligned with the assay purpose (See Sections 3.1 and 3.2). For this reason, compendial methods are scarce. However, through the standardisation of flow-cytometry best practices and an ever-increasing knowledge of the relationship between product CQAs and clinical safety and efficacy, it is possible that more cell type or product specific compendial standards will be developed in the future.

4. SYSTEM SELECTION, QUALIFICATION, AND COMPARABILITY

For the best results to be obtained, selection and qualification of a suitable flow cytometer is key. A suitable flow cytometer is one that enables the user to perform all required testing to an appropriate standard based on the user's intention and the properties of the therapeutic. Consideration should also be given at this stage as to whether the software running the flow cytometer and whether this needs to be 21 CFR part 11 compliant; typically, in the GMP setting, compliant software is desirable. This addresses the key concerns of audit trail availability and data integrity. A systematic and documented procedure should be applied when defining the minimum user requirements. Suitable methodology should be employed to perform flow cytometer qualification at different operational levels to ensure the flow cytometer performs as per the manufacturer's specifications, and in a way that is suitable for its intended purpose.

System qualifications are required for laboratories that operate within a quality management system, comply with global standards, and/or follow country-specific regulations. Qualifications provide documented verification that laboratory instruments are installed and operating according to the manufacturer's performance specifications, as well as meeting users' needs and expectations.

Initial system selection / qualification and subsequent re-qualification activities (e.g. following hardware replacement and/or software upgrade) should be recorded and managed under the facility's quality management system using the change control procedure and applying risk-based approaches.

As part of the change control procedure, specific requirements regarding training of personnel on how to operate the flow cytometer should be assessed. Training may be provided by the flow cytometer manufacturer and/or local subject matter experts and fully documented in the relevant operators' training records.

4.1 User-specific requirements for system selection

The first step in selecting a new instrument is the generation of a URS document. A URS is the document which details all the requirements, in this case, for a new flow cytometer. For example, it is defined in Annex 15 of EudraLex volume 4 as follows:

"*The specification for equipment, facilities, utilities or systems should be defined in a URS and/or a functional specification. The essential elements of quality need to be built in at this stage and any GMP risks mitigated to an acceptable level. The URS should be a point of reference throughout the validation life cycle*."

As well as an introduction defining the scope of use, the regulatory requirements of the laboratory, should also be detailed. The following list of operational requirements that should be considered is not exhaustive:

- **Essential requirements**:
  - Number of lasers.
  - Number of parameters.
  - Number and type of detectors.
  - Acoustic/hydrodynamic focussing.
  - Meeting regulatory data integrity requirements.
  - Fluidics shut off in case of a fluidics failure.
  - Equipment manufacturer to carry out IOQ.
  - Ability for the flow cytometer to be connected to a network.
  - Uninterruptible power supply (UPS).
- **Optional requirements**:
  - Option to add lasers at a later stage.
  - Option to link to a cell separation device.
  - Option to add / replace filters, detectors, and lasers.
  - System ergonomics.
  - File format compatible with non-manufacturer supplied analysis software.
- **Environmental requirements:**
  - Benchtop space.
  - Temperature of operation.
  - Vibration tolerance.
- **Cleaning/disinfection requirements**:
  - Defined cleaning cycles.
  - Ease of cleaning components and equipment.

Additional, non-operational, requirements can be set out in the URS in relation to:

- **Maintenance/calibration:** *(Unless maintenance is to be performed only by the manufacturer.)*
  - Manufacturer should define the maintenance and calibration procedures.
  - Manufacturer should provide a spare parts list.
  - Manufacturer should provide a call out service.
- **Qualification:**
  - IOQ documentation.
  - IOQ to be performed by the manufacturer or internally.
  - PQ to be performed internally.
- **Training:**
  - Manufacturer should provide training and resources.

Once complete, the URS should be reviewed and approved by the users and by a responsible person of appropriate expertise to ensure that regulatory requirements have been met. The completed URS can be used internally to support the purchase of a flow cytometer or it can be used as part of a tender process.

4.2 Design qualification (DQ)

Once all potential suppliers provide their feedback, design qualification (DQ) should be performed, at a minimum, for the selected piece of equipment. This involves verifying whether all requirements in the URS are fully met and risk assessing those that are not. It also requires documentation of the stage of system qualification where each requirement will be assessed.

The result of DQ should be a traceability matrix linking requirements and risks (if applicable) with relevant stages of system qualification – see Table 6 for an example of such a traceability matrix.

Table 6. Example stages of system qualification / traceability matrix.

User requirement specification		**Document**			
Requirement number	**Specific requirement**	**IQ**	**OQ**	**PQ**	**RA**
5.3.4	Three lasers – blue, violet, red	X			
5.3.5	Defined cleaning cycles		X		

In situations where a pre-acquired/installed piece of equipment is to be used and operators have no input on URS drafting and system selection, DQ should still be performed. In this case, the traceability matrix should be generated prior to/during PQ. If the system is to be used for regulated bioanalysis then it is essential that the system meets the regulatory requirements applicable to its intended use.

Please consult Rules and Guidance for Pharmaceutical Manufacturers and Distributors 2017 (The Orange Guide) and Eurdralex Volume 4 for further information.

4.3 System qualification

To ensure result reliability and robustness, assay validation (See Section 8) should only be conducted on a qualified flow cytometer. It is, however, acknowledged that assay validation may have to be performed in conjunction with/as part of PQ; in this instance the minimum requirement is for assay validation to be performed on a cytometer that has passed full IQ/OQ.

A multi-disciplinary team should be involved in flow cytometer qualification, including representatives from scientific management, quality assurance, information technology and the “system owner”. The “system owner” is the individual who is ultimately responsible for the equipment; they must have adequate knowledge of the instrument and its intended use, as they will be responsible for drafting all qualification plans and associated SOPs, as well as defining the overall acceptance criteria the qualification exercise must meet.

Flow cytometer qualification protocols should be developed and approved prior to the launch of the qualification. They should describe responsibilities, timelines, deliverables, and detailed testing protocols for IOQ and PQ as exemplified in ICCS Quality & Standards Modules.

IOQ and PQ protocols should provide documented evidence that the qualified flow cytometer was correctly installed and operates according to both the manufacturer’s and the end user’s specifications and requirements. Upon IOQ/PQ completion, the decision to make the flow cytometer available for general use must be conditional to the following conditions:

- All acceptance criteria have been met and all specifications verified.
- Any significant deviations encountered have been resolved and applicable corrective actions have been taken and documented.

4.3.1 Installation qualification (IQ)

An example instrument installation qualification template is given in Annex 1.

IQ is the first phase of flow cytometer qualification, which verifies that the equipment, along with its sub-systems and any ancillary components, have been installed and configured according to the manufacturer's specifications. IOQ and PQ should meet a set of pre-determined criteria, failure to do so would require actions to be taken to resolve faults and assess impact.

IQ aims at providing documented evidence that:

- The instrument and its components are received as designed and specified.
- All key aspects of hardware and software adhere to the manufacturer's specifications.
- The instrument is installed properly in the selected environment.
- The environment where the equipment is installed is suitable for its operation and use.

IQ typically comprises two stages: pre-installation and physical installation. Any specific regulatory requirements, as well as the methodology used for IQ, must be detailed thoroughly in a VMP.

4.3.1.1 Pre-installation

During pre-installation and ideally prior to flow cytometer receipt, the site is checked for the fulfilment of the manufacturer's recommended minimum requirements for optimal equipment operation (for example electrical requirements, network connectivity, temperature, relative humidity, and vibration levels). It should be checked that sufficient space is available for the flow cytometer and the availability of any required ancillary modules (such as fluidics carts, UPS units, computer workstations, and additional monitors).

4.3.1.2 Physical installation

Physical installation consists of the verification that the flow cytometer and all its components are received undamaged and in working condition. It also involves confirming that all fluidics, electrical and communication connections are established for the system components as per the manufacturer's recommendations.

Comprehensive flow cytometer IQ should cover the following areas:

- Location of install and necessary floor space.
- Documentation of all computer-controlled instrumentation.
- Compilation of all instrument certifications and manuals.
- Proper unpacking and cross-checking of instruments.
- Examining instruments and components for damage.
- Ensuring correct power supply.
- Installing ancillary instruments.
- Verifying connections and communications with peripheral units.
- Documenting firmware versions and serial numbers.
- Assessing environmental and operating conditions.
- Checking software system installation and accessibility.
- Recording calibration and validation dates of any tools used.

Note: in the event that subsequent software tools/updates are installed, IQ must be repeated as part of the instrument re-qualification activities.

4.3.2 Operational qualification (OQ)

An example operational qualification template is given in Annex 2.

OQ provides documented evidence that all parameters are functioning according to the manufacturer's specifications and approved design criteria. Importantly, it verifies that equipment performance is consistent with user requirements within the manufacturer-specified operating ranges. It also confirms that any documented recommendations made by the manufacturer have been applied, and that the environmental setting is compatible with the operation of the flow cytometer.

OQ should only be conducted once IQ has been finalised. It serves as a detailed review of hardware and software start-up/shut-down, operation, maintenance, cleaning, and safety procedures. However, it is important to note that, although OQ usually follows IQ completion and sign-off, the two may also be performed as a combined IOQ procedure. OQ can be performed by FSEs, qualified internal staff and/or contracted external consultants.

OQ should include but is not limited to the following:

- Tests that have been developed from the knowledge of processes, systems, and equipment to ensure the flow cytometer is operating as designed.
- Tests to confirm upper and lower operating limits, and/or "worst case" conditions.

Successful OQ completion should allow the finalisation of:

- Detailed SOPs and forms for flow cytometer operation, performance check and maintenance.
- A training protocol providing instructions for operation of the instrument, the workflow in the laboratory, quality control, instrument maintenance, clinical flow assays using the instrument, and competency assessment after initial training.
- A preventative maintenance protocol in line with the manufacturer's recommendations.

4.4 Performance qualification (PQ)

PQ normally follows the successful completion of IQ and OQ. However, it may be appropriate to perform it in conjunction with OQ and/or Assay Validation.

PQ constitutes the most time-consuming part of the overall flow cytometer qualification. It is intended to provide documented evidence that the equipment performs consistently for the intended purpose of the end user. It should be performed by the key operator and the lab personnel who will be the primary users of the flow cytometer.

Basic PQ includes evaluation of instrument performance through integrated QC applications (involving the use of multi-peak calibration beads) and monitoring linearity and sensitivity over a period of time, typically using Levey-Jennings charts.

It is recommended that basic PQ includes data from any appropriate flow cytometry assay verification or validation. In this case, there are three main possible scenarios:

- For IVD assays, where the reagent manufacturer has already validated the assay and received clearance from regulatory bodies, a verification that the laboratory can reproduce those specifications using the new flow cytometer may serve as additional PQ (e.g. ability to match the "Intended Use" specified in the "Instructions for Use" for IVDs).
- If a laboratory already has an existing validated LDT and plans to run that assay on a new instrument, a verification showing that the assay specifications can be reproduced on the new instrument may serve as additional PQ.
- For new LDTs, flow cytometer comprehensive PQ and initial method validation may be combined. Assay validation requirements are independent of the equipment used (See Section 8).

Regardless of the scenario and the validation scope of the PQ, the experiments detailed below should also be included to evaluate basic instrument performance.

After the initial IQ/OQ/PQ, re-qualification must be performed following any major maintenance, or whenever the equipment or software controlling the equipment is modified/updated (e.g. following

addition of extra lasers, detectors, or filters). Re-qualification must also be performed as part of routine quality assurance processes. Extended PQ includes inter-instrument comparison, inter-laboratory comparison and longitudinal performance.

4.4.1 Instrument stability and precision

Most instrument vendors provide integrated quality-monitoring systems that allow the end user to establish the optimal instrument settings and performance over time. As a starting point for PQ, it is important to demonstrate and document that the instrument performance passes such internal quality assurance programmes.

As precision refers to the closeness of two or more measurements, it is also recommended that, as part of PQ, the same control sample is analysed with the same settings at distinct time-points (See Section 8).

4.4.2 Instrument linearity and sensitivity

Multi-peak fluorescent calibration beads are useful tools to assess the flow cytometer's performance in terms of linearity and sensitivity.[7] These beads consist of various size particles that are dyed to multiple fluorescent intensities. Because every bead contains a mixture of fluorochromes that are excited at a wide range of wavelengths (typically at any wavelength from 365 – 650 nm), the multi-peak fluorescent beads feature an array of emission spectra, with fluorescence being observed in all applicable detection channels (See Figure 2).

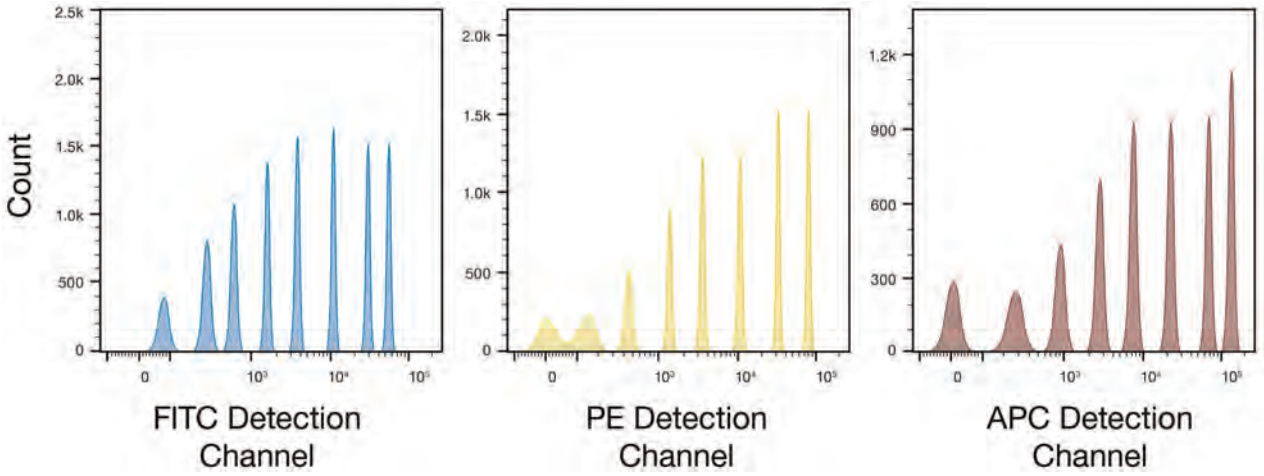
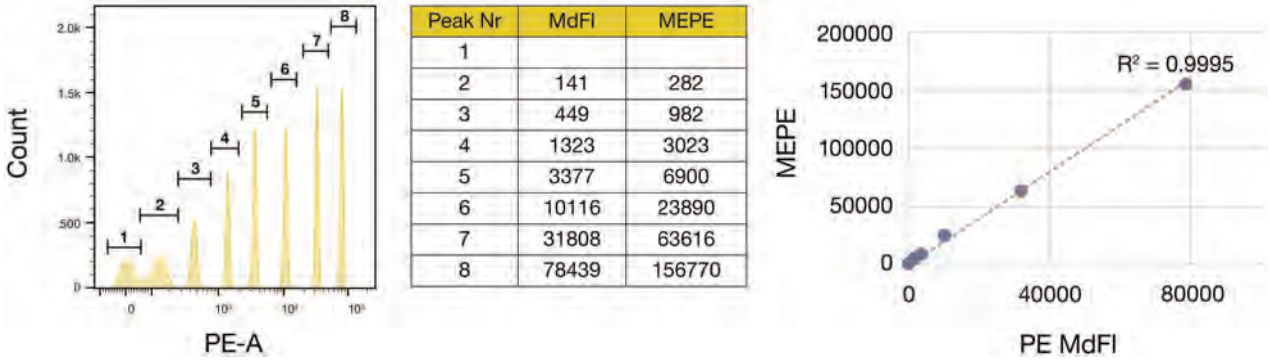


Figure 2: Illustrative example of 8-peak fluorescent bead acquisition. Histograms show the eight different fluorescence intensities detected in each one of the detection channels used. Compensation must be set to "off" whenever acquiring multi-peak fluorescent beads.

Linearity refers to the relationship between the detected fluorescence signal and the real (known) fluorescence intensity over a given range. There are different methods to assess instrument linearity. One method consists in plotting the median fluorescence intensity (MdFI) obtained from multi-peak fluorescent beads against the reference values provided by the manufacturer for each peak (See Figure 3). If the instrument demonstrates good linearity, the R^2 value of the plotted regression line will be close to 1.

Note: when assessing instrument linearity, the results of non-fluorescent bead populations (e.g. peak number 1 in Figure 3) is not to be taken into account.

[7] *Du L et al. The evolution of guidelines for the validation of flow cytometric methods. Int J Lab Hematol. 2015; 37 Suppl 1: 3-10*



Peak Nr	MdFI	MEPE
1		
2	141	282
3	449	982
4	1323	3023
5	3377	6900
6	10116	23890
7	31808	63616
8	78439	156770

Figure 3: Example of flow cytometer linearity assessment. The MdFI was obtained for each fluorescence peak (numbered 1-8) and plotted against the corresponding number of MEPE provided by the manufacturer. Linear regression was then performed.

The sensitivity of a particular instrument is mainly demonstrated by the ability to resolve different populations, especially dim populations (See Figure 4). Sensitivity depends both on the measured difference in fluorescence intensity between dim and negative peaks, and their respective spreads (usually expressed as their RSD). In general, the intrinsic RSD of bright beads (i.e. one of the peaks with the highest MdFIs) should be ≤3% at low flow rate or ≤5% at high flow rate. Sensitivity can be quantified as mean channel separation of the negative and dimmest peak, which is calculated by taking the difference of fluorescence intensities of the negative and dim peaks divided by a pooled SD of both populations. For a given set of PMT targets, an instrument will have characteristic sensitivities in each fluorescence parameter.

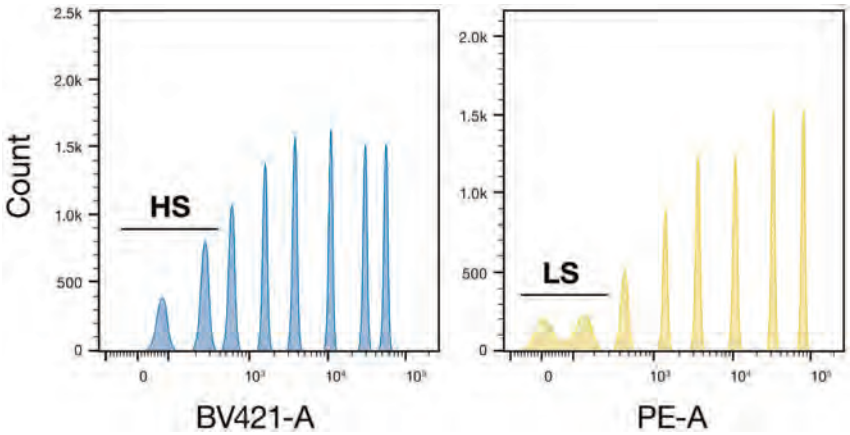


Figure 4: Illustrative example of sensitivity assessment using multi-peak fluorescent beads. Discrimination between the negative and the dimmest peaks in the BV421 channel is good (high sensitivity, HS), whilst it is more difficult to clearly distinguish between the negative and dimmest peaks in the PE channel (low sensitivity, LS).

4.4.3 Small particle sensitivity, debris optimization, and resolution of cell populations

Small particle sensitivity is typically tested by the FSE during OQ by running appropriate size beads (e.g. 0.2 µm). The data should support that the system is able to separate these beads from debris. However, the laboratory should confirm this by running samples containing the cells / particles of interest. Additionally, because the resolution of cell populations by light scatter is one of the critical parameters measured by the flow cytometer, it is also always necessary to verify light scatter performance using the cells of interest.[8]

[8] *Welsh JA et al. MIFlowCyt-EV: The Next Chapter in the Reporting and Reliability of Single Extracellular Vesicle Flow Cytometry Experiments. Cytometry A. 2021 Apr;99(4):365-368.*

Sample carryover may be measured by acquiring a fixed volume of sample, followed by acquiring a fixed volume of a buffer-only solution such as PBS. The concentration of particles detected in the buffer blank divided by the concentration of particles in the sample, multiplied by 100, yields the percent carryover.

Assessment of sample carry-over is particularly important in the context of adherent cells. Adherent cells may adhere to the internal tubing of the flow cytometer and therefore require more extensive SIT flushing and/or cleaning to be performed between samples. Another scenario where sample carry-over is important is in the context of rare population analysis, as any read-outs may become contaminated by adjacent samples.

4.5 System comparability

If considering multi-centre and longitudinal clinical trials, it is critical to ensure that flow cytometry data can be combined. This is particularly relevant when assay results are based on fluorescence intensity analysis. It is, therefore, recommended to include inter-instrument and/or inter-laboratory comparability assessment as extended PQ testing.

Cross-instrument standardisation has been adopted as a solution in many laboratories to ensure consistency. Use of multi-peak fluorescent beads can improve results' stability.

Multi-peak fluorescent beads allow a fixed output value (target MdFI) for each fluorescent channel to be set. The target MdFI should be set before each experiment, to ensure that all readings are taken in the standardised settings. This is more accurate than using the same PMT settings through different experiments (See Figure 5). In some flow cytometers, an automated calibration process is integrated within the instrument, this enables calibration across different days, or across different cytometers from the same model. Laser intensity can change during the day, this should be considered when many samples are being analysed. Multi-peak fluorescent beads can be used, not only to standardise readings between experiments, but also to monitor flow cytometer performance during data acquisition. Reading the beads and adjusting acquisition voltages to obtain target MdFIs ahead of running the samples eliminates the possibility of deviations due to flow cytometer performance. This is also a useful method to employ when transferring assays between flow cytometers and/or facilities.

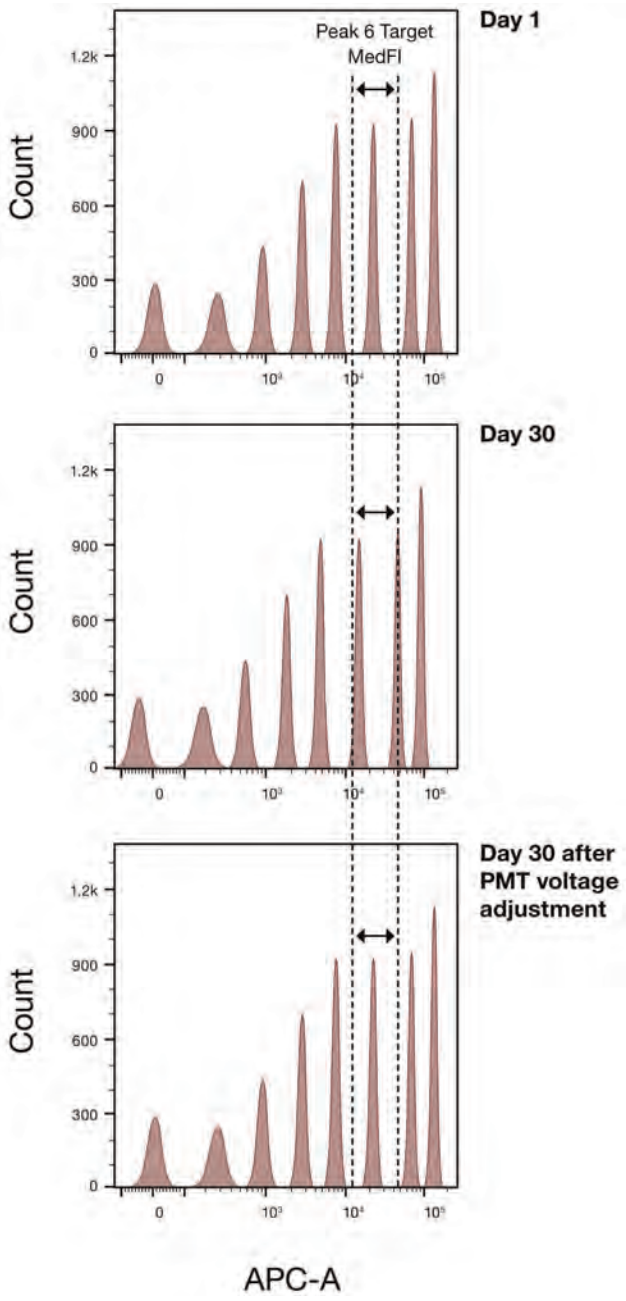


Figure 5: Illustrative example of the use of multi-peak fluorescent beads for flow cytometer standardisation.

Another method for standardisation of MdFI is converting it to known fluorescent units, such as MESF.[9] The MESF concept is based on the fact that a sample labelled with a specific fluorochrome has the same MdFI value as a fluorochrome solution with an equivalent number of free fluorochrome molecules. By this principle, designated MESF beads can be used to create a standard curve of MdFI vs MESF values that can then be used to translate MdFI into more universal MESF units. This also allows comparisons to be made between analysis on different days and/or different flow cytometers.

Similarly, MdFI can be transformed into ABC units.[10] In this approach, beads with a known number of antibody binding sites are stained together with the cells/blood samples. Under optimal conditions, the MdFI of the beads should reflect the number of molecules bound to a single cell.

[9] *Wang, L. and Hoffman, R.A. 2017. Standardization, calibration, and control in flow cytometry. Curr. Protoc. Cytom. 79:1.3.1-1.3.27*
[10] *Mizrahi O et al. Quantitative flow cytometry: Concerns and recommendations in clinic and research. Cytometry Part B Clin Cytom 2018; 94 (2): 211–218*

5. SAMPLE KNOWLEDGE

It is important to recognise that biological starting and process materials may be highly variable between and within individuals and according to disease status (biological variability), and that the measurement of such materials at the start or during the process compounds this variability dependent upon the methods and procedures used (process variability).

5.1 Starting and process material variability

It is important that the test sample to be analysed is representative of the sample it has been collected from, whether it represents starting material (such as bone marrow, peripheral blood mononuclear cells or blood), bulk drug substance or final product. Typical sample types in the context of cell and gene therapy products include cell cultures, biological fluids, and single-cell suspensions obtained from dissociation of solid tissues.

A variety of cell populations may be present in the cell suspensions including dead cells and debris, and some cells may not be relevant for the analysis (e.g. exclusion granulocytes when analysing lymphocytes). Steps taken during sample preparation, such as staining, lysis, fixation, cryopreservation, thawing, and paraffin-embedded tissue preparation may lead to the presence of debris. In addition, processes such as cryopreservation can alter the expression of cell surface markers leading to further processing artifacts.

The material the test sample is being collected from, whether it is from a whole blood bag, apheresis bag, or cell suspension of final product should be adequately mixed before sampling. Care should be taken to obtain sufficient sample volumes for analysis. For adherent cell cultures, for example, it is difficult to collect a small representative sample of a whole population by taking a defined area from a culture vessel. The use of enzymatic chelating agents such as Trypsin, to transitorily lift cells, may preferentially select cells on the basis of cell type or other biological properties (e.g. viability) of cells, and the use of such procedures should be validated in house where necessary. Enzymatic treatment of cells may also affect cell surface marker expression of the cell. As a result, the sample of cells selected may not be representative of the population of cells in culture.

Primary cells from blood that are stained and analysed immediately following collection will behave differently to primary cells that have been in cell culture for some time (See Section 4).

It can be useful in the early phases of development to test incoming starting materials for quality attributes to begin to acquire data on CQAs. Starting material CQAs can be used to create a specification for testing starting material to ensure material is only processed if the minimum quality standard is attained. It may not be possible for all types of starting material to have CQAs determined, for example cells obtained from solid tumour tissue may need to be cultured and expanded for several days before QC sampling can be performed. Cells cultured for several days are susceptible to cell culture variability. This adds variability to the quality of the starting material and makes CQAs difficult to determine.

5.2 Patient to patient variability

Patients receiving ATMPs tend to be in an advanced disease stage and in the case of oncology patients, have experienced different treatment pathways. They will have different comorbidities depending on the indication for the ATMP. Age and gender may also vary between patients (See Section 5.4). The immune system of paediatric and adult patients is known to vary significantly. Clinical trial exclusion criteria and the licensed use of a drug product will be defined to ensure patients fit into a sub-population, but the disparity between patients can be wide. These variations will affect the range of results likely to be obtained and can make setting specifications challenging, for example setting CQAs for starting material. When used for process development or as controls in assays, the choice of healthy donors should match patient demographics as much as feasible.

5.3 Healthy versus diseased donor

ATMPs are currently used to treat serious life-threatening conditions such as monogenic defects which cause symptoms such as severe combined immunodeficiencies, SMA1, and oncology patients who are refractory to standard therapy options. As a result of the late stage of the diseases, and the use of standard patient treatment pathways being exhausted, each patient will have received unique treatments. Treatments can include steroids, haematopoietic stem cell transplant and cytotoxic drugs. These treatments can affect the haematological cell compartment and may result in the patients having abnormal cell differentiation populations compared to the reference range.

Disease can also directly impact the cells, for example patients with adenosine deaminase deficiency have severely reduced or absent lymphocytes. If certain white cell compartments are ablated this can lead to difficulties in designing assays and templates for defining these patients' populations of cells. Similarly, assay development using healthy tissue can also be difficult in non-blood disorders. These considerations must be carefully planned in assay development to ensure quality by design see SC X Supplementary Chapter on the use of Analytical Quality by Design concepts for analytical procedures. Healthy donor material can be used for development and validation, when patient material is scarce, however relevance to the intended material should be demonstrated with bridging studies for example.

5.4 Distribution and transport effects

5.4.1 Collection containers and buffers

Specimens derived from human blood/apheresis can be anticoagulated using many agents, commonly used agents include citrate-based anticoagulants, EDTA or heparin. Lithium heparin and EDTA anticoagulant sample tubes will preserve samples for several hours to days depending upon sampling methods and handling and studies on the stability of cells in anticoagulants should be undertaken. The exact length of time different anticoagulant samples can be in transport and stored before processing should be validated by the user. Where feasible and appropriate, a healthy donor control sample, sent alongside the patient sample, can provide assurance as to the preservation of the patient sample through transport and handling, however this is not always practical. For longer-term transport or storage, specific media may be required, and validation studies should be performed to ensure that those samples are equivalent to fresh samples at the time of flow cytometric analysis or limitations should be defined. Laboratories should have written procedures describing protocol when specimens are found to have degraded during transport or storage. Care should be taken that steps such as washing or other cell handing are minimized so that the cell sample being assayed is as representative of the cell population as possible. Where cell handling is required, this should be fully validated.

5.4.2 Transport

Transport of samples can affect the quality of cells being analysed by flow cytometry and quality controls should be included in assays where possible. Many clinical trials in early phase are initiated with one manufacturing centre with associated QC laboratories. The logistics of receiving starting material (patient or healthy donor cells) from clinical sites and of transporting QC samples and final product should be well defined. Patient monitoring samples are also usually shipped from the clinical sites to a single hub of QC laboratories. As the product development moves into later phase clinical development, multiple manufacturing sites may be opened to meet clinical supply and demand. These additional manufacturing and clinical sites may be overseas which will add complexity to the logistical management of clinical starting materials and patient monitoring samples. Comparability studies will be required for addition of new manufacturing sites to a clinical trials authorisation or market authorisation, and validation of transport will be required.

Transport from site of collection of a sample to a laboratory where flow cytometry analysis is to be performed should be validated and a sampling plan generated. A sampling plan should include an SOP that covers collection, transport, and storage of the sample before analysis. For monitoring of

patients post infusion of ATMP, blood samples are taken directly from patients. In these scenarios, any specialist training on sample handling for the phlebotomists who procure the samples is key, as is the understanding of the sample journey from patient to laboratory.

Samples that are to be lysed, such as whole blood lysis, and surface antigen stained should be transported and stored, preferably with continuous mixing at ambient temperature. Fixed samples (e.g. formaldehyde-based fixatives) or live cell preparations are typically stored at 4°C. To achieve ambient or chilled conditions, insulated transport boxes with appropriate cooling strategies should be used. Calibrated temperature monitors that have data point logging intervals appropriate to the journey should be included in the transport pack to ensure the intended temperature was maintained.

5.5 Media, buffers, and excipients

It is important that any media, buffers and excipients used need to be evaluated closely during process development of assays and every component weighted and risk assessed to understand impact. The sample matrix in which cells are suspended can affect flow cytometry analysis significantly, there can be interference and matrix effects.

An example of a common excipient used in ATMP formulation is DMSO. DMSO can affect the analysis of cells by flow cytometry in several ways. DMSO is also known to affect the expression of some surface markers such as CD34 and may give different results to equivalent samples that do not contain DMSO. If final product formulation contains DMSO, transport of QC samples to the testing laboratory in a timely manner is critical because DMSO will cause the cells to lose viability. To avoid this issue, in some instances, final drug product characterisation is performed on test samples taken from the drug substance, while formulated in non-cryopreservative buffer in the penultimate step before formulation. However, it is possible to test the final drug product formulated in cryopreservative containing DMSO if the transport to the testing laboratory is within a timeframe and a temperature range that has been determined not to detrimentally affect sample stability. Once in the QC laboratory, the sample can either be diluted in an appropriate diluent or the sample can be spun down and the DMSO washed out and resuspended in appropriate diluent. This type of handling should be validated to ensure it produces results that are representative of the original sample. However, transporting samples in DMSO to QC laboratories and sample handling may lead to reduced cell viability results that are not truly representative of the final product. It is important to understand in these scenarios that the viability results may be a worst-case scenario compared to the final product which will provide contingency to the specification of the product.

Freeze/thaw of final product will also affect cell viability. Freezing a vial of the frozen final drug product may not be a representative sample of a frozen final drug product in a bag due to the number of cells present, and the volume and material of each container. The final drug product and samples should be stored in the same environment for example, if the final product requires storage in vapour phase liquid nitrogen, then samples should be stored in vapour phase liquid nitrogen to be truly representative. Thawing of samples and final product should be as controlled as possible and validated to ensure the sample thawing is equivalent to the final product thawing.

A commercial lyse/fix solution can increase storage time but may impact cell viability. For specimens separated by density-gradient centrifugation, storage in a solution of buffered paraformaldehyde (0.1% to 2.0%) is recommended after cell labelling has occurred. Sample processing, staining, and fixation reagents used should be qualified for their intended use. Further guidance is available in ICH Q6B, Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products. When using reagent kits, follow the manufacturer's sample processing instructions; otherwise validate any use outside of these instructions.

5.6 Prevalence of target cell population

The number of events should be increased depending on how rare the cell population that defines the product is; the ideal minimum is 500 cells of interest. If the cell product is rare, cell events may require greater than 2 million total events to count a significant (100 or more) number of the desired population (See Section 6.2). This may require live gating and the amount of sample available for testing becomes a limiting factor.[11] Long sample processing times can have implications on flow cytometry analysis if the stability of the fluorophores used is negatively impacted or the stability/ conformation of the sample is impacted resulting in cell clumping or reduced cell viability.

Rare populations should be defined by brighter fluorochromes to aid the limit of detection.

5.7 Sample preparation effects

5.7.1 Sample processing

Suitable records to support sample storage, sample processing, and critical assay steps should be maintained.

Extended sample processing can sometimes be necessary for analysing ATMPs using flow cytometry for instance in cases where the cell of interest is a rare population, such as invariant natural killer T cells or CD34+ stem cells. Extended sample processing involving spinning in a centrifuge, washing, RBC removal or lysis, or density-gradient separation is common but can introduce error. Several techniques and reagents are available for RBC removal and lysis. IVD reagents are usually of high quality, but artefacts can still occur. Density-gradient centrifugation can suffer from variable cell losses among subpopulations that are being measured. These sources of error and artefact are avoided when analysing whole blood but use of whole blood will only apply to starting / raw materials. Most whole blood lysate instructions recommend staining at room temperature and in the dark. Many methods include a dilute fixative to prevent capping and internalization of cell surface antigens preventing binding of fluorochrome.

In contrast, cell preparations (density-gradient cell preparations, apheresis specimens, tissue culture) can additionally be stained at 4°C, washed with cold buffer, and stored cold until analysed but this methodology should be fully validated. Whatever storage temperature is selected, the length of storage of samples should be validated to determine maximum storage time for samples. Validation is also important to determine optimum temperature for processing each product in each laboratory. Fixation that also preserves cell surface antigens can be accomplished using commercial leukocyte preservatives or with buffered methanol-free formaldehyde. Formalin contains added methanol which can affect both cells and fluorochromes. Any laboratory that considers batch analysis of fixed specimens should validate these techniques thoroughly before implementing them.

Excessive cell handling such as long sample mixing times on a vortex, improper fixation of samples, or bacterial contamination of the cells can give a high particulate background. Careful sample preparation and in-date reagents used a set number of times before discarding can ensure consistent side scatter profiles.

5.7.2 Sample dilution

The dilution method used to alter the concentration of a sample can impact the flow cytometric results, particularly quantification:

- Inadequate mixing and settling of the cell sample can result in high cell event rates. High event rates may be attributed to high cell densities during antibody staining or in the final cell sample.

[11] *U. Sommer et al. High-sensitivity Flow Cytometric Methods: Considerations for Design Control and Sensitivity Validation. Cytometry Part B, Clinical Cytometry. 100: 42-51, 2021. DOI: 10.1002/cyto.b.2194*

- Low event rates can result from cell clumping and low final sample cell densities. This can be prevented by consistent staining protocols.

5.7.3 Reverse pipetting

Accurate pipetting is essential and even trained operators have a pipette error rate of +/- 2% RSD. Reverse pipetting is more precise and may be used if appropriate, especially when performing cell counts. To ensure accuracy of pipetting it is essential that a uniform temperature is maintained throughout the pipetting procedure and all pipettes should be routinely calibrated using a method and frequency as defined in a written laboratory procedure.

5.7.4 Antigen labelling

Cell surface (extracellular) antigen staining, and intracellular staining techniques are applied to the identification of cell subsets to define function. For example, stimulation of NK cells with Lipopolysacharide (LPS) can result in the expression of intracellular activation markers and cytokines that can then be measured in parallel with antibodies defining phenotypic markers on the surface of target cells. The use of secretion inhibitors allows the intracellular accumulation of cytokines. The cells are then fixed, permeabilized, and detected by a flow cytometric method. Permeabilization methods vary upon the protein of interest, but common reagents include 2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol and polyoxyethylene (20) sorbitan monolaurate (See Section 6.5). Such assays are useful for monitoring T-cell subpopulations that respond to a target antigen, such as CAR-T cells targeting cancer antigens.

Common issues with improper antigen labelling of samples include:

- High fluorescence background intensity: Excessive addition of antibody or poor cell washing. Consistent antibody concentration and cell density, adequate washing and blocking will help avoid abnormally high fluorescence background.
- High fluorescence intensity: As in the case for high fluorescence background, high mean cell fluorescence can result from too much labelled antibody, inadequate or inconsistent cell washing, or inadequate blocking. Including detergent in the wash buffer, especially during intracellular staining, can help prevent non-specific antibody binding.
- Weak fluorescence intensity: In addition, cell physiology or reagent preparation issues, such as insufficient antibody concentration, labile or secreted target antigen, poor-quality or improperly stored reagents (resulting in fluorochrome fading), or inaccessible target antigen, can all result in a weak signal.

5.7.4.1 Use of controls

Ideally, fluorochrome-conjugated bead preparations are used for standardizing PMTs, compensation, and for quantifying the expression of specific markers. The use of biological controls should be used. Cell samples can be stained with primary antibodies, and secondary antibodies to assess non-specific binding, unless the laboratory has determined that non-specific binding does not interfere with assay results. Antigen-positive and antigen-negative cell populations (prepared and stained in a manner identical to that for the test articles) provide internal system suitability standards. Such control cell populations also allow the laboratory to assess lot-to-lot variations in antibody preparations and staining reagents.

5.7.4.2 Cell surface antigen staining

Techniques for surface antigen staining vary with the type of specimen. Whole blood lysis techniques generally require surface labelling at room temperature in darkness for 15 to 30 minutes, followed by RBC lysis and, if desired, fixation. Published techniques use ammonium chloride or formaldehyde

lysis of whole blood or marrow specimens, followed by washing before antibody labelling for leukaemia or other immunophenotyping.

Mononuclear cell or cultured cell samples that are stained live should be kept at 4°C or in an azide-containing buffer to prevent capping and internalization of the antibodies.

5.7.4.3 Intracellular staining

Several standardized procedures exist for labelling intracellular antigens and cytokines. The operator should consult the manufacturer's protocol and standardized reagents for these procedures. Care should be taken when using reagents from different manufacturers due to variability in properties. For cytokine labelling it is often necessary to use an activating step and a Golgi complex blockade or endoplasmic reticulum block to allow enough cytokine to accumulate in the cytoplasm for detection. If standardized reagents or procedures are not available from the manufacturer or if analysis of specialised functions is required, many common procedures and techniques can be found in sources such as Current Protocols in Immunology.[12]

Once samples are stained, specimens should be acquired and analysed as soon as possible. SOPs should define validated time limits for both sample stability post-collection before sample staining and also processed sample stability, which is the time taken for acquisition after collection. Paperwork should include details of when a sample was collected and when it was stained and analysed. To facilitate the reproducible labelling and processing of samples the system should preferably use automated sample staining/handling where possible.

6. PANEL DEVELOPMENT AND STAINING PROTOCOL

6.1 Purpose of panel

The purpose of the flow cytometry panel should be clearly defined. It should be decided whether the panel is determining primary release criteria or secondary criteria. If the information from the panel forms part of release criteria, then the minimum values set for release must be achievable and clinically relevant. The values may be percentages of a particular makers, cell types, viability, function or absolute number. For example, for some investigational products where engraftment or proliferation are required for function, viability is important as dead cells would fail to engraft or proliferate. Primary release criteria often include cell viability, composition of the product, and percentage or number of cells transduced. Secondary criteria usually provide additional characterisation of the product that may be used as primary criteria in future trials.

It is important to note that successful target cell population characterisation and contaminant exclusion are dependent on the use of suitable positive and negative controls, which may be challenging to establish when designing a flow cytometry panel. For example, when looking for the presence of contaminants, the use of associated positive controls is critical to ensure that the assay would be able to detect the presence of contaminants/impurities, even at very low levels. Alternatively, if the purpose of the panel is merely to evaluate the purity of the final cell product, the use of appropriate negative controls becomes important to allow correction for non-specific background.

The flow cytometer that is being used may influence panel design. The more lasers and detectors the cytometer has, the more fluorochromes that can be run. Some have automated set up and acquisition that may restrict choice; however most offer open software as well. Many now have partially or fully automated instrument set up using beads or other calibrators. Some manufacturers offer the ability to correct and apply settings in software across multiple sites

[12] *Du L et al. The evolution of guidelines for the validation of flow cytometric methods. Int J Lab Hematol. 2015; 37 Suppl 1: 3-10.*

6.2 Cell type

The cell type being examined needs to be considered. Larger cells such as fibroblasts and epithelial cells will have different size and granularity than smaller cells such as T cells and CD34+ stem cells. As cells are cultured, they can increase in size and granularity. Instrument settings (FSC and SSC voltages) should be altered to adjust for any increase in size and granularity. The frequency of the modified cell within the population will affect the amount of sample to be stained and the number of cells to acquire. The rarer the cell the more events need to be collected at acquisition. The more cells of interest collected the more robust the statistical analysis; however as sample may be limited this may not be possible. As a guide if you decrease the frequency of cells of interest by one log you need to increase the number of events by one log. Reliable data depends on the cell population being analysed compared to control samples (e.g. untransduced cells or cells not expressing the marker(s) of interest).[13] If events are extremely rare several tubes may be set up and run with each data file being combined to the previous one so that sufficient events are obtained. In addition, a time gate may be used to remove autofluorescence and spurious events enabling better quantification of ultra-rare events. The rarer the event the more events that will need to be acquired and the better defined the population of interest should be.

6.3 Cell markers

Cell markers are used to identify the cell type and modifications to the cells. Well defined markers exist for most haematopoietic cells and some epithelial and fibroblast cells. Although some cell characteristics can be detected with common markers, research antibodies often need to be validated or bespoke antibodies made. If multiple tubes are required to characterise the cells, it is essential that there are common markers across the tubes to ensure comparability. For example, for T cells, CD3 may be common to each tube. In addition, it is easier to identify rarer populations/proteins if brighter fluorochromes are used for their detection. The polymer fluorochromes as well as APC and PE are brightest. For common markers (e.g. CD3) there is often a range of fluorochromes to choose from but for less commonly used antibodies there may be a limited choice.

6.4 Antibodies

Pre-conjugated antibodies are preferable due to ease of use and are less variable due to the fluorochrome already being attached. Although in research CE / UKCA marked IVD antibodies are not required, they are preferred in GMP environments. In research they may not be required, but it is dependent upon the phase of the clinical trial and amount of validation undertaken. CE / UKCA marked reagents are recommended to ensure reliability and performance since the quality of their manufacture is verified. All antibodies should be evaluated prior to use and a system for verifying new lots established. Laboratories often prefer lyophilised antibody tubes as these come with the required antibodies dried down into a single tube and have long shelf lives so that verification of new lots is less likely to be required. Where lyophilised tubes are not available, antibody premixes may be available. These reduce error as the required antibodies are combined on a single vial and only this mix needs to be added to each tube. Premixes or antibody cocktails can also be made by the laboratory if they are not available otherwise, although it is advisable to start with concentrations of antibody recommended by the manufacturer, antibodies should be titred out to ensure maximal sensitivity with minimal non-specific staining.

6.5 Permeabilisation

As part of designing the panel, it is important to decide if cell population relative frequency assessment or exact enumeration is required. For extracellular flow cytometry where washing the cells is not mandatory, counting beads can be added with a precise volume of product so that the cell concentrations can be calculated. Some software does this automatically for common cell types (e.g.

[13] *Cossarizza A et al Eur J Immunol 2019 Oct 49 (10): 1457-1973 - Guidelines for flow cytometry*

CD3+ or CD34+). Alternatively, cells can be added to tubes already containing counting beads and cell number can be calculated based on bead number and sample volume. For washed samples, enumeration is not accurate as the beads are either washed out or are added later, the cell numbers may have decreased during the washing step. For intracellular analysis, which is required to examine intracellular markers, the cells are permeabilised with detergents such as saponin, triton, tween, or alcohol. The antibodies are added and then a series of wash steps are required before acquisition. Percentage analysis is accurate for intracellular analysis, but absolute enumeration is not accurate because of the permeabilization and washing steps. A combination of a single platform assay (beads) to enumerate the cell type of interest and a second tube with permeabilised samples can be used to calculate the percentage of the cell(s) of interest. This percentage can then be multiplied by the absolute count to obtain a count for these cells.

To design a flow cytometry panel the purpose of the panel (primary or secondary criteria), the flow cytometer available, the cell types, the frequency of the cell, as well as the markers required and the reagents involved must all be considered.

7. DATA ACQUISITION AND ANALYSIS

A variety of sources have addressed data acquisition and analysis of cellular therapies by flow cytometry. This field may be divided into four broad categories – design, set up, measurement and post-acquisition analysis. The result is a cellular analysis which confirms either one or all of the following: identity, purity, and viability quality attributes of the product. The cellular analysis may also provide evidence of "activity", which would more likely be designated as "potency" today.

The techniques used in multi-parameter research flow cytometry – time gates, doublet gates, debris exclusion, viability dyes, exclusion of unwanted cells from analysis to support the detailed characterisation of a specific cell population are also applicable when characterizing cellular therapy products. The use of these techniques must be critically evaluated where the objective is to fully describe the content of samples representative of a cultured cellular therapy (See Figure 6). A systematic evidence-based approach is required.

The .FCS file format, used by most cytometers, is a standardised format which allows for multiple data analysis software to be used. Initial data should be captured in an unalterable format and a chain of custody can be demonstrated for subsequent conversion for use in analysis packages. This will allow for files to be acquired in the native format of the instrument, but also can allow conversion for specific analysis. The gated and ungated data should be saved in the format that is fit for purpose, in case the population gates defined at acquisition are investigated at a later date by the regulator. The MHRA data integrity guidance document should be adhered to.

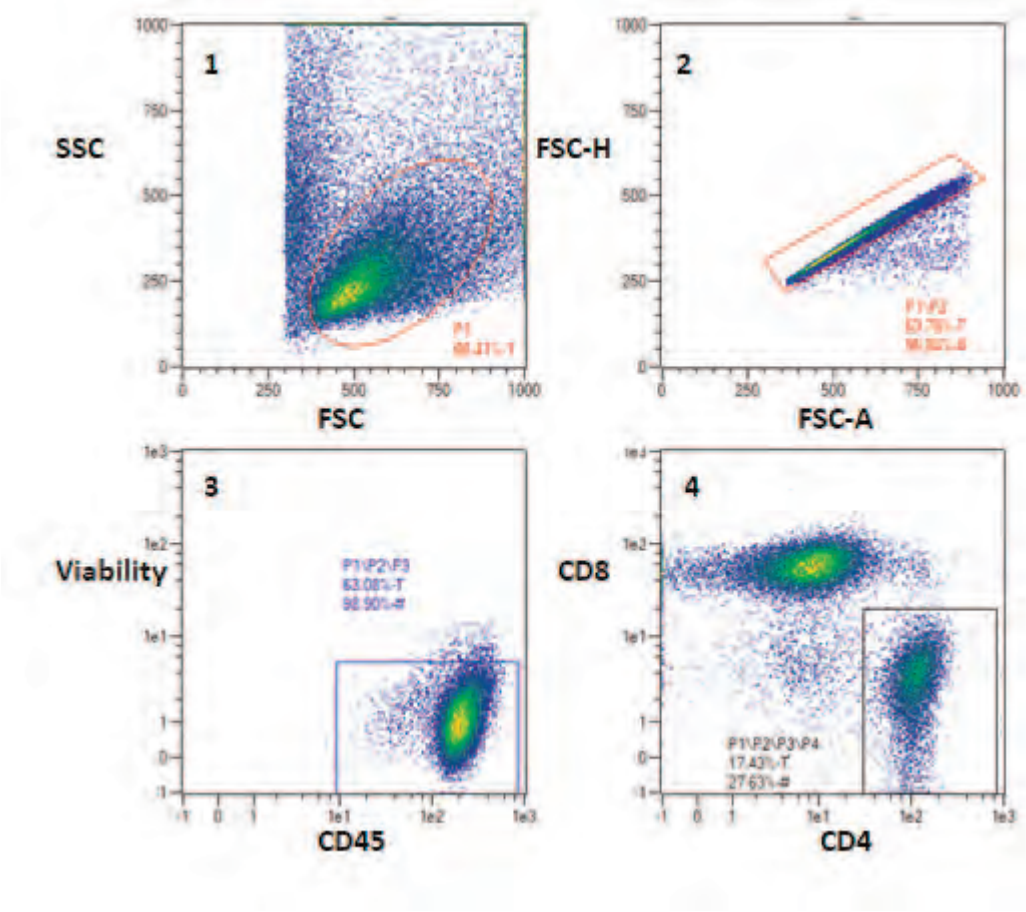


Figure 6: Exemplar application of flow cytometry for routine analysis using cultured T cells. The gating strategy typically follows 1. FSC/SSC gate set by the user by eye, with debris excluded. 2. Singlet gate FSC-A vs -H for doublet exclusion 3. Positive identification of leukocytes with CD45 and exclusion of dead material 4. Positive identification of live CD45+ singlet CD4+ cells. Gated on CD4+ CD8-ve.

7.1 Design

7.1.1 Pre-clinical development and biological relevance.

The assays chosen for a cellular therapy must capture a minimum data set to satisfy the identity, purity, and viability of the product. The exact assay will be product-specific but commonly includes positive and negative elements for example differentiating between a T cell and a B cell, or a monocyte and a neutrophil. There are well understood cell-family markers to aid this identification for example CD45 for leukocytes (See Figure 6) and SSEA-3 for pluripotent cells (See Table 2). While the principal cell product will require positive identification (e.g. is a CD45+ CD3+ CD45RO+ memory T cell), contaminating cells which must be below a certain threshold may not require to be phenotyped in detail (e.g. CD56 may suffice to exclude all NK cell types from a T cell product). It is generally desirable to characterise a product using as simple an assay as possible.

The design of the assay must consider the overall process, characterizing the starting material, the end product, and potentially, critical manufacturing intermediates. There will therefore be a requirement to design and validate several assays and templates for each stage of the manufacturing pathway.

7.1.2 Cultured cells

Templates and phenotyping approaches suitable for freshly isolated cells in starting material may not apply to a final cultured product (See Figure 7). As an example, T cells or monocytes/macrophages can increase greatly in FSC and SSC measurements. This can often be best captured using

logarithmic rather than linear scatter plots, ensuring that no cells are "lost" from the analysis (See Figure 8). Memory T cell markers suitable for fresh material are often not applicable to rapidly growing T cells due to shedding and downregulation as part of the proliferation, but then re-express and normalise once expansion is complete. Similarly, some exhaustion markers can transiently appear during proliferation but not be found in a final product. It is therefore essential to assay the cells at the end of a proliferation cycle to ensure that the memory T cells state is accurately determined or add additional markers such as intracellular cytokine expression to confirm the surface marker staining.

7.1.3 Delta MFI

An assay may need to consider state changes in the cells associated with up or downregulation of markers, rather than a positive or negative result. The assay may then seek to measure delta median, mean, or geometric mean fluorescence intensity for particular markers – e.g. decrease in CD14 expression and increase in CD1c expression for monocyte-derived DCs. These changes in expression may also be captured during manufacturing if in-process assays are required.

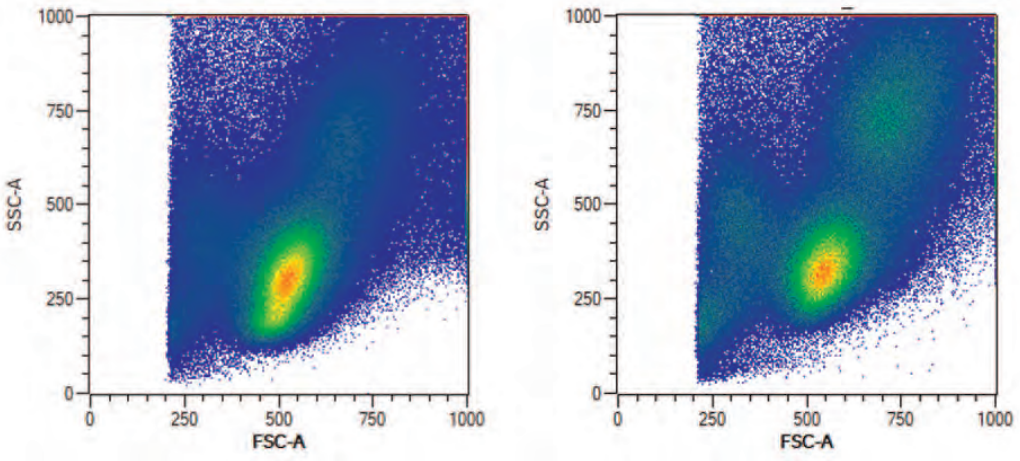


Figure 7: Cultured T cells. At start (left) the main population is easily identified, with some noise in the scatter plot. At harvest (right) there is a clear blast population that an original gate may not encompass, in addition the main population has shifted slightly.

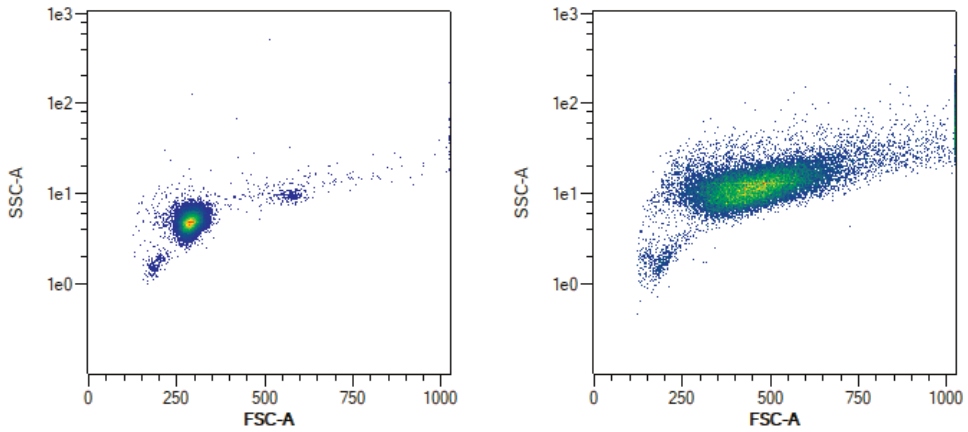


Figure 8: CD14-selected monocytes (left) cultured into macrophages (right). CD14+ selected monocytes. Using a logarithmic SSC scale allows the population of large and complex cells to be focussed on in the scatter plot and debris/large cells easily resolved. Note: on culture to macrophages the

cells double in size and become more complex but remain easily focussed within the same parameters as the starting material.

7.1.4 Cellular standards

A cell population of known composition should be used to determine the accuracy of positive or negative staining. A ruler or a reference standard material – e.g. blood cells staining positively for haematopoietic markers (ruler) in an assay showing negative results for MSC ; stabilized blood products for example CD-Chex (stabilised whole blood controls) staining positively for CD45 used to determine positive/negative cut off for blood cells (standard). Each laboratory will commonly have their own reference material and lab to lab standardisation can be inconsistent, necessitating extensive in-house validation.

In figure 9 whole blood is used as a ruler material for MSC. To "prove" that MSCs do not stain with leukocyte markers, antibodies are used in the same concentration on whole blood. In this example blood cells stain strongly with CD45 and CD34. There is confidence that the lack of stain with the same antibodies on MSC is due to genuine absence of expression of CD34 and CD45.

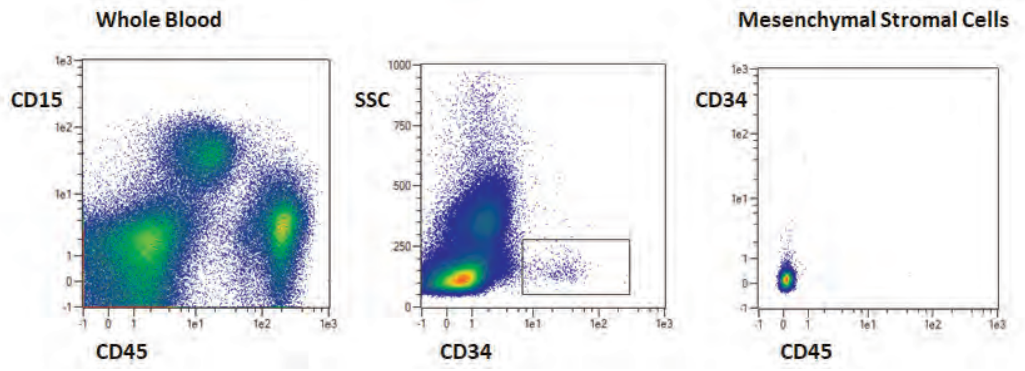


Figure 9: Ruler material – whole blood for MSC.

7.2 Set up

7.2.1 Initial voltage and scatter settings

Initial voltage and scatter settings should be created to suit the population being studies. This can be done manually, but many flow cytometers have automated voltage, compensation and gating software packages which can perform set up. Logarithmic or bi-exponential (part linear, part logarithmic) scales can be used to resolve populations with a wide range of sizes, scatters and intensity of fluorescent staining. Digital instruments do not "lose" data not displayed on plot axes, but it is desirable to design a scaling strategy to visualise all events for analysis. Very high PMT voltages should be avoided, and fluorochrome choice re-evaluated if high voltages are required.

7.2.2 Gating strategy

Attention should be paid to generating clear, well separated populations of cells with distinct boundaries, wherever possible, to enable clear gating for description of the population. While simple positive / negative gates (commonly seen on histograms, or as quadrants on dot plots) can be appropriate, care must be taken to ensure that clear populations are described, rather than a whole population shift in fluorescence bisecting a threshold being described as a certain percentage positive and negative. Analysis of true cell lines such as iPSC are commonly presented as histograms, but this contains limited information. Dot or density plots allow the same analysis of as histograms (positivity, mean fluorescence etc.) but provide a more data rich data presentation (See Figure 10).

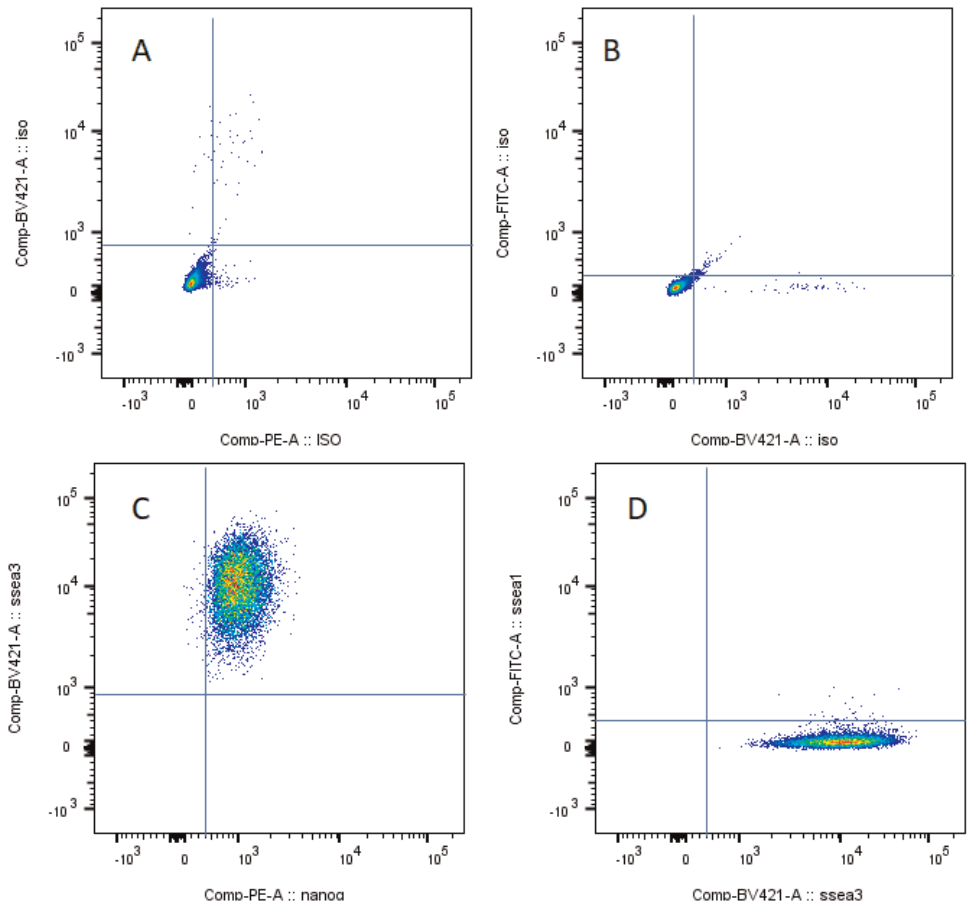


Figure 10: Pluripotent Cells. A and B – Isotype control staining on human pluripotent cells. C and D – healthy culture identified positively – co-expression of SSEA-3 and Nanog. Cells are also not expressing the differentiation marker SSEA-1, and thus pluripotency is maintained. Quadrants A and C are relative to one another and so are quadrants B and D.

7.2.3 Use of beads for compensation

Use of beads is useful for multi-colour panels and compensation, but is not suitable for all antibodies, especially with engineered FC domain. Even when beads are used for set-up, the unstained controls should be the cells of interest to account for the auto-fluorescence of the cells when calculating compensation. Compensation values generated using beads should be verified with cells.

7.2.4 Debris thresholds

Good practice in research is to set an appropriate threshold such as <1 μm to eliminate the inclusion of small debris in the acquired data file. This is controversial in ATMP manufacture as the amount of debris (which may include non-viable cells) in the product may influence meeting the product specification. Any threshold set should take account of the size of e.g. cell counting beads added to the samples. If a threshold is set that does include debris in the analysis, this may be mitigated by rigour in doublet and viability gating as well as positive identification of true cells events – e.g. CD45 staining on blood cells.

7.2.5 Viability

Viability should be determined using spectrally-suitable vital dyes e.g. DAPI, PI, or 7AAD. A range of fixable viability dyes are also available for use to stain cells before fixation for e.g. biological safety or intracellular analysis.

7.2.6 Isotypes

Use of isotypes is controversial particularly for gate setting as they are only sufficiently useful if truly Fluorescence/Protein (F/P) matched to the test antibody. Isotype controls are used to test unspecific binding of antibodies. However, it should be noted that unspecific binding of antibodies to isotype controls can be due to the antibody binding to the Fc receptor expressed on the cell. This can be mitigated by Fc receptor blocking reagents. Many commercially sold isotypes are more concentrated than the test antibodies. In addition, it should be noted that isotype controls are distinct from the antibodies they are used in conjunction with. The isotype control varies in numbers of fluorochromes bound, and the variable region amino acid sequence - thus isotypes are an imperfect control and should be avoided where possible. Properly matched isotypes have greater use in intracellular staining where “trapping” of fluorescence inside the cell is possible.

7.2.7 Compensation

Cytometer settings should be suitably compensated for spectral overlap using reference beads and/or cells stained with single fluorochromes. Compensation must be performed whenever there are significant laser fluctuations, replacement of lasers and / or the use of new antibody lots. Compensation is performed regardless of the number of fluorochromes used and should be assessed regularly.

7.2.8 FMO controls

The use of FMO limits constitutes good research practice which should be applied for control of cellular therapies. FMOs enable the analysis of any potential fluorescence spillover by other fluorescent stains into the channel being analysed. FMOs should be used for gate setting as they are often more effective than isotype controls. FMOs are mandatory in the assay development phase, they may or may not be needed during production.

7.2.9 Appropriate use of isotypes and FMO controls

The user should also apply their understanding of the cellular population in question to place the gating accordingly regardless of whether isotype or FMO controls are being used. When the greatest stringency in the assay is required, separate FMO and isotype control samples could be used for the same fluorescence channel. However, this is not necessary in many situations.

7.3 Measurement

7.3.1 Time as a parameter

Scatter versus time plots are very informative in detecting any anomalies such as cell aggregates in the analysed samples. These plots can be used to exclude these events from the analysis, often at the beginning and end of acquisition.

7.3.2 Cell counts

Commonly achieved using IVD-standard counting beads but need to be accommodated into scatter and thresholding strategy. Some flow cytometers produce accurate volumetric cell counts but may require to be regularly verified against bead and or controlled cellular reference material standards.

7.3.3 Data acquisition

The instrument should be regularly monitored for PMT drift and a policy developed to adjust voltages and check compensation accordingly. Some flow cytometers will apply such adjustments based on the regular calibration check.

A policy should be in place for when results following measurement of a sample fall outside of the expected ranges for one or more of the parameters being measured. Analysis of further samples would be good practice in this scenario. For example: a second unstained sample that can be stained and re-analysed by a second user using different laboratory equipment (e.g. pipettes). If the results then appear correct, a third sample should then be re-tested by the first user using the second set of equipment and result confirmed. This will confirm any issues of equipment failure or operator error when generating the first result, versus a true out of specification performance of the biological material. This may not be feasible in every setting e.g. with limited sample availability.

7.3.4 Retention of sample for reanalysis

When a product is cryopreserved, sufficient QC vials should be retained to allow for re-analysis as well as any ongoing stability testing. If sufficient material is available, stability should be determined on stocks of the product, as there may be differences in stability depending on the volume and type of cryopreservation container used. When a product is issued fresh, a sample of the final product should be retained for the validated shelf life. Retention of stained (fixed) samples should be treated with caution due to uncontrolled sample deterioration.[14]

7.4 Post-acquisition data analysis

Flow cytometry data can be infinitely re-analysed and re-gated. As discussed above, the templates for analysis for cellular therapies, particularly release criteria, should be set during assay validation and ideally not altered throughout the course of the manufacturing campaign.

The total cell population and count is determined from the number of cells determined after application of an FSC/SSC gate, and a doublet exclusion e.g. FSC-H versus FSC-A (other area vs. height measurements may also be appropriate e.g. SSC). The total viable count is determined by application of a vital dye exclusion gate. These strategies may be augmented by inclusion criteria such as CD45+ staining on blood cells to allow discrimination of live leukocytes.

Any such strategy must be based on evidence and risk. The strategy should capture all relevant events for analysis. If a “dump channel” is used to improve discrimination of measurement of a positive marker, this must be carefully applied to ensure that the contents are included in any analysis of the overall cellular composition.

Data acquired for information only should be acquired with clear reasoning and with the same rigour as release criteria data.

8. ASSAY VALIDATION

8.1 Validation foreword

A significant amount of regulatory guidance exists with respect to analytical method development and validation. Key guidance documents relevant to the validation of a flow cytometric assay are listed in Table 7. These should be consulted prior to commencing any assay development plan, to ensure the correct validation strategy is employed. The volume of guidance reflects the importance of ensuring patient safety through the ability to characterise ATMPs in terms of their quality, safety, and efficacy

[14] *EudraLex. The Rules Governing Medicinal Products in the European Union. Volume 4 EU Guidelines to Good Manufacturing Practice Medicinal Products for Human and Veterinary Use. Annex 19 Reference and Retention Samples.*

prior to use. The timing of assay validation activities (e.g. before or in parallel to process validation) will be dependent on the specific assay and the context in which it is being used. It is important to note from the outset that:

"*The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose*" (ICH Q2(R1)).

Thus, identifying the purpose of the assay being developed (See Section 3.2) is critical from the outset. The effort expended on method development and validation should be reflective of the phase of development, so the appropriate level of validation rigour can be applied.

In part, this is shaped by an appreciation of sample material availability; often insufficient amounts of material are available for analytical development during the early stages of development. Additionally, it reflects the understanding that material available during early stages of development may not always represent the cell population characteristics expected for clinical trial or commercial samples at later stages of development. However, there are still very clearly defined expectations in terms of assay validation during early stage development, especially for those assays associated with a product's safety. Ultimately however, it is mandatory that by product registration (e.g. marketing authorisation application in Europe) all assays are validated to ICH standards.

In terms of ATMP development, Eudralex Volume 4, Part IV, Annex 15 guidelines on GMPs for ATMPs provides information regarding which assays need to be validated at which stage of development:

- *First-in-man and exploratory clinical trials: Sterility and microbial assays should be validated. In addition, other assays that are intended to ensure patient's safety should also be validated (e.g. when retroviral vectors are used, the analytical methods for testing for replication competent retrovirus should be validated).*
- *Throughout the clinical development, the suitability of analytical methods used to measure critical quality attributes (e.g. inactivation/removal of virus and/or other impurities of biological origin) should be established but full validation is not required. Potency assays are expected to be validated prior to pivotal clinical trials.*
- *Pivotal clinical trials: Validation of analytical methods for batch release and stability testing is expected.*

In general, for phase I and II clinical trials, the suitability of the analytical methods used should be confirmed prior to the commencement of the trial. The relevant regulator can be approached for scientific advice. The acceptance limits (e.g. acceptance limits for the determination of the content of impurities, where relevant) and the parameters (specificity, linearity, range, accuracy, precision, quantification and detection limit, as appropriate) for performing validation of the analytical methods should be presented in a tabulated form or otherwise. If validation studies have been undertaken for early phase trials, a tabulated summary or otherwise of the results of analytical method validation studies could be provided for further assurance.

For phase III clinical trials, validation of the analytical methods used for release and stability testing should be provided. A tabulated summary of the results of the validation carried out should be submitted (e.g. results or values found for specificity, linearity, range, accuracy, precision, quantification, and detection limit, as appropriate). By the end of phase III full method validation must be completed, including confirmation of robustness. It is not necessary to provide the full validation report within the regulatory dossier but if it exists then this may be helpful to include.

For the purpose of this chapter, it is assumed that prior to commencing any assay validation activities, that associated equipment has been suitably qualified (See Section 4).

Table 7: Regulatory guidelines associated with analytical method development and validation.

Guideline / Issuing Body	Guideline Title
CLSI	Validation of Assays Performed by Flow Cytometry, 1st ed. CLSI document H62. Wayne, PA: Clinical Laboratory Standards Institute; 2021.
ICH Q2 (R1)	Validation of Analytical Procedures: Text and Methodology
ICH Q6B	Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products
ICH Q14*	Analytical Procedure Development and Revision of Q2 (R1) Analytical Validation
FDA	Analytical Procedures and Methods Validation for Drugs and Biologics
IUPAC	Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report)

*Guidance in draft.

8.2 Assay performance characteristics

The assay performance characteristics assessed during analytical validation are defined in ICH Q2(R1) and summarised illustratively in Figure 11. Considerations with respect to the individual performance characteristics, when validating flow cytometry-based assays, are detailed below.

8.2.1 Linearity and range

This section describes the linearity assessment of the assay and not the instrument, which should be a part of System Qualification. The linearity of the assay should be evaluated across the intended assay range. The specified validated range is then normally derived from these linearity studies (where the linear relationship holds true). It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy, and precision (See Section 8.2.2) for samples within or at the extremes of the specified range. The acceptance criteria for the range typically depends on the intended application of the procedure. ICH Q2 (R1) proposes minimum specified ranges depending on the assay purpose. For example:

- For active substance or finished drug product: 80-120% of the test concentration;
- For the determination of an impurity: from the reporting level of an impurity to 120% of the specification; for impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects, the detection/quantification limit should be corresponding with the level at which the impurities must be controlled.

However, when employing flow cytometry techniques to support analysis of cell-based products, especially those derived from autologous material where significant donor to donor variability exists, developers should define acceptable assay operating ranges to support the potential diversity expected within the broader population. It should be noted that depending on the type of assay this may require the ability to detect cells over several orders of magnitude within a population. Examples of assay range considerations,

- If developing an assay for clinical samples to detect the active substance (e.g. CAR+ CD3+ T-cells) of the IMP (post-administration), then the potential range that assay may need to detect could be extremely wide (several orders of magnitude), depending on the expansion of the cells *in-vivo* and the duration post-administration.

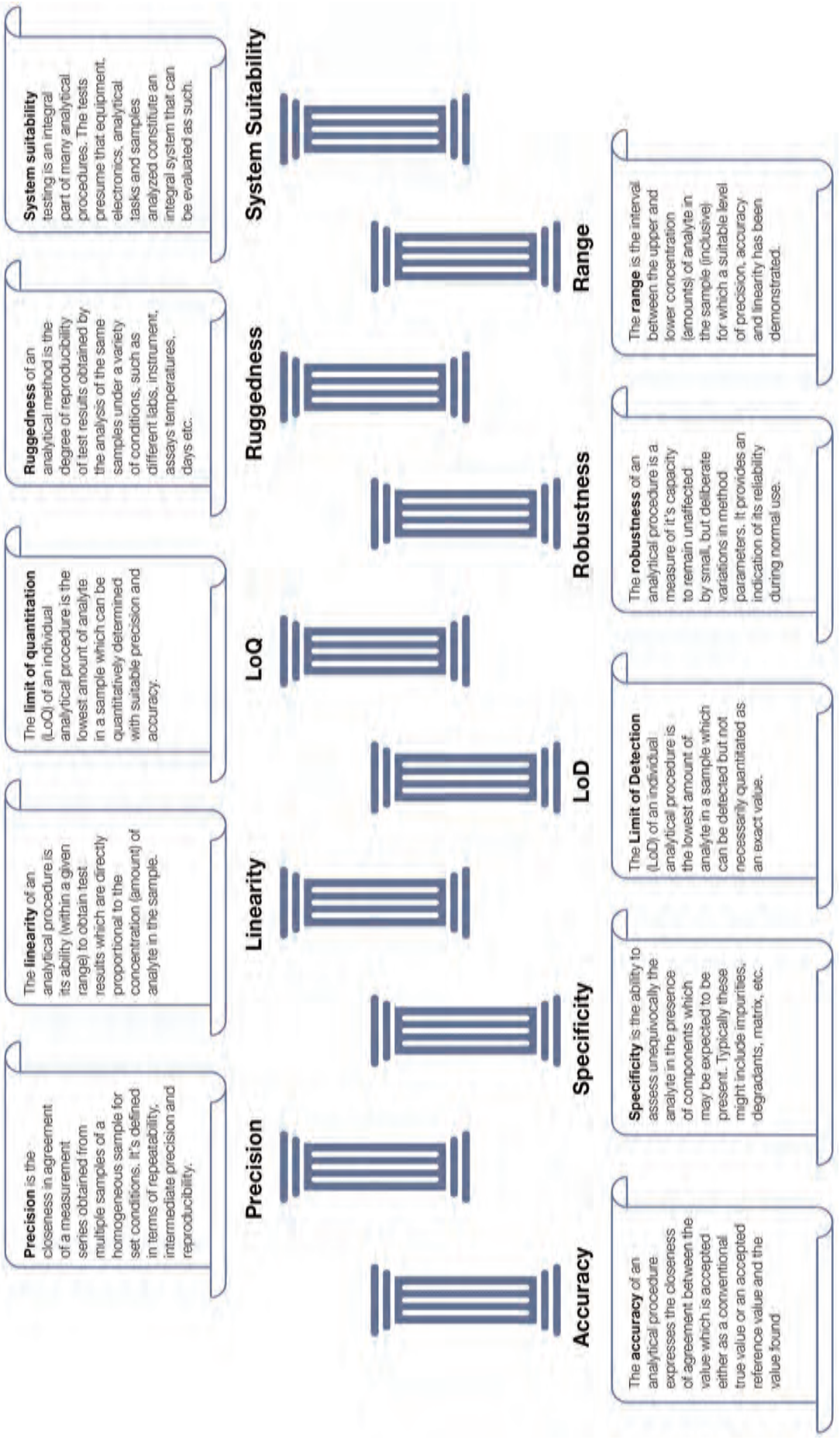


Figure 11: The assay performance characteristics of analytical validation.

- If developing an assay for product release to detect the active substance (e.g. CAR+ CD3+ T-cells) of the IMP. Then whilst the specification may be set at $>40\%$, the assay as a minimum, should be able to quantify 40-100% (not just 80-120% of the target value commonly used for chemical formulations e.g. 32-48%) and ideally a much broader range from say 10-100%.

If assays do demonstrate a linear relationship, the test results should be evaluated and reported using appropriate statistical methods. For example, a common method is by calculation of a regression line by the method of least squares, although other methods such as standardised residuals can be used in conjunction with the regression line. The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity.

For the establishment of linearity, a minimum of five concentrations is recommended (any other approaches should be justified), but typically eight concentrations are used. The required cell concentrations are often achieved by performing a limited dilution, but effects of pipetting accuracy should be taken into account. For chemical analytes, the target is simply diluted in an appropriate buffer. However, for flow cytometry analysis of cell based samples, demonstrating the ability to detect the target cell (at different percentage levels) within a fixed population of cells/events (fixed number of cells) means that the positive cell population should be diluted in an appropriate medium (negatively expressing cell population, rather than buffer). It can sometimes be very challenging to acquire 100% positively or 100% negatively expressing samples to support assay development and therefore it should be recognised that the range of the assay may be limited by sample availability. Where possible, samples used to support linearity assessment should be well characterised and fluorescence intensity signal should be quantified with calibration beads (e.g. MESF). It is good practice to retain sufficient samples from very successful and/or very poor development assays (or undertaken the generation of reference banks) to support assay development and validation. For example:

- If developing an assay for product release to detect the active substance (e.g. CAR+, CD3+, T-cells) of an IMP. The manufacturing process may have been run many times with transduction efficiencies ranging in the 20-75% range. Thus, if using the best representative sample available, the assay may only be validated to an upper range limit of 75%;
- Similarly, when considering products comprised of differentiated cell lineages from stem cell progenitors, differentiation efficiency may be variable and below 100%.

If developing an assay, ideally the positive and negative samples used for creating the dilution series are donor matched and have been through the same manufacturing process, except for the key step (e. g. addition of the vector). The length of expansion should be maintained so that activation markers or other cell attributes are likely to be the same on both the negative and positive populations. It is just expression of the transduced gene that can vary. However, it is recognised that this is not always feasible and therefore consideration should be given to selection of appropriate positive and negative samples to create the dilution series.

This data should be reported accordingly to minimise the impact on assay performance as the linearity assessment in semi-quantitative flow cytometry assays is not always possible and therefore it may not be practical to set assay acceptance criteria based on assay linearity.

8.2.2 Accuracy and precision

The distinctions between the accuracy (sometimes termed trueness) and the precision (the repeatability and reproducibility) of data yielded from an assay are illustrated in Figure 12.

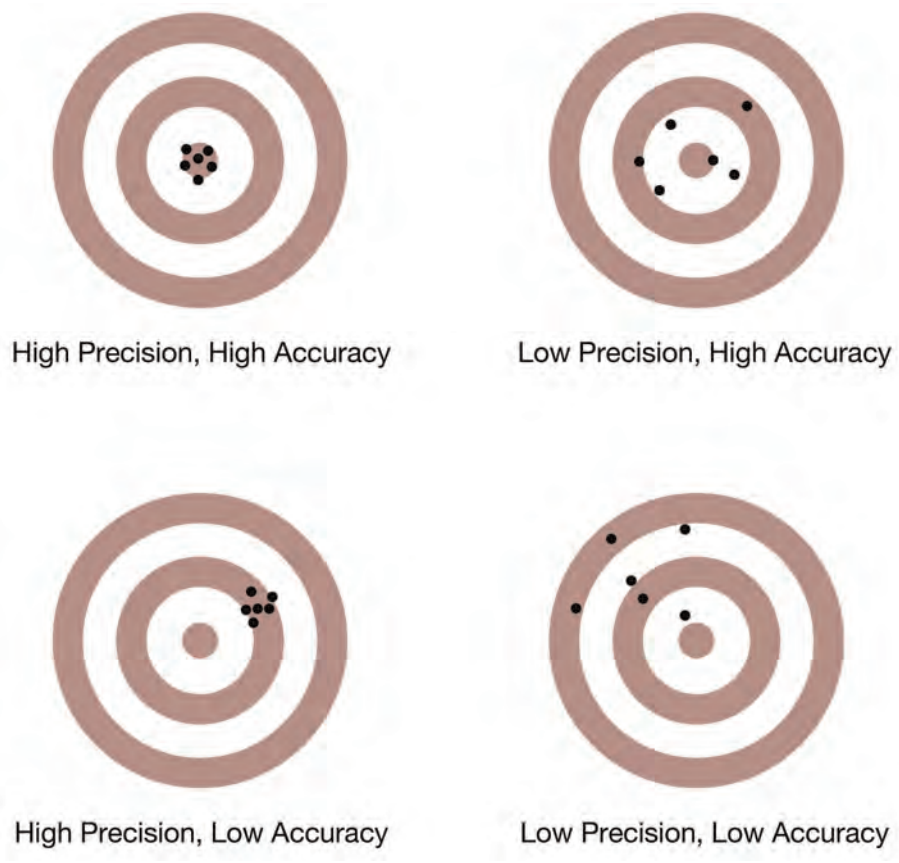


Figure 12: Distinctions between assay accuracy and precision. The bullseye on the target is assumed to be the "true" or reference value for the assay.

Accuracy expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. A key challenge of cell-based flow cytometry assays is the very limited number of certified reference materials to act as the "true" value. For example, NIBSC has a single cell-based reference material, which is for the cell surface marker CD4. Whereby "*the material is stabilised lyophilised human blood leukocytes pooled from donations to the UK National Blood Service. In the hands of expert laboratories, material 15/270 returned an overall mean of 336 CD4 T cells/μL, with an intra-laboratory RSD between 4 and 6% for most laboratories and a maximum intra-laboratory RSD of 16%. The mean value obtained by an individual laboratory upon repeat testing is expected to fit within the range of 272-400 CD4 T cells/μL with a maximum RSD of 16%.*" Thus, even with a well-controlled reference material the accuracy of cell-based analysis can still encompass a broad range. NIBSC additionally provides several reference samples for cytokines and growth factors that may be useful in the development of intracellular flow-based assays. There are also commercial control samples available that can be used for assay validation as well as performance monitoring.

Alternatively, developers may need to develop independent internal sources of reference samples such as reference cell banks reflecting positive (or negative) marker expression, to support assay validation. These reference cell banks are important tools and therefore careful consideration should be given to the quality systems by which they are produced and whether orthogonal approaches are necessary to confirm that cell banks demonstrate the required quality attributes. If developing a reference bank of non-viable cells, you may wish to confirm the degree of non-viability by different methods. Furthermore, they can prove highly useful when comparing cross-platform comparisons e.g. for vector copy number assay by qPCR or dPCR.

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same material under the prescribed conditions. Precision may be considered at three levels: repeatability, inter-assay variability and reproducibility with respect to different sources of variation (e.g. sample, run, instrument, operator, day etc) (See Figure 13). Selection of sources of variation / measurement uncertainty to be considered within the validation protocol, should be determined based on the intended use of the assay and can be supported internal risk assessment exercises, which may leverage insight from an extensive wealth of literature in the public domain.

- Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.
- Inter-assay variability (intermediate precision) expresses variations within laboratories: different days, different analysts, different equipment, etc.
- Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

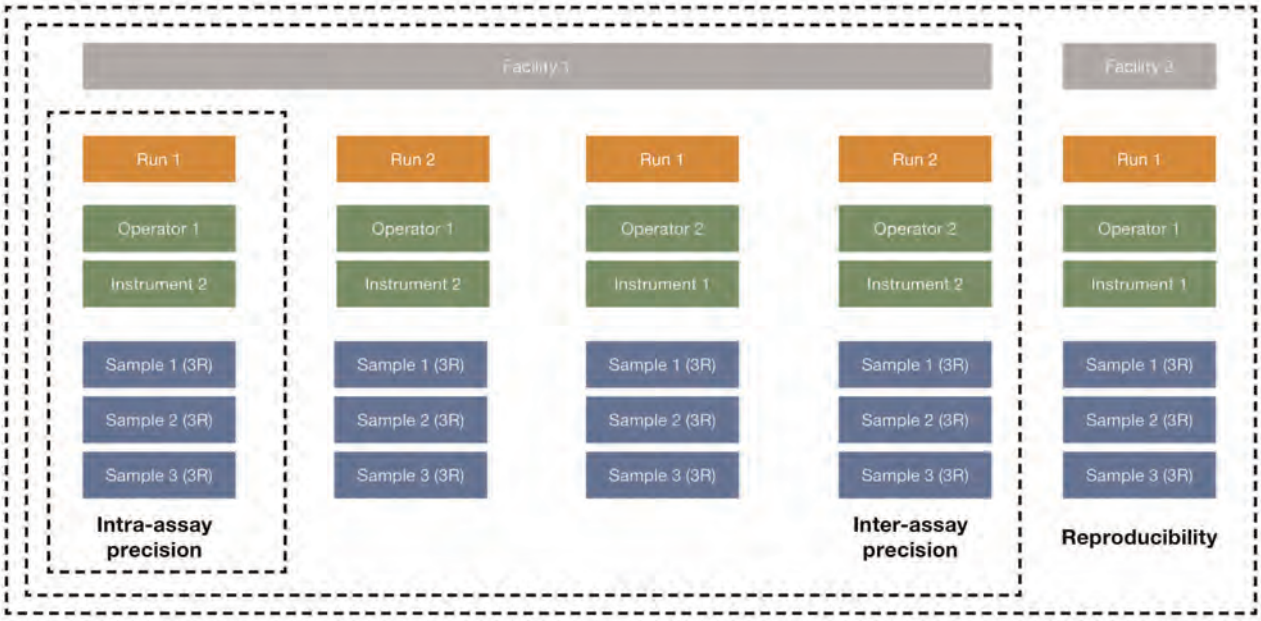


Figure 13: Precision: repeatability (intra-assay precision), inter-assay variability / intermediate precision and reproducibility.

Precision should be investigated using authentic samples where possible. The precision of an analytical procedure is usually expressed as the variance, standard deviation, or RSD of a series of measurements. It is important to understand what an appropriate acceptance level for your assay will be. Ideally this should be as low as possible, but it is recognised that for flow cytometry-based assays this can be challenging, especially with respect to reproducibility between laboratories, but even in terms of the inter-assay precision between different instruments (same supplier) operated within the same facility, or just due to inherent biological variability within the test samples. It is good practice to report any statistically different results generated using the assay with an annotation on the assay imprecision. This highlights the need for Certified Reference Materials to qualify the equipment and appropriate Reference Standards to be developed & adopted for Assays.

Acceptance criteria for precision measurements are commonly considered in the context of percentage CVs. Suitable acceptance criteria ranges for flow cytometry methods are dependent on the assay being developed. For example, assays targeting the measurement of common events may have acceptance criteria of %CV $< 10\%$, whilst for rare event analysis it might be as high as 30-40%. It is important to

note that there are multiple statistical methods for calculating the mean %CV and appropriate statistical models for the protocol design should be employed.

8.2.3 Specificity and sensitivity

Specificity is the ability to assess unequivocally the analyte in the presence of matrix components which may be expected to be present. In the context of cell-based flow cytometry assays it is interpreted as the ability to analyse or detect the intended target cell population(s) and antigens of interest. Typically, these populations fall into one of two tests: identification or impurity.

Suitable tests should be able to discriminate between closely related cell phenotypes or cell markers/components with similar structures that are likely to be present in the test sample. The ability of an assay to discriminate between the cell phenotype/cell marker/cell component of interest with background matrix components may be confirmed by obtaining positive results from samples containing the analyte, coupled with negative results from samples which do not contain the analyte. Thus, confirming the antibody specificity and cell phenotype are pre-requisites to performing qualification or validation activities. If required, expression of a specific marker can be confirmed using orthogonal methods such as Next Generation Sequencing (NGS) or PCR.

In flow cytometry assays, specificity is typically achieved through optimising the panel design to detect relevantly expressed markers of the targeted cell type(s) (See Section 6.2). For example, to identify NK T-like cells in blood, it would be important to be able to discriminate between T cells (CD3+CD56−), NK cells (CD3−CD56+), and NK T-like cells (CD3+CD56+). Therefore, in this example, you would need to stain for at least two different surface markers (CD3 and CD56), and have an appropriate gating strategy during data analysis, to be able to specifically identify and quantify the target population. It is also necessary to identify non-specific interactions within the potential assay (e.g. non-specific binding of antibodies), and to employ relevant controls to the assay being developed, which may include isotype control antibodies, or the simultaneous measurement of negative control/reference cell types, or to normalise data to background levels of noise so as to explicitly confirm that the assay identifies only the cell type(s) of interest.

Closely related to the specificity of the assay is the assay sensitivity; LoD and LoQ of the target cell population. Note: the requirement to determine an assay's LoQ and/or LoD will be assay type dependent. For example, LoQ and LoD may be required for assays measuring impurities, whilst LoQ may be sufficient for other assay types, such as potency.

8.2.3.1 Limit of detection (LoD)

ICH Q2(R1) describes two methods for determining the limit of detection for instrument-based assays, such as flow cytometry: 1. standard deviation of the response and the slope (more commonly used) or 2. signal-to-noise.

1. Standard Deviation of the Response and the Slope: ICH Q2(R1) states that the detection limit may be expressed as:

$$LoD = 3.3 \quad \sigma / S$$

where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte and σ may be carried out based on either:

- The Standard Deviation of the Blank Measurement - the magnitude of analytical background response is performed by analysing an appropriate number of blank samples (e.g. unstained cells) and calculating the standard deviation of these responses.

- Based on the Calibration Curve - A specific calibration curve should be studied using samples containing an analyte in the range of DL (e.g. by performing a dilution series of the stained cells). The residual standard deviation of a regression line, or the standard deviation of y-intercepts of regression lines may be used as the standard deviation (See exemplar validation report for an example of calculating the limit of detection).

2. Signal-to-Noise: This approach can only be applied to analytical procedures which exhibit baseline noise. Whereby determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte to those of blank samples. The minimum concentration at which the analyte can be reliably detected is then established; an acceptable detection limit is typically reflective of a signal-to-noise ratio between 3 or 2:1.

8.2.3.2 Quantitation limit (LoQ)

Similar to LoD, ICH Q2(R1) describes two methods for determining the LoQ for instrument-based assays, such as flow cytometry; Signal-to-Noise or Standard Deviation of the Response and the Slope.

- Signal-to-Noise: Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified; a signal-to-noise ratio of 10:1 is typically accepted.
- Standard Deviation of the Response and the Slope - LoQ may be expressed as:

$$LoQ = 10 \quad \sigma / S$$

where σ = the standard deviation of the response

S = the slope of the calibration curve.

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways based on

- Standard Deviation of the Blank Measurement - the magnitude of analytical background response is performed by analysing an appropriate number of blank samples and calculating the standard deviation of these responses.
- Calibration Curve - A specific calibration curve should be studied using samples, containing an analyte in the range of LoQ. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

When determining the sensitivity of the assay, the validation protocol and report should state the methods used to determine the LoD and LoQ. The limit should then be experimentally validated by the analysis of a suitable number of samples known to be near or prepared at the quantitation limit.

It is important to note that depending on the purpose of the assay, the LoD and LoQ should be fit-for-purpose, as for all performance characteristics i.e. aligned to the expectations of the acceptance criteria for the quality attribute or critical quality attribute (CQA) being assessed, as exemplified below:

- If the assay will support the release of ATMP drug substance or drug product then the validated assay should be capable of supporting the requirements of the specification i.e. if the release specification is set to ≥40% CAR positive cells, then the LoQ for the CAR marker should be well below this value, if the LoQ is 45%, then the assay is unable to quantify the result in the required range of the specification and is therefore not fit-for-purpose.
- If the assay purpose is to demonstrate that an undesirable cell population is below a pre-defined limit such as residual Pluripotent Stem Cell in a differentiated cell population is below one in a million, then the LoD of the assay should be less than one in a million.

8.2.4 Robustness

Robustness demonstrates the reliability of an analysis with respect to deliberate variations in method parameters. During method development, a risk assessment based approach can be useful in identifying assay parameters or variables to evaluate as part of the Robustness studies. If measurements are considered to be sufficiently susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled / assessed for impact, or a precautionary statement should be included in the procedure. Examples of typical method parameters that can cause variations in assay performance include:

- Stability of analytical solutions,
- Sample processing or incubation times,
- Environmental conditions (temperature, humidity etc.)

One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

8.2.5 System suitability

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analysed are fit for purpose. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated.

8.3 Assay qualification, validation, and verification

8.3.1 Assay verification

Analytical procedures, which are either described in the Pharmacopoeia, or are linked to a product specific monograph, and are performed according to the monograph, are normally considered as validated. In such cases, the user ensures compliance of the system suitability requirements to demonstrate the verification of the validated method. Verification is the demonstration that conditions under which the user employs the monograph are suitable for use with the validated method. For verification of the pharmacopoeial method the user should ensure compliance with the System Suitability requirements.

8.3.2 Assay validation

To ensure the assay is fit-for-purpose it should be noted that it is not essential to validate it with respect to all performance characteristics; selection should be tailored to use.

8.3.3 Assay qualification

If not validated, assays should still be qualified for use. A useful overview with respect to "What is Test Method Qualification".[15] In brief, qualified test methods have:

• No pre-determined performance specifications; however, there may be performance capability requirements based on the intended application. Whereas for validated assays, specifications should be established before validation begins and be met by every validation run.

• The qualification studies are used to determine method performance capabilities for parameters such as specificity, linearity, accuracy, and precision, as required for the intended application. Whereas validation studies define each of the assay characteristics in detail, demonstrating reproducible and

[15] *Ritter N et al. Bioprocess International (Technical) What Is Test Method Qualification? Proceedings of the WCBP CMC Strategy Forum, 24 July 2003.*

robust performance in response to expected variations in analytical conditions through the control strategies employed.

• A method cannot fail qualification, it can (and should) be reoptimized until it can achieve the required performance. If it cannot achieve the required performance, it should be rejected for the application. Conversely, a method can fail validation; if it does then the cause should be investigated and resolved before the method can be considered fully validated.

8.3.4 Quality system considerations

The environment in which the assay is being developed will determine the quality system considerations to be implemented. This will include factors such as the level of operator training, traceability etc. When the assay is intended for the analysis of GxP samples (e.g. product release testing) or patient clinical samples (e.g. in-vivo persistence assays) then the expectation is that the assay implementation conforms to the appropriate pharmaceutical quality system guidelines (e.g. Eudralex Volume 4, GMPs specific to ATMPs) or regulatory agency reflection papers (e.g. "Reflection paper for laboratories that perform the analysis or evaluation of clinical trial samples"). In GxP environments one key element of the quality system is document control:

"*The main objective of the system of documentation utilized must be to establish, control, monitor and record all activities which directly or indirectly may affect the quality of the medicinal products*" *(Eudralex Volume 4, GMPs specific to ATMPs)*.

The guidance should be consulted to understand the full expectations, but particular attention should be paid to the following two specific points:

1. Section 6.4. Other documentation

Paragraph 6.28. There should be appropriate documentation of policies and procedures to be applied by the manufacturer with a view to safeguard the quality of the product, including:

(i) Qualification of premises and equipment.

(ii) Validation of manufacturing process.

(iii) Validation of relevant analytical methods.

(iv) Maintenance and calibration of equipment.

(v) Cleaning procedures.

(vi) Environmental monitoring.

(vii) Investigations into deviations and non-conformances.

(viii) Procedures for handling of quality complaints and recall of products.

The above statement implies that all equipment qualification, analytical validation and equipment maintenance and calibration procedures must be documented.

2. Paragraph 6.13. Irrespective of the form in which data is kept, suitable controls should be implemented to ensure data integrity, including:

(i) Implementation of measures to protect data against accidental loss or damage, e.g. by methods such as duplication or back-up and transfer to another storage system.

(ii) Implementation of measures to protect the data against tampering or unauthorised manipulation. Physical and/or logical controls should be in place to limit access to computerised system to authorised persons. Suitable methods of preventing unauthorised entry to the system may include e.g. the use of keys, pass cards, personal codes with passwords, biometrics, or restricted access to computer equipment and data storage areas. The extent of security controls depends on the criticality of the computerised system

(iii) Implementation of measures to ensure the accuracy, completeness, availability, and legibility of documents throughout the retention period.

Typically, a data integrity risk assessment will be performed to determine the most appropriate mitigation strategies to be employed to ensure compliance. This may include institutions ensuring all raw data files generated to support assay running (including validation) being duplicated and stored appropriately (either manually via user lead actions or automatically through regular mirroring of data storage servers onto back up servers). Alternatively, implementation of security measures to avoid data deletion and corruption may be sufficient.

Raw data files from modern flow systems are automatically generated as read only. However, some older systems may generate raw data files that can be amended; if such a system is employed, then users must implement a policy, which "locks" files and safeguards them against data manipulation. It is generally recommended where supported by equipment manufacturers, to export raw data files in an FCS format for storage – a read only file type that can then be used by multiple software analysis tools for data processing. Additionally, systems should be appropriately configured to remove the ability of operators to manipulate data origination and maintain full traceability e.g. operators should not be able to delete a file and rerun a sample; it should be clear that the sample was acquired multiple times if this occurs, the associated documentation for the run should then capture and explain these deviations. Mitigation of such risks can be achieved through establishing user-specific password access to systems.

8.4 Assay validation and verification - protocols and reporting examples

Considering the points highlighted in the previous sections, prior to validating any assay it is essential to document an assay validation plan or protocol (and any supporting SOPs) that capture the performance characteristics being assessed, the methodology undertaken, raw materials to be used, control samples, gating strategies, acceptance criteria etc. necessary to perform the assay. If the assay validation is successful, this will be exactly how the assay must be performed going forward.

Data generated from executing the assay validation plan, must then be analysed, and reported in accordance with the plan, clearly detailing any deviations from the plan and the success/failure against any acceptance criteria.

Exemplar document structures for worked examples of a validation protocol and report a are presented in the Annexes detailed below.

ANNEX 1 - IQ EXAMPLE CASE STUDY

An illustrative example of IQ report template is shown in Table 6 below. Notes on physical integrity and effectiveness should be taken for all components. Test parameters meeting specified criteria should be indicated with “Pass”, and parameters not meeting criteria with “Fail”. If any deviations are observed, an exception report must be completed including all corrective actions taken.

Flow cytometer IQ and associated report drafting are typically performed by specialised FSEs. In this case, IQ results and actual readings need to be further reviewed by the instrument qualification team, who must verify if each result is acceptable, and ensure that the generated documentation meets all relevant regulatory requirements.

Table 6: Example IQ report template. All installed components should be detailed, and corresponding installation requirements and specifications listed. Specifications should be set in line with the manufacturer's instructions.

Flow cytometer	**Model**			
	Catalogue number			
	Serial number			
	Manufacturing date			
Workstation	**Model**			
	Catalogue number			
	Serial number			
Software	**Name**			
	Version			
Installation requirements	**Specification**	**Pass/Fail**	**Initials**	**Date (dd/mmm/yyyy)**
Space for flow cytometer and workstation	Dimensions (W x D x H)			
Additional space required for ancillary modules and accessibility	Additional dimensions (W x D x H)			
Total space required	Total dimensions (W x D x H)			
Power specifications	Voltage: ___ V Frequency: ___ Hz Current: ___ A Power: ___W			
Humidity	Relative Humidity: ___ %			
Operating temperature	__ to __ ^{o}C			
Drainage	Waste line connected to a waste container			
Network connection	Available			
Lasers available	Blue laser: ___nm Red laser: ___nm Violet laser: ___nm			
Comments				

ANNEX 2 - OQ EXAMPLE CASE STUDY

An illustrative example of OQ report template is shown below. The minimum battery of tests performed should typically involve:

- Determination of laser power.
- Testing of filter characteristics.
- Evaluation of signal synchronisation.
- Laser delay determination.
- Sensitivity of PMTs.

Test parameters meeting specified criteria should be indicated with "Pass", and parameters not meeting criteria with "Fail". If any deviations are observed, an exception report must be completed including all corrective actions taken. Calibrated testing equipment must be used to verify all operational specifications. Items in the test plan must be defined as per the equipment's specifications provided by the manufacturer.

Flow cytometer	**Model**			
	Catalogue number			
	Serial number			
	Manufacturing date			
Workstation	**Model**			
	Catalogue number			
	Serial number			
Software	**Name**			
	Version			
Etc.				
OQ parameter	**Specification**	**Pass/Fail**	**Initials**	**Date (dd/mmm/yyyy)**
Software operation	Software connects to the instrument			
	Software executes with no error messages			
Fluidics start-up	All tanks are filled and there are no leaks			
	Level/volume detectors are operational			
	The system is primed when the option is selected			
System alerts	System detects problems and gives appropriate warnings			

Sample flow rate	Volume of fluid consumed in 5 minutes at "Low", "Medium" and "High" meets manufacturer's specifications			
Carryover	Meets the manufacturer's specifications			
Laser power	After 20 minutes of warmup, measured laser power meets the manufacturer's recommended output			
Fluorescence sensitivity	Sensitivity of each fluorescence detector is greater than the value specified by the manufacturer			
Fluidics shutdown	Fluidics shutdown procedure is executed without errors			
Comments				

ANNEX 3 - FLOW CYTOMETRY VALIDATION PROTOCOL - WORKED EXAMPLE

READ THIS STATEMENT FIRST

The worked example for a validation protocol presented herein, is intended to support an understanding of the practical translation of expectations outlined in regulatory documents and guidelines. The objective is to assist readers having limited experience of undertaking assay validation activities with the development of their own internal procedures, processes, and protocol styles.

[Black italicised text provides some points for consideration with each section]

Blue text provides an exemplar in the context of a flow cytometric assay aimed at detecting TCR-X expression in transduced T cells.

This document is not intended to present a gold standard to be copied or cloned. It is important readers develop their own protocols that are fit-for-purpose for the assay being developed. Any information and data presented herein is provided as an exemplar to illustrate possible content. It is not necessarily exhaustive of expectations, and where multiple approaches are potentially applicable (e.g. different statistical techniques / approaches) it is the readers responsibility to ensure the correct methods are employed for their specific assay / validation plan design.

Readers with existing procedures and processes for assay validation activities are invited to reflect on the content but encouraged to continue using their own internally approved procedures, processes and documentation styles where they have been shown to be fit-for-purpose already.

Validation plan for a flow cytometric assay detecting TCR-X expression in transduced T cells

Project code:	
Study title:	Validation plan for a flow cytometric assay detecting TCR- X expression in transduced T cells
Protocol number:	XXX-XXXX
Experimental start date:	DD-MMM-YYYY
Experimental end date:	DD-MMM-YYYY
Study locations: **Address:**	
Experimental lead: **Name:** **Signature:**	
Reviewed by: **Name:** **Job Title:** **Signature:**	
Approved by: **Name:** **Job title:** **Signature:**	

Amendments to version X.X of the validation plan

The following amendments were made to version X.X of the flow cytometry assay validation plan to create this document:

1	**Change**	
	Reason for change	
2	**Change**	
	Reason for change	
3	**Change**	
	Reason for change	

All changes were made at the beginning of validation activities.

1. Assay principle

[Describe in relevant sub-sections the principles underlying the assay development. For example, the background to the assay (e.g. the assay purpose, what is being detected, why and/or how it is being detected); the objective of the assay (e.g. what it needs to demonstrate); the sources of cellular material / samples for performing the validation; and/or any other relevant considerations]

1.1 Background

[Points to consider for inclusion in the background sub-section include sample source (e.g. clinical or manufacturing); the purpose of the assay (e.g. starting material characterisation, final product release testing or in-process controls testing to determine viability, identity, purity, potency, product or process impurities characteristics etc.)]

This validation plan will describe the procedures necessary to validate a multi parameter flow cytometric assay that detects the transduction of gene modified T cells directed against antigen X (AntX). AntX peptides are present at abnormally high levels on the surface of tumour cells. AntX expressing cells can be specifically recognised by T cells transduced with TCR-X. These transduced T cells have been shown, during development and in pre-clinical studies, to specifically kill tumour cells by recognising AntX expressed on their surface.

Note: The antigen specific binding region of the TCR lacks a suitable staining antibody and therefore an antibody targeting the β chain of TCR-X is employed. Thus, all TCR-X transduced cells will express the β chain, but not all β chain expressing cells will express the TCR-X. Therefore, non-transduced cells are also expected to have low levels of positive expression using the β chain targeting antibody.

1.2 Objective

[Points to consider for inclusion in the objective sub-section include what the assay needs to detect (e.g. specific cell makers, cytokines, growth factors etc.) to determine specific characteristics of the cell population (e.g. for T cells, this may incorporate T cell sub-class, activation status, proliferative status, TCR or CAR-T expression, viability), and if relevant any particular range or sensitivity, LoD or LoQ required.]

The assay needs to demonstrate the ability to specifically detect transduced T cells by their expression of TCR-X. This is essential for the measure of transduced cell persistence *in vivo* and important for efficacy assessment during future clinical trials. PBMC that have been transduced with TCR-X will be serially diluted and spiked into samples of un-transduced T cells before being measured by flow

cytometry using the antibody panel listed in Table 1. This panel will, in addition to measuring TCR-X expression classify the cells by T cell subclass (CD4, CD8) and memory/activation status (CD45RO / CD62L) by gating on the respective CD markers. In addition, the proliferative status of the cells will be measured through the expression of Ki67.

Table 1. Antibody Panel to be used for detection of transduced T cells.

Cell Marker	Fluorophore
Live/Dead	Yellow
CD3	Alexa Fluor 700
CD4	V500
CD8	APC-H7
CD45RO	PerCP-Cy5.5
CD62L	BV450
TCR-X	FITC
Ki67	PE

1.3 Sample sources

[Points to consider for inclusion in the sample source sub-section include a description of cell samples used in the assay validation and how the cells will be prepared for the protocol, any thawing, cell counting, resuspension at set concentrations etc.]. Also, if sample preparation for assay validation will vary compared to the actual samples analysed this should be described e.g. to measure % transduction it may be necessary to create transduced and un-transduced samples that can be mixed in different proportions to create samples of different transduction levels for analysis.]

Two sources of frozen T cell material are to be used for this validation study as follows:

- TCR-X transduced cells that have undergone stimulation, viral transduction and expansion, followed by cryopreservation. These are referred to in this document as “transduced cells”.
- Non-transduced cells that have undergone stimulation, mock transduction (no virus used) and expansion, followed by cryopreservation. These are referred to in this document as “non-transduced cells”.

Both cell materials are to be derived from a single donor source. The cells are to be thawed, counted using the Trypan Blue assay on an automated Vi-Cell cell counter to calculate viable cell number. Based on the viable cell count, the cell concentration of both the transduced and un-transduced cells will be adjusted to 5 x 10^6 cells/mL. All samples are to be labelled with antibodies in accordance with SOP-YYY.

2. ICH classification of assay

[Points to consider for inclusion in this sub-section of the document include reference to any guidance documents used to support the validation protocol generation (e.g. ICH Q2(R1)) and if aspects of the guidance are not being followed then an explanation for any deviation must be recorded. For example, the intention may be to only validate certain assay characteristics (e.g. accuracy or linearity etc. at this point in development. It may also be pertinent to state the regulatory status of the assay.]

The validation of the assay will be carried out according to ICH guidelines for the Validation of Analytical Procedures (ICH Q2(R1)) with respect to those assay performance characteristics detailed in Table 2.

Table 2. ICH recommendations for the characteristics to be assessed during validation of an analytical assay/procedure.

Type of analytical procedure	**Functional Assay Validation requirements**
Accuracy	No
Precision • **Repeatability** • **Intermediate Precision**	 Yes Yes
Specificity	Yes
Detection limit	For Information Only
Quantitative limit	For Information Only
Linearity	Yes
Robustness	No
Range	Yes

3. Amendment and Deviation Reporting

[Points to consider for inclusion in this sub-section include how teams should manage and report deviations from the protocol, including deviations from pre-set acceptance criteria. Depending on the Quality system in place where the assay is being performed, reference to the related quality management procedures may be included.]

Any amendments / deviations to the protocol that have the potential to impact the integrity of the study should be reported (this should include an assessment of the impact of the deviation to the study).

4. Controls

[Points to consider for inclusion in this sub-section include a description the controls (e.g. negative and specificity controls or quality controls) that will be used to support validation of the assay].

The following controls will be used to validate the performance of the assay.

4.1 Negative and specificity controls

Non-transduced T cells will be used as both assay negative and specificity controls. They will be the equivalent of background controls for biochemical assays or immunoassays and will be used accordingly.

4.2 Quality control through TCR-X expression

Quality control requirements, established from data obtained in the clinical trials, for the transduced cells will be the assessment of TCR-X expression as previously used in pre-clinical studies and as criteria for the Investigational Medicinal Product (IMP) release. Therefore, the acceptance criteria for this marker will be the same, $>$ X% TCR-X positive cells.

5. Reagents and Equipment

[List all the antibodies and reagents (buffers, calibration beads etc) required in relevant sub-sections, including variables such as antibody or reagent name, conjugate type (for antibodies), supplying company, catalogue numbers etc. The equipment used should also be detailed.]

5.1 Antibodies

Antibodies used in this study are shown in Table 3.

Table 3. List of antibodies to be used in the validation study.

Antibody	Conjugate	Company	Catalogue Number #
Live/Dead	Yellow	Company A	AA-BB-CC-DD
CD3	Alexa Fluor 700	Company B	AA-BB-CC-DD
CD4	V500	Company A	AA-BB-CC-DD
CD8	APC-H7	Company B	AA-BB-CC-DD
CD62L	BV450	Company A	AA-BB-CC-DD
CD45RO	PerCP Cy5.5	Company B	AA-BB-CC-DD
TCR-X	FITC	Company A	AA-BB-CC-DD
Ki67	PE	Company B	AA-BB-CC-DD

5.2 Reagents and buffers

Reagents used in this study are shown in Table 4.

Table 4. List of reagents and buffers required for the validation study

Reagent	Company	Catalogue number #
Compensation Beads	Company A	AA-BB-CC-DD
Stain Buffer	Company A	AA-BB-CC-DD
Rainbow Calibration Particles (6 Peaks)	Company A	AA-BB-CC-DD
Reagent X	Company A	AA-BB-CC-DD
Reagent Y	Company A	AA-BB-CC-DD
Reagent Z	Company A	AA-BB-CC-DD

5.3 Equipment list

Equipment used in this study is shown in Table 5.

Table 5. List of equipment required for the validation study

Equipment	Company	Serial number #
Flow cytometer	Company A	AA-BB-CC-DD
Pipette A	Company B	AA-BB-CC-DD
Pipette B	Company B	AA-BB-CC-DD
Pipette C	Company B	AA-BB-CC-DD
Vortex	Company C	AA-BB-CC-DD

6. Procedure

[Describe in relevant subsections the individual procedures being followed to evaluate the different assay performance characteristics. Where assay performance characteristics are not being evaluated, provide justification.]

The following assay performance criteria will be measured in this validation study:

6.1 Accuracy

Define accuracy – Where appropriate use the definition as provided by relevant guidelines being followed e.g. ICH Q2(R1).]

Accuracy expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

For this study, no reference standard is available for the target of interest and therefore accuracy is not being validated.

6.1.1. Accuracy assessment

[Describe how the accuracy will be assessed, including which attributes or characteristics of the sample are the focal point of assessment (e.g. all cell types or just one specific target cell type). Provide details of reference standards used or how the "true" value is determined if a reference standard is not used.]

Not applicable

6.1.2. Accuracy results reporting

[Describe how the linearity results will be reported and any statistical approaches used]

Not applicable

6.1.3. Acceptance criteria

[Define the acceptance criteria for the assessment]

Not applicable

6.2 Linearity

[Define Linearity – Where appropriate use the definition as provided by relevant guidelines being followed e.g. ICH Q2(R1).]

The definition of linearity is the ability to elicit test results that are directly, or by defined mathematical transformation, proportional to the concentration of analyte in the samples within a given range.

6.2.1. Linearity assessment

[Describe how the linearity will be assessed, including which attributes or characteristics of the sample are the focal point of assessment (e.g. all cell types or just one specific target cell type). Provide details of how dilution curves will be set up including the calculations, the plate layout for the assay, any controls etc. Furthermore, indicate the number of times the procedure is to be repeated and by how many operators in order to support assessment of other assay characteristics.]

Linearity will be assessed by analysing TCR-X expression in transduced cells diluted into a non-transduced cell population at a dilution ratio of 1:2. The highest cell density (5 x 10^6 cell/mL) will be diluted as shown in Table 6 to create an eight point dilution curve.

Only TCR-X will be assessed for linearity as the non-transduced cells used as a diluent for the study will have undergone processing and activation and therefore will be expected to express comparable levels of ki67 and have a similar immune-phenotype to the transduced cells.

Table 6 – Set up of the cell dilution curve for the linearity study

[insert appropriate table]

A negative or blank control (NC) will be generated by using 5 x 10^6 cells/mL of no n-transduced cells. Fluorescence minus one (FMO) controls for TCR-X and a Ki67 Isotype control will be generated using the highest cell density 5 x 10^6 cells/mL of the transduced cell sample. Each dilution/sample will be generated as triplicate 200µL volume per wells giving a total 1 x 10^6 cells/well.

Figure 1 below illustrates the plate layout for the linearity study, black numbers show the amount of transduced cells per well, and red numbers show the amount of non-transduced cells per well.

Figure 1. Plate layout for the linearity study.

[insert appropriate diagram of plate layout]

The linearity experiment will be repeated by a single operator five times over five different days to allow the data to also be used for assessment of specificity and repeatability. A second operator will also perform the linearity experiment three times over three different days so that the intermediate precision can be determined.

6.2.2. Linearity results reporting

[Describe how the linearity results will be reported and any statistical approaches used]

Linearity will be reported as

- Correlation coefficient of the regression line plotted for percentage TCR-X positive cells against
  1. the CD3+ cell population
  2. the CD4+ cell population
  3. the CD8+ cell population

6.2.3. Acceptance criteria

[Define the acceptance criteria for the assessment]

A correlation coefficient will be generated by plotting the measured expression of TCR-X cells on the y-axis against CD3+, CD4+ or CD8+ cell numbers on the x-axis. The assay will be described as linear across the range of analysed samples when the correlation coefficient is ≥ 0.90.

6.3 Specificity

[Define Specificity in the context of the assay being performed - Where appropriate use the definition as provided by relevant guidelines being followed e.g. ICH Q2(R1).]

Specificity is defined in this assay as the ability to accurately measure TCR-X cells in transduced cell samples in the presence of non-transduced cells.

6.3.1. Specificity assessment

[Define how the specificity assessment will be performed (e.g. this may be through use of the data generated from the linearity assessment), describe the specific type of to be detected, and the concentration range within which this detection should occur.]

Specificity will be assessed using the data generated in the linearity study by comparing the number of TCR-X positive cells in transduced and non-transduced cell samples. The specificity of the assay is confirmed when specific TCR-X positive cells in the transduced cell sample at the highest cell concentration (5×10^6 cells/mL), are considered to be significantly different from non-transduced cells at the same concentration.

6.4 Specificity results reporting

[Define how specificity will be reported and any statistical techniques employed]

Specificity will be reported as the mean and standard deviation for TCR-X expression in the transduced and non-transduced cells from at least four assay runs completed by a single operator.

6.4.1. Acceptance criteria

[Define the acceptance criteria used to assess specificity]

Specificity will be statistically determined by paired T test where the p value ≤ 0.05 will be considered to be significant.

6.5 Detection Limit (LoD) and Quantification Limit (LoQ)

[Define Sensitivity and whether the aim is to determine the LoD and/or LoQ - Where appropriate use the definition as provided by relevant guidelines being followed e.g. ICH Q2(R1).]

Sensitivity is defined as the ability to consistently identify – detection limit (LoD) and quantify the analyte – quantification limit (LoQ) and are to be evaluated.

Note: This performance characteristic is being assessed for information only. As described previously in the background section of this protocol non-transduced cells are also expected to have low levels of positive expression using the β chain targeting antibody. The level of background expression will be patient specific and therefore the LoD and LoQ for the assay will be patient dependent. Measurements are being performed to understand the approximate values for the LoD and LoQ.

6.5.1. LoD and LoQ assessment

[Determination of LoD and LoQ, should be performed according to ICH Q2(R1)]

The LoD as prescribed in ICH Q2(R1) guidelines will be calculated by the mean plus three times the standard deviation (SD) of TCR-X in the non-transduced samples using the data obtained from the results from the linearity assessment.

The LoQ as prescribed in ICH Q2(R1) guidelines will be calculated by the mean plus ten times the standard deviation (SD) of TCR-X in the non-transduced using the data obtained from the results from the linearity assessment.

6.5.2. Acceptance Criteria

Assay is for information only and therefore no pre-defined acceptance criteria are set.

6.6 Precision

[Define Precision - Where appropriate use the definition as provided by relevant guidelines being followed e.g. ICH Q2(R1).]

Precision can be determined by repeat testing of a sample. The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample. Precision will be evaluated at two levels:

- **Repeatability:** the variation between replicates of all dilutions within one assay run or occasion by the same operator.
- **Inter-assay variability / intermediate precision**: Variation within a laboratory to include, tests performed on different days and by a different analyst.

6.6.1. Precision Assessment

[Detail the assessment approach for each of the types of precision being assessed (e.g. repeatability, intermediate precision or reproducibility), how the data will be generated, any statistical techniques employed.]

In order to determine precision, the data from the assay linearity assessments for TCR-X will be used to record the mean, standard deviation, coefficient of variation and confidence intervals of each assay run.

The three different levels of precision will be assessed as follows:

- **Repeatability:** Mean % CV generated from each assay.
- **Inter-assay variability / intermediate precision**:
  - Mean %CV generated for four assay runs as performed by one operator.
  - Mean %CV generated for at least five assay runs as performed by two operators.

6.6.2. Precision results reporting

[Detail how the results will be reported, any statistical techniques employed]

The results for the precision measurements will be reported as follows:

Repeatability will be based on the results for all eight dilutions from a single run and will be reported as follows:

i. Repeatability will be reported as the mean, standard deviation, and coefficient of variance for the TCR-X measurements in the CD3, CD4 and CD8 cell populations.

Inter-assay variability / intermediate precision

Variability on different days - will be based on the highest concentration of the transduced cells from four independent runs performed by a single operator and will be reported as follows:

i. Mean, standard deviation and coefficient of variance for the TCR-X measurement in the CD3, CD4 and CD8 cell populations.

ii. Mean, standard deviation and coefficient of variance for the Ki67 measurement in the CD3, CD4 and CD8 cell populations.

iii. Mean, standard deviation and coefficient of variance for the immune-phenotype measurement using CD45RO and CD62L in the CD3, CD4 and CD8 cell populations.

Variability as a function of operator/analyst - will be based on the highest concentration of the transduced cells from at least 5 runs performed by two operators and will be reported as follows:

i. Mean, standard deviation and coefficient of variance for the TCR-X, Ki67 and immune-phenotype measurement in the CD3 population.

ii. Mean, standard deviation and coefficient of variance for the TCR-X, Ki67 and immune-phenotype measurement in the CD4 population.

iii. Mean, standard deviation and coefficient of variance for the TCR-X, Ki67 and immune-phenotype measurement in the CD8 population.

6.6.3. Acceptance criteria

[Define acceptance criteria]

The acceptance criteria for all precision measurements is the percentage coefficient of variation ≤ 20%.

6.7 Robustness

[Define Robustness - Where appropriate use the definition as provided by relevant guidelines being followed e.g. ICH Q2(R1).]

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

6.7.1. Robustness assessment

[Define proposed assessment of robustness or provide rationale for not including]

The robustness of the assay will not be addressed in this validation plan. This assay is being developed to support a Phase I/II clinical trial and confirmation of robustness is not required at this stage of development.

6.7.2. Acceptance criteria

[Define acceptance criteria]

Not applicable

6.8 Range

[Define Range - Where appropriate use the definition as provided by relevant guidelines being followed e.g. ICH Q2(R1).]

The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision and linearity.

6.8.1. Range assessment

[Define how the range will be assessed based on the results of different assay characteristics such as the *linearity, accuracy, sensitivity and precision assessments]*

The range will be determined for transduced cells (TCR-X expressing cells; CD3, CD4 and CD8) that demonstrate suitable levels of precision and linearity. It will use the data from the four independent assay runs performed by a single operator over 5 days as detailed in the inter-assay / intermediate precision analysis section.

6.8.2. Range results reporting

[Detail how the results will be reported, any statistical techniques employed]

The results are reported as follows:

1. Percentage CV for TCR-X expression in each of the dilutions in the CD3 population
2. Percentage CV for TCR-X expression in each of the dilutions in the CD4 population
3. Percentage CV for TCR-X expression in each of the dilutions in the CD8 population

6.8.3. Acceptance criteria

[Define acceptance criteria]

The acceptance criteria for the range will be determined based on a %CV ≤ 20% for individual dilutions.

7. Reporting results

[Define how the results of the validation protocol will be reported, who has responsibility for producing the report, and how the raw data will be stored or maintained for future reference if needed. It may be valuable to include a summary of all acceptance criteria from the individual sections above as a summary for clarity.]

Results will be reported in a validation report written by author/designee. The data for each individual experiment will be included in tables in the validation report. The raw data will be maintained as support for the validation report.

8. References

[Include any references used in the design of the validation plan. Including internal SOPs etc]

The following references were used for the design of this validation plan:

- Validation of Analytical Procedures: Text and Methodology Q2 (R1). ***International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use***.
- An Approach to the Validation of Flow Cytometry Methods. ***Pharmaceutical Research (2009) 26; 12 2551-2557***. Cunicliffe J, Derbyshire N, Keeler S and Coldwell R.
- Validation of Cell-based escence Assays: Practice Guidelines from the ICSH and ICCS – Part III – Analytical Issues. ***Cytometry Part B (Clinical Chemistry) (2013) 84B; 315-323***. Tanqri S, Vall H, Kaplan D, Hoffman, Purvis N, Porwit A, Hunsberger B and Shankey VT
- Validation of Cell-based escence Assays: Practice Guidelines from ICSH and ICCS – Part V-Assay Performance Criteria. ***Cytometry Part B (Clinical Chemistry) (2013) 84B; 315-323***. Wood B, Jevremovic D, Bene MC, Yan M, Jacobs P and Litwin V
- Flow Cytometry. New Considerations for Validating Laboratory-Developed Tests. ***Clinical Laboratory News (2013) 39, 12***. Litwin V, Salkowitz-Bokal J and Steele P.

ANNEX 4 - FLOW CYTOMETRY VALIDATION REPORT - WORKED EXAMPLE

READ THIS STATEMENT FIRST

The worked example for a validation report presented herein, is intended to support an understanding of the practical translation of expectations outlined in regulatory documents and guidelines as presented in the exemplar report structure document. The objective is to assist readers having limited experience of undertaking assay validation activities with the development of their own internal procedures, processes and report styles.

[Black italicised text provides some points for consideration with each section]

Blue text provides an exemplar in the context of a flow cytometric assay aimed at detecting TCR-X expression in transduced T cells.

This document is not intended to present a gold standard to be copied or cloned. It is important readers develop their own protocols that are fit-for-purpose for the assay being developed. Any information and data presented herein is provided as an exemplar to illustrate possible content. It is not necessarily exhaustive of expectations, and where multiple approaches are potentially applicable (e.g. different statistical techniques / approaches) it is the readers responsibility to ensure the correct methods are employed for their specific assay / validation plan design.

Readers with existing report styles for assay validation activities are invited to reflect on the content but encouraged to continue using their own internally approved procedures, processes and documentation styles where they have been shown to be fit-for-purpose already.

Validation report - Flow cytometry assay for the detection of TCR-X expression in transduced T cells

Project code:	
Study title:	Validation report - flow cytometric assay detecting TCR-X expression in transduced T cells
Protocol number:	XXX-XXXX
Experimental start date:	DD-MMM-YYYY
Experimental end date:	DD-MMM-YYYY
Study locations: **Address:**	
Experimental lead: **Name:** **Signature:**	
Reviewed by: **Name:** **Job title:** **Signature:**	
Approved by: **Name:** **Job title:** **Signature:**	

1. Principle

1.1 Background

[This section should briefly describe the background to the assay (e.g. the assay purpose, what is being detected, why and/or how it is being detected); the objective of the assay (e.g. what it needs to demonstrate); any important points for consideration the reader should take into account about the assay (e.g. availability of suitable antibodies etc.); and provide any cross-references to the validation protocol from which the report is based]

This report describes the procedures undertaken to validate a multi parameter flow cytometry assay as detailed in protocol XXX-XXX "*Validation plan for the flow cytometric assay for the detection of TCR-X expression in transduced T cells*"

The flow cytometry assay will be used on clinical trial samples to detect the persistence of gene modified T cells which are directed against Antigen X (AntX) following infusion. AntX peptides are present at abnormally high levels on the surface of tumour cells. AntX expressing cells can be specifically recognised by T cells transduced with TCR-X. These transduced TCR-X cells have been shown, during development and in pre-clinical studies, to specifically kill tumour cells by recognising AntX expressed on their surface.

Note: For this assay, the antigen specific binding region of the TCR lacks a suitable staining antibody and therefore an antibody targeting the β chain of TCR-X is employed. Thus, all TCR-X transduced cells will express the β chain, but not all β chain expressing cells will express the TCR-X. Therefore, non-transduced cells are also expected to have low levels of positive expression using the β chain targeting antibody.

1.2 Assay Objective

[Recap the assay objective from the validation plan / protocol, reconfirming who the assay was performed by and where it was performed.]

The primary aim of the assay is to measure the persistence of transduced T cells through their expression of TCR-X. However, the assay is designed to characterise three components in the clinical trial samples:

1. The persistence of TCR-X transduced T cells measured through the expression of TCR-X in the CD3, CD4 and CD8 cell populations.
2. The proliferative status of the T cells measured through the expression of Ki67 in the CD3, CD4 and CD8 cell populations.
3. The immune-phenotype of the T cells measured through the expression of CD45RO and CD62L in the TCR-X specific CD3, CD4 and CD8 cell populations.

Scientists at *[insert Company]* performed the assay according to SOP-XXX.

2. Amendments and deviations

[Describe any amendments or deviations to the approved validation plan / protocol on which the report is based and the potential impact of each, specifically noting any specific assay characteristics e.g. linearity, specificity etc. that may be affected.]

During validation, the following amendments and deviations from the SOP were noted.

2.1 Amendments (planned changes)

[This section should list changes that were planned and agreed prior to the study commencing with a short evaluation of the potential impact.]

The assay is normally run on patient samples; therefore the following amendments need to be considered:

1. The transduced and non-transduced cells used in this validation study were derived from samples taken from healthy volunteers.
    - **Impact minimal** – it is not expected that cells obtained from healthy volunteers and transduced with the TCR-X vector will perform differently from cells donated by cancer patients.
2. The transduced and non-transduced cells used in this study were from non-matched healthy donors.
    - **Impact moderate** – the activation status, immune-phenotype and proliferation status of the transduced and non-transduced cells is expected to be different due to the variability of the starting material and process induced differences. This could increase variability in the measurements particularly for the linearity and precision studies.

2.2 Deviation (unplanned changes)

[This section should list changes that were unplanned and the corrective action that was taken during the validation protocol execution and a short evaluation of the potential impact.]

1. Poor cell number recovery of non-transduced cells required extra vials to be thawed then pooled to ensure the required cell numbers.
    - **Impact minimal** – sufficient live non-transduced cells were recovered from the extra thawed vials to allow all experiments to be completed.

3. Materials and methods

[Detail materials and methods used (e.g. a list of reagents and lot numbers to be used / instrument models / serial numbers), where relevant cross-reference to SOPs, the validation protocol or other relevant documents, detail correct version numbers etc. where applicable.]

1. Description of the methods and materials used are within SOP-XXX vX.X.
2. A detailed description of validation process is contained protocol XXX-XXXX vX.X "Validation plan for the flow cytometric assay for the detection of TCR-X expression of transduced T cells".
3. Statistics were performed using (software XXX and methods YYY).

4. Results

4.1 Assay linearity

4.1.1 Overview

[Provide brief overview of the assay linearity assessment method, this could be paraphrased from the protocol document, include information on how dilution curves were set up including the calculations, the plate layout for the assay, any controls etc. Furthermore, describe how many times the procedure was repeated and by how many operators in order to support assessment of other assay characteristics].

Linearity was assessed by measuring the ability of the assay to detect TCR-X positive transduced cells serially diluted into a non-transduced cell population (Table 1).

Table 1. Serial dilution scheme.

	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Dilution 6	Dilution 7	Dilution 8
Transduced cells	$1x10^6$	$5x10^5$	$2.5x10^5$	$1.25x10^5$	$6.2x10^4$	$3.1x10^4$	$1.5x10^4$	0
Non-transduced cells	0	$5x10^5$	$7.5x10^5$	$8.75x10^5$	$9.38x10^5$	$9.69x10^5$	$9.85x10^5$	$1x10^6$

Only TCR-X was assessed for linearity as the non-transduced cells used as a diluent for the study underwent processing and activation and therefore are expected to express comparable levels of ki67 and have a similar immune-phenotype to the transduced cells.

The mean number of TCR-X positive cells for each dilution generated from four independent assay runs performed by a single operator were used as the reportable result.

Linearity is reported as correlation coefficient of the regression line plotted from the percentage TCR-X positive cells against total number of CD3, CD4, and CD8 positive transduced cells.

4.1.2 Acceptance criteria

[Re-iterate the acceptance criteria for the assessment, this should match that defined in the protocol.]

The assay is described as linear when the correlation coefficient (R^2) is $\geq$ 0.90 (Table 2).

Table 2. Linearity acceptance criteria.

Assay Parameter	Acceptance criteria
R^2 value	$\geq$ 0.90

4.1.3 Gating strategy

[Describe gating strategy utilised for linearity assessment, to keep this section of the document manageable a simple process flow of the gating strategy can be presented with exemplars of real gating plots in an appendix. However, it would be equally suitable to present the actual plots of the gating strategy here.]

Figure 1 illustrates the gating strategy used to measure the percentage TCR-X positive cells in each of the dilutions. Exemplar dot-plots are shown in Section 6 – Gating Strategies.

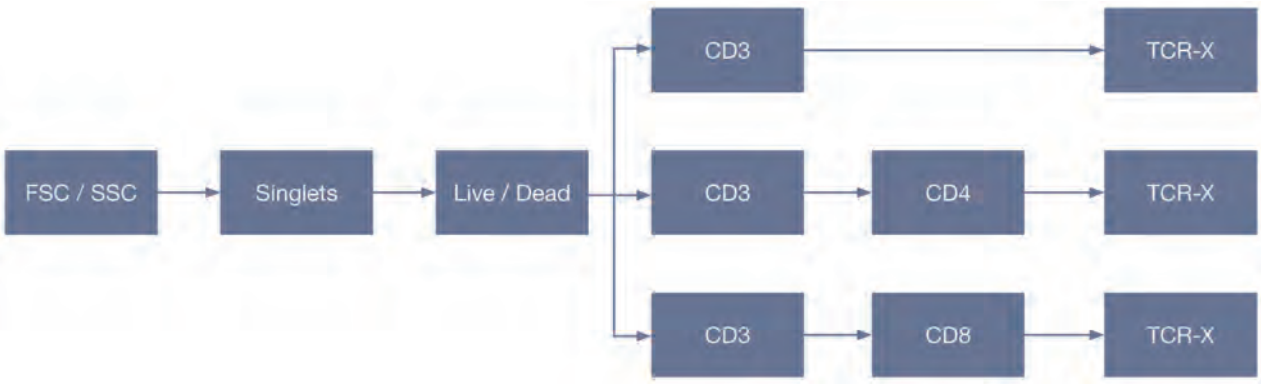


Figure 1. Gating strategy to determine linearity.

4.1.4 Results

[Report the results (including any relevant tables of data, graphs, statistical analysis performed etc.) state how the assay performed against the acceptance criteria and if the assay characteristic is considered to have passed or failed against the acceptance criteria.]

The results for the linearity assay are reported as follows:

1. The linearity of the CD3+TCR-X+ cells over the eight dilutions (Figure 2).
2. The linearity of the CD4+TCR-X+ cells over the eight dilutions (Figure 3).
3. The linearity of the CD8+TCR-X+ cells over the eight dilutions (Figure 4).

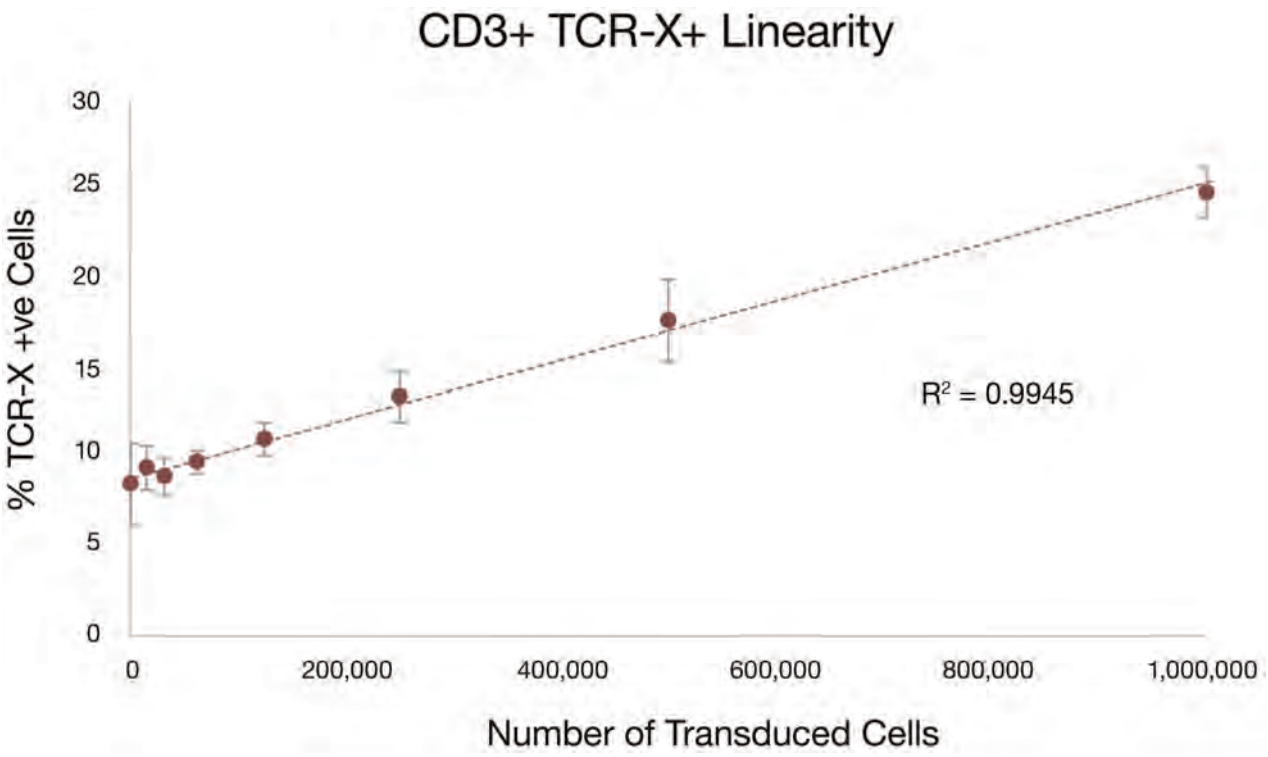


Figure 2. Linearity of the CD3+TCR-X+ cells over the eight dilutions.

Dilution	% CD3 TCR-X +
1	24.75
2	17.61
3	13.35
4	10.94
5	9.70
6	8.85
7	9.36
8	8.42
Acceptance criteria (R^2)	**$\geq$ 0.90**
Result (R^2)	**0.9945**
Pass/Fail	**Pass**

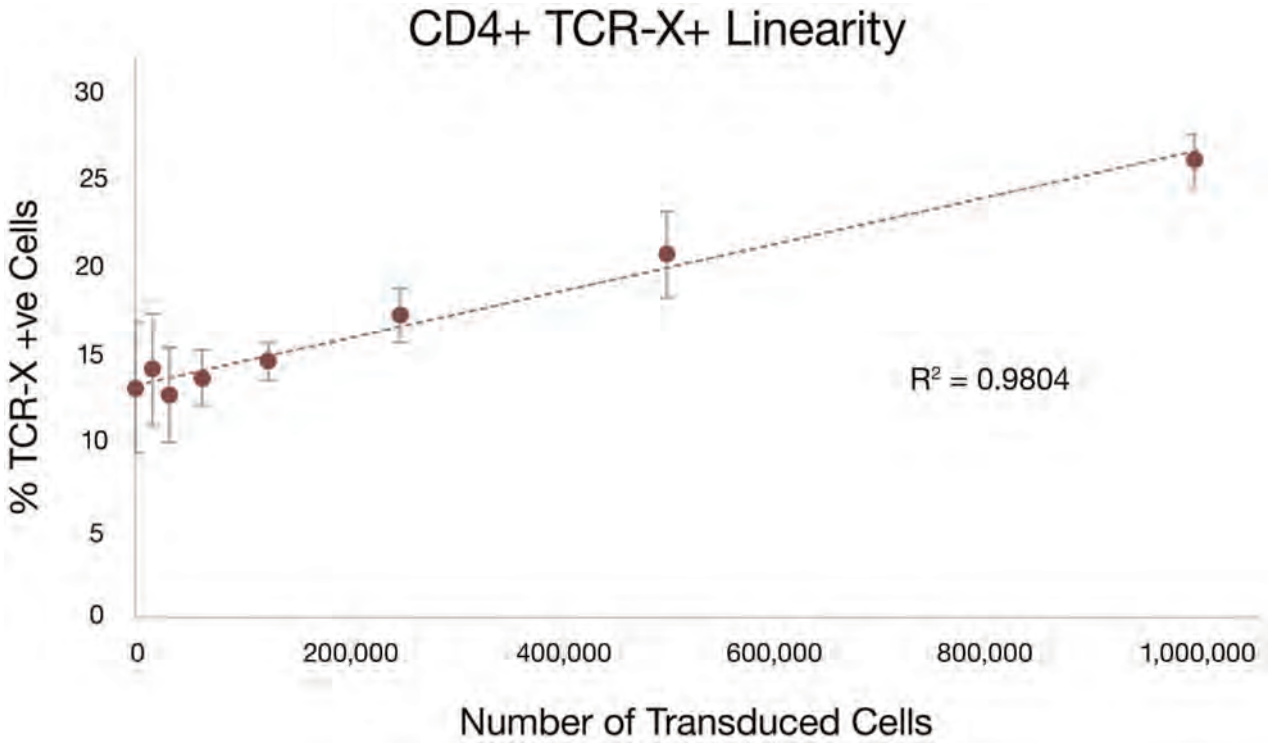


Figure 3. Linearity of the CD4+TCR-X+ cells over the eight dilutions.

Dilution	% CD4 TCR-X +
1	26.22
2	20.83
3	17.32
4	14.75
5	13.82
6	12.80
7	14.31
8	13.26
Acceptance criteria (R^2)	**$\geq$ 0.90**
Result (R^2)	**0.9804**
Pass/Fail	**Pass**

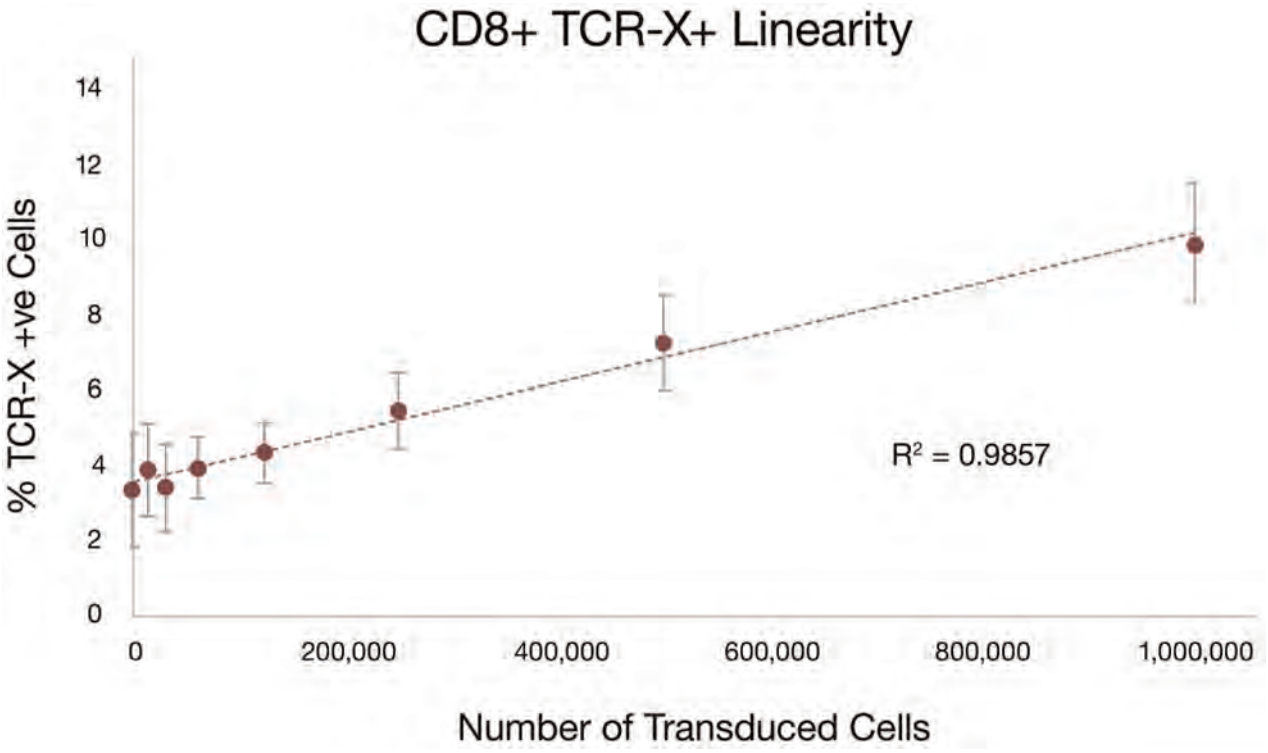


Figure 4. Linearity of the CD8+TCR-X+ cells over the eight dilutions.

Dilution	% CD8 TCR-X +
1	9.81
2	7.17
3	5.40
4	4.38
5	4.01
6	3.51
7	3.78
8	3.22
Acceptance criteria (R^2)	**$\geq$ 0.90**
Result (R^2)	**0.9857**
Pass/Fail	**Pass**

4.1.5 Summary

[State any final conclusions about linearity results]

The assay demonstrates acceptable levels of linearity across the eight dilutions of transduced cells for the CD3, CD4, and CD8 populations.

4.2 Assay precision

4.2.1 Overview

[Provide brief overview of the assay precision assessment method for each of the types of precision being assessed (e.g. intra-assay, inter-assay / intermediate precision / reproducibility), how the data was generated, any statistical techniques employed.]

Precision was evaluated at two levels:

- **Repeatability:** the agreement between replicates of all dilutions within one assay run or occasion by the same operator.
- **Inter-assay variability / intermediate precision**: the agreement between replicates within and between assay runs by the same operator over a short period of time and variation within the laboratory to include tests performed on different days and by a different analyst.

Reproducibility was not evaluated as the assay is only intended to be performed at a single facility.

4.2.2 Acceptance criteria

[Re-iterate the acceptance criteria for the assessment, this should match that defined in the protocol.]

For each of the precision assessments the assay will be considered precise if the CV ≤ 20% (Table 3).

Table 3. Precision acceptance criteria.

Assay Parameter	Acceptance criteria
CV	≤ 20%

4.2.3 Gating strategies

[Describe gating strategy for precision assessment – exemplars of real gating plots can either be shown here or placed in appendices; if placed in appendices, then cross-references should be inserted into text. If different gating strategies are used to assess the precision with respect to different markers / cell phenotypes etc. then gating strategies for each should be presented. Furthermore, if combinatorial marker expressions correlate to specific cell sub-populations then these can be defined here for reference e.g. for immune phenotypes see Figure 8 below.]

Three gating strategies were used to assess the precision of the assay. Exemplar dot-plots are shown in Section 6 – Gating Strategies.

Gating strategy 1

Figure 5 depicts the gating strategy used to assess the precision of the TCR-X positive measurements in the CD3, CD4 and CD8 populations.

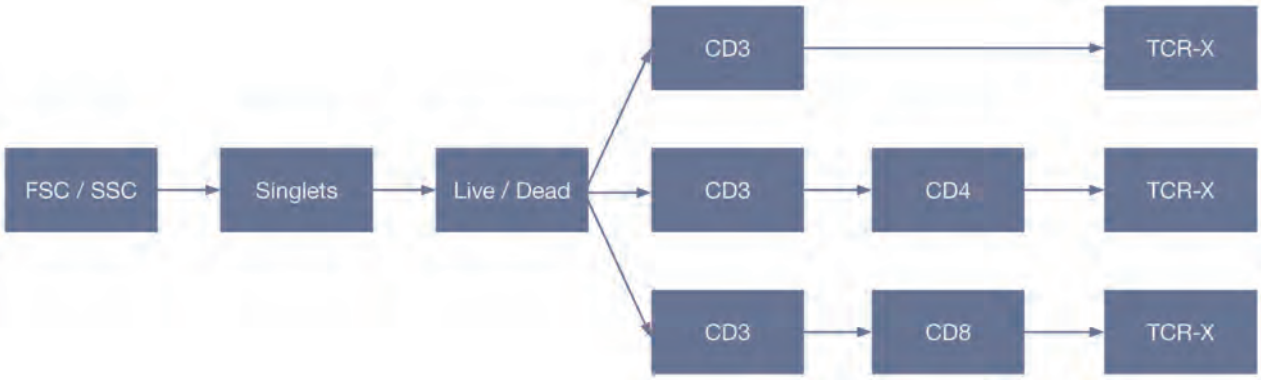


Figure 5. Gating strategy 1.

Gating strategy 2

Figure 6 depicts the gating strategy used to assess the precision of the Ki67 measurements in the CD3, CD4, and CD8 populations.

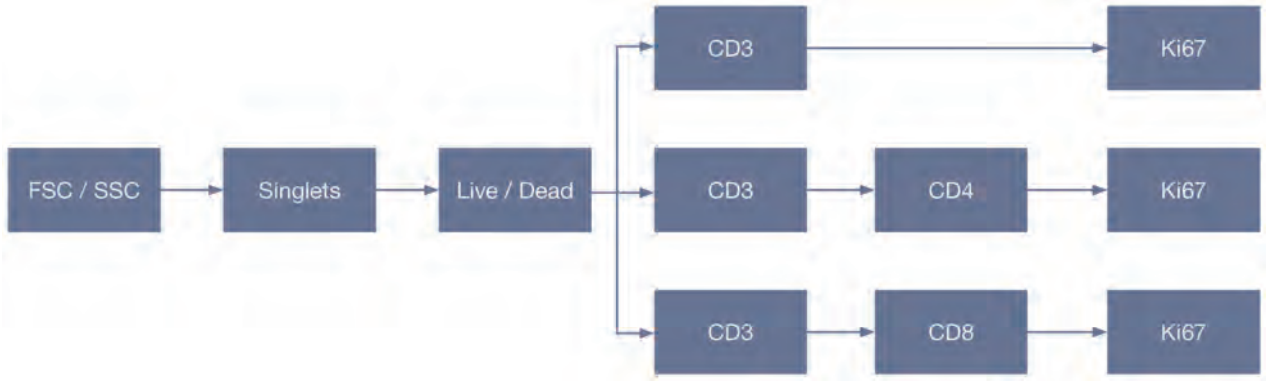


Figure 6. Gating strategy 2.

Gating strategy 3

Figure 7 depicts the gating strategy used to assess the precision of the immune-phenotype measurement using measurements of CD45RO and CD62L in the CD3, CD4, and CD8 populations.

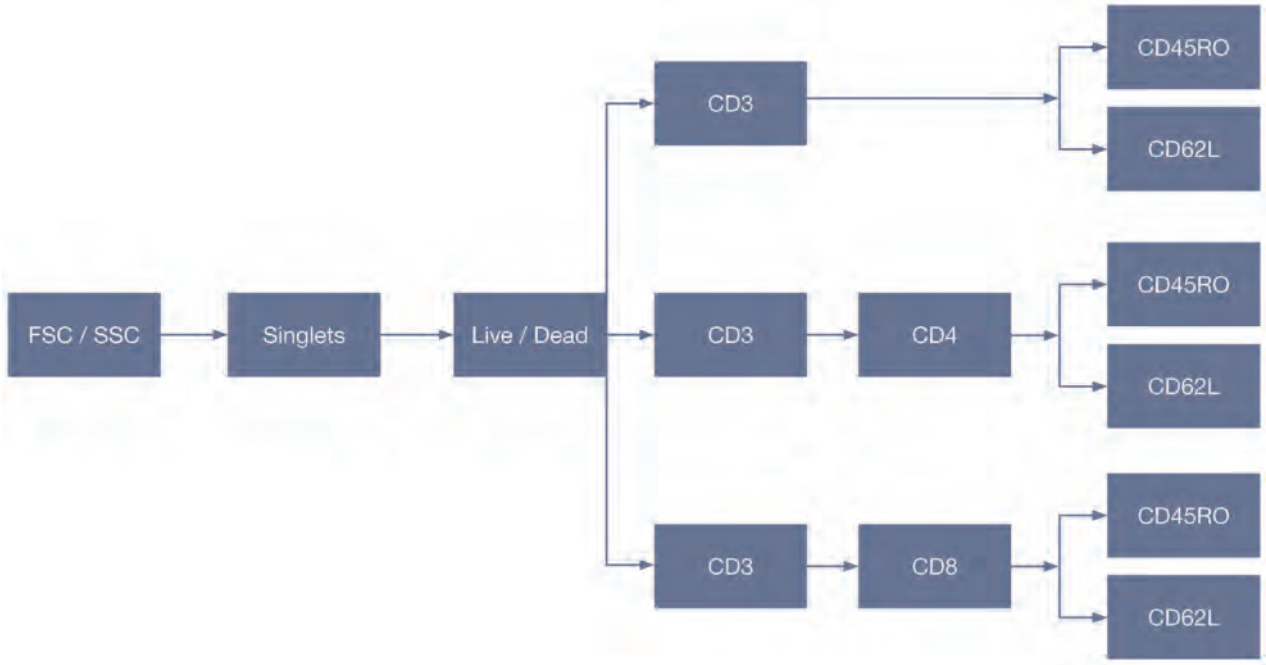


Figure 7. Gating strategy 3.

The immune-phenotype of the cells was then assessed using the expression marker categorisation in Figure 8:

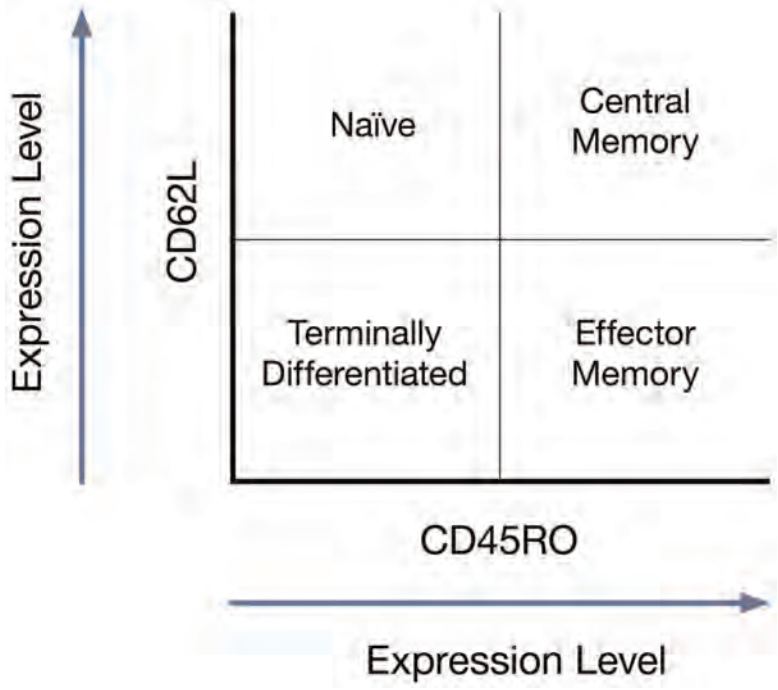


Figure 8. CD62L and CD45RO correlation to immune phenotype.

4.2.4 Intra-assay precision results

[Report the results, making it clear how the assay performed against the acceptance criteria and if it is considered to have passed or failed.]

Intra-assay precision was determined using the 8 dilutions outlined in the linearity study (Table 1) for TCR-X from a single run (run one) performed by a single operator.

The results, using gating strategy 1 (Figure 5), are reported as the mean, standard deviation and %CV of the triplicate samples for each dilution (Table 4). The repeatability is assessed by measuring the mean of the CV value.

Table 4. Intra-assay precision results.

Dilution	CD3 TCR-X +			CD4 TCR-X +			CD8 TCR-X +		
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
1	25.71	0.37	1.4	27.92	0.13	0.5	10.80	0.11	1.1
2	20.10	0.28	1.4	24.58	0.47	1.9	8.61	0.38	4.4
3	14.01	0.92	6.6	18.00	2.45	13.6	5.39	0.94	17.4
4	11.00	0.13	1.2	14.14	0.08	0.5	3.95	0.13	3.4
5	8.99	0.35	3.9	11.46	0.43	3.8	2.87	0.15	5.3
6	8.31	0.22	2.6	10.81	0.30	2.8	2.64	0.26	9.8
7	10.99	0.21	1.9	18.45	0.51	2.8	5.33	0.29	5.4
8	10.08	0.17	1.7	17.44	0.75	4.3	4.75	0.27	5.6
Acceptance criteria (Mean CV)	**≤ 20%**			**≤ 20%**			**≤ 20%**		
Result (Mean CV)	**3.1%**			**5.5%**			**8.1%**		
Pass/Fail	**Pass**			**Pass**			**Pass**		

Mean %CV = 100(mean $CV^2)^{1/}2$

Summary

The assay demonstrates an acceptable levels of intra-assay precision across the eight dilutions of transduced cells for TCR-X expression in the CD3, CD4, and CD8 populations.

4.2.5 Inter-assay variability / intermediate precision

[Report the results, making it clear how the assay performed against the acceptance criteria and if it is considered to have passed or failed.]

Variability across different days

Inter-assay variability was determined using dilution one (Table 1) from four independent assay runs performed by a single operator over 5 days. Each result was run in triplicate and the reported results are the mean, standard deviation and percentage CV of all twelve replicates (triplicate samples from each of four assay runs).

The results are reported as follows:

1. Inter-assay variability of the TCR-X measurement in the CD3, CD4 and CD8 cell populations using gating strategy 1 (Table 5).
2. Inter-assay variability of the Ki67 measurement in the CD3, CD4 and CD8 cell populations using gating strategy 2 (Table 6).
3. Inter-assay variability of the immune-phenotype measurement using CD45RO/CD62L in the CD3, CD4 and CD8 cell populations using gating strategy 3 (Tables 7 to 10).

Table 5. Inter-assay variability of TCR-X measurements.

Dilution 1	CD3 TCR-X +			CD4 TCR-X +			CD8 TCR-X +		
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
Run 1	25.71	0.37	1.4	27.92	0.13	0.5	10.80	0.11	1.1
Run 2	24.87	1.73	7.0	26.77	1.35	5.0	7.51	0.94	12.5
Run 3	22.86	0.68	3.0	23.92	0.59	2.5	11.21	0.39	3.4
Run 4	25.55	0.43	1.7	26.26	0.30	1.1	10.07	0.47	4.6
Acceptance criteria (CV)	≤ 20%			≤ 20%			≤ 20%		
Result (CV)	5.8%			6.3%			15.9%		
Pass/Fail	Pass			Pass			Pass		

Table 6. Inter-assay precision of Ki67 measurements.

Dilution 1	CD3 Ki67 +			CD4 Ki67 +			CD8 Ki67 +		
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
Run 1	64.81	0.91	1.4	40.59	0.88	2.2	18.08	0.49	2.7
Run 2	67.97	1.68	2.5	48.40	1.44	3.0	24.87	1.73	7.0
Run 3	62.29	1.18	1.9	34.36	0.64	1.9	19.18	0.25	1.3
Run 4	67.09	0.53	0.8	44.99	1.18	2.6	19.14	0.88	4.6
Acceptance criteria (CV)	≤ 20%			≤ 20%			≤ 20%		
Result (CV)	3.8%			13.2%			14.3%		
Pass/Fail	Pass			Pass			Pass		

Table 7. Inter-assay precision for immune-phenotype 1 - Terminally Differentiated population (Phenotype = CD45RO- / CD62L-)

Dilution 1	CD3 CD45RO- CD62L-			CD4 CD45RO- CD62L-			CD8 CD45RO- CD62L-		
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
Run 1	5.46	0.35	6.4	3.50	0.40	11.3	10.94	0.39	3.6
Run 2	4.83	0.25	5.8	3.29	0.32	9.7	11.80	0.34	2.9
Run 3	4.57	0.39	8.5	2.77	0.18	6.5	10.18	1.30	12.7
Run 4	4.59	0.32	6.9	2.57	0.37	14.4	7.76	0.39	5.0
Acceptance criteria (CV)	≤ 20%			≤ 20%			≤ 20%		
Result (CV)	11.0%			15.8%			16.6%		
Pass/Fail	Pass			Pass			Pass		

Table 8. Inter-assay precision for immune-phenotype 2 Naïve population (Phenotype = CD45RO- / CD62L+)

Dilution 1	CD3 CD45RO- CD62L+			CD4 CD45RO- CD62L+			CD8 CD45RO- CD62L+		
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
Run 1	2.75	0.57	20.9	3.99	0.53	13.4	4.28	0.48	11.2
Run 2	3.25	0.37	11.2	5.18	0.17	3.2	5.43	0.44	8.1
Run 3	3.73	0.35	9.4	5.98	0.28	4.7	4.50	0.45	10.0
Run 4	2.83	0.49	17.2	4.15	0.27	6.4	4.45	0.55	12.3
Acceptance criteria (CV)	≤ 20%			≤ 20%			≤ 20%		
Result (CV)	17.9%			18.5%			13.4%		
Pass/Fail	Pass			Pass			Pass		

Table 9. Inter-assay precision for immune-phenotype 3 Effector Memory population (Phenotype = CD45RO+ / CD62L-)

Dilution 1	CD3 CD45RO+ CD62L-			CD4 CD45RO+ CD62L-			CD8 CD45RO+ CD62L-		
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
Run 1	60.55	1.33	2.2	56.09	1.45	2.6	57.68	0.69	1.2
Run 2	57.26	1.38	2.4	53.07	1.87	3.5	55.90	0.98	1.8
Run 3	69.14	0.65	0.9	63.63	0.94	1.5	67.46	0.48	0.7
Run 4	65.07	1.98	3.0	59.34	1.70	2.9	66.85	1.54	2.3
Acceptance criteria (CV)	≤ 20%			≤ 20%			≤ 20%		
Result (CV)	7.7%			7.1%			8.9%		
Pass/Fail	Pass			Pass			Pass		

Table 10. Inter-assay precision for immune-phenotype 4 Central Memory population (Phenotype = CD45RO+ / CD62L+)

Dilution 1	CD3 CD45RO+ CD62L+			CD4 CD45RO+ CD62L+			CD8 CD45RO+ CD62L+		
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
Run 1	31.24	1.03	3.3	36.42	1.11	3.0	27.10	1.45	5.3
Run 2	35.12	1.73	4.9	37.84	2.06	5.4	26.87	1.18	4.4
Run 3	22.56	1.35	6.0	27.62	1.27	4.6	17.86	1.26	7.1
Run 4	31.17	1.56	5.0	37.27	1.23	3.3	25.26	0.65	2.6
Acceptance criteria (CV)	≤ 20%			≤ 20%			≤ 20%		
Result (CV)	16.5%			13.0%			16.7%		
Pass/Fail	Pass			Pass			Pass		

Summary

The assay demonstrates an acceptable levels of inter-assay precision for TCR-X, Ki67 and CD45RO/CD62L expression in the CD3, CD4 and CD8 populations when the assay is operated on different days by the same operator/analyst using the same equipment.

Variability as a function of operator/analyst

Intermediate precision was determined using dilution 1 (Table 1) from six independent assay runs performed by two operators over five days. Runs 1, 3 and 4 from operator 1 and runs 5, 6 and 7 from operator two were included in the analysis. Each result was run in triplicate and the reported results are the mean, standard deviation, and percentage CV of all 18 replicates (triplicate samples from each of six assay runs).

The results are reported as follows:

1. Intermediate precision of the TCR-X, Ki67 and immune-phenotype measurement in the CD3 population (Table 11).
2. Intermediate precision of the TCR-X, Ki67 and immune-phenotype measurement in the CD4 population (Table 12).
3. Intermediate precision of the TCR-X, Ki67 and immune-phenotype measurement in the CD8 population (Table 13).

Table 11. Intermediate precision of TCR-X, Ki67 and immune-phenotype measurements in the CD3 population

Dilution1	Immuno-Phenotype	Assay Results			Acceptance Criteria	Pass / Fail
		Mean	SD	%CV	%CV	
TCR-X		25.83	2.13	8.3	≤ 20%	**Pass**
Ki67		64.73	2.23	3.4	≤ 20%	**Pass**
CD45RO- CD62L-	Terminally Differentiated	3.59	1.44	40.1	≤ 20%	**Fail**
CD45RO- CD62L+	Naïve	2.20	1.06	48.0	≤ 20%	**Fail**
CD45RO+ CD62L-	Effector Memory	69.21	6.02	8.7	≤ 20%	**Pass**
CD45RO+ CD62L+	Central Memory	25.72	5.08	19.7	≤ 20%	**Pass**

Table 12. Intermediate precision of TCR-X, Ki67 and immune-phenotype measurements in the CD4 population

Dilution1	Immuno-Phenotype	Assay Results			Acceptance Criteria	Pass / Fail
		Mean	SD	%CV	%CV	
TCR-X		26.24	2.12	8.1	≤ 20%	**Pass**
Ki67		39.98	4.69	11.7	≤ 20%	**Pass**
CD45RO- CD62L-	Terminally Differentiated	2.02	1.03	51.1	≤ 20%	**Fail**
CD45RO- CD62L+	Naïve	3.24	1.69	52.2	≤ 20%	**Fail**
CD45RO+ CD62L-	Effector Memory	63.43	5.61	8.8	≤ 20%	**Pass**
CD45RO+ CD62L+	Central Memory	31.86	5.10	16.0	≤ 20%	**Pass**

Table 13. Intermediate precision of TCR-X, Ki67 and immune-phenotype measurements in the CD8 population

Dilution1	Immuno-Phenotype	Assay Results			Acceptance Criteria	Pass / Fail
		Mean	SD	%CV	%CV	
TCR-X		11.97	1.72	14.3	≤ 20%	**Pass**
Ki67		18.80	0.75	4.0	≤ 20%	**Pass**
CD45RO- CD62L-	Terminally Differentiated	7.29	2.8	38.4	≤ 20%	**Fail**
CD45RO- CD62L+	Naïve	3.08	1.48	48.1	≤ 20%	**Fail**
CD45RO+ CD62L-	Effector Memory	62.27	6.28	9.3	≤ 20%	**Pass**
CD45RO+ CD62L+	Central Memory	22.53	4.05	18.0	≤ 20%	**Pass**

Summary

The assay demonstrates an acceptable level of intermediate precision for TCR-X and Ki67 expression in the CD3, CD4 and CD8 cell populations. Measurement of immune-phenotype using CD45RO and CD62L demonstrates that the assay has an acceptable level of precision for analysis of the effector memory and central memory cells in the CD3, CD4 and CD8 population. However, the assay failed to pass the acceptance criteria for intermediate precision in the terminally differentiated and Naïve cell populations (Table 14).

The failure of the assay to pass the acceptance criteria (CV ≤ 20%) is due to the low percentage of positive cells present in these samples. When the intra-assay CVs are measured for the terminally differentiated and naïve cell populations for each of the 6 runs (see table below) this show that in only two instances is the CV above 20%. It can therefore be assumed that while the assay does not pass the acceptance criteria for the measurement of terminally differentiated and naïve cells in the CD3, CD4 and CD8 populations the impact on the overall performance of the assay is minimal unless the assay is being used to detect rare events.

Table 14. Percentage CVs (intra-assay precision) for measurement of terminally differentiated and Naïve cells in CD3, CD4 and CD8 populations

		CD3		CD4		CD8	
		Term Diff	Naive	Term Diff	Naive	Term Diff	Naive
Operator 1	**Run 1**	6.4	20.9	11.3	11.2	3.6	11.2
	Run 3	8.5	9.4	6.5	4.7	12.7	10.1
	Run 4	6.9	17.2	14.4	6.4	5.0	12.3
Operator 2	**Run 5**	7.3	7.4	15.1	9.7	11.1	9.8
	Run 6	14.6	6.5	8.1	8.5	17.1	25.3
	Run 7	2.5	15.2	9.6	19.2	5.4	10.2

4.3 Assay specificity (TCR-X)

[Provide brief overview of the assay specificity assessment method, this could be paraphrased from the protocol document.]

Specificity was defined as the assay's ability to accurately measure and distinguish between the percentage TCR-X % positive expression in transduced (dilution one) and non-transduced (dilution eight) cells (Table 1). Specificity was assessed by using unpaired student T test.

4.3.1 Acceptance criteria

[Re-iterate the acceptance criteria for the assessment, this should match that defined in the protocol.]

The assay is considered to be specific if the resulting p value ≤0.05 indicating the two cell populations were significantly different.

4.3.2 Gating strategy

[Describe gating strategy utilised for specificity assessment, to keep this section of the document manageable a simple process flow of the gating strategy can be presented with exemplars of real gating plots in an appendix. However, it would be equally suitable to present the actual plots of the gating strategy here.]

Figure 9 depicts the gating strategy used to measure the percentage TCR-X positive cells in the transduced and non-transduced cells. Exemplar dot-plots are shown in Section 6 – Gating Strategies.

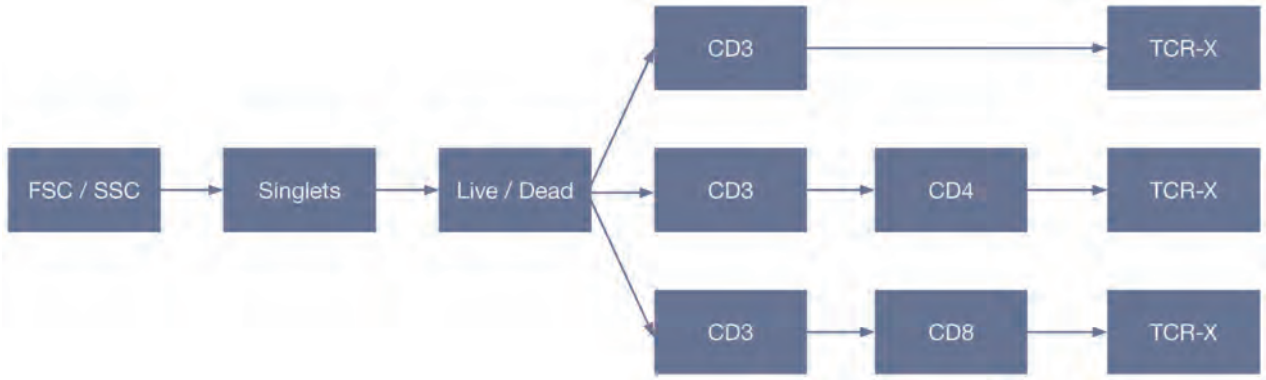


Figure 9. Specificity gating strategy.

4.3.3 Results

[Report the results (including any relevant tables of data, graphs, statistical analysis performed etc.) state how the assay performed against the acceptance criteria and if the assay characteristic is considered to have passed or failed against the acceptance criteria.]

Table 15 presents a summary of the precision results.

Table 15. Precision Results

Cell population	Transduced cells		Non-transduced cells		Acceptance criteria		
	Mean	SD	Mean	SD	p Value	Result	Pass / Fail
CD3	24.75	1.44	8.42	2.28	≤ 0.05	**0.00001**	**Pass**
CD4	26.22	1.65	13.26	3.74	≤ 0.05	**0.00086**	**Pass**
CD8	9.90	1.58	3.34	1.51	≤ 0.05	**0.00118**	**Pass**

Summary

The assay demonstrates an acceptable level of specificity for detection of TCR-X expression in the transduced cells.

4.4 Detection Limit (LoD) and Quantification Limit (LoQ)

[Provide brief overview of the LoD and LoQ assessment methods utilised, this could be paraphrased from the protocol document.]

The LoD and LoQ of the assay were calculated as the mean plus three times the standard deviation (LoD) and the mean plus ten times the standard deviations (LoQ) respectively of the background TCR-X+ expression in non-transduced cells.

4.4.1 Acceptance criteria

[Re-iterate the acceptance criteria for the assessment, this should match that defined in the protocol.]

No pre-defined acceptance criteria were set for this assay as information on LoD and LoQ was for information only.

4.4.2 Results

[Report the results (including any relevant tables of data, graphs, statistical analysis performed etc.) state how the assay performed against the acceptance criteria and if the assay characteristic is considered to have passed or failed against the acceptance criteria.]

The data for the TCR-X expression in non-transduced (dilution eight - Table 1) cell samples from the four independent assay runs performed by operator one (runs 1-4) were used to calculate the LoD and LoQ for the assay. The LoD and LoQ values are reported for the CD3, CD4, and CD8 populations (Table 16).

Table 16. LoD and LoQ results for non-transduced TCR-X+ cells.

	Mean	St Dev	LoD	LoQ
CD3+ TCR-X+	8.42	2.28	15.26	32.11
CD4+ TCR-X+	13.7	4.27	26.50	56.36
CD8+ TCR-X+	3.57	1.81	9.01	21.71

Summary

Non-transduced cells naturally express TCR-X. The main application of this assay is to detect transduced cell persistence measured through TCR-X expression in the CD3 population. Therefore, it

is important to understand the LoD and LoQ for non-transduced cells. In this assay the detection limit was 15.26% and quantification limit 32.11% of TCR-X positive cells respectively. However, the transduced and non-transduced cells used in this study are from non-matched donors and so the limit of detection is only an indicative value and is likely to differ from sample to sample.

4.5 Assay range

[Provide brief overview of the assay range assessment methods utilised, this could be paraphrased from the protocol document.]

The range of the assay was described as the interval between the upper and lower cell numbers (dilutions) of TCR-X expression of transduced cells which demonstrated a suitable level of precision and linearity. Linearity was within the pre-defined acceptance criteria for CD3, CD4 and CD8 cells for all dilutions (see section 4.1).

This assessment is based on the data from the 4 independent assay runs performed by a single operator used in the inter-assay / intermediate precision analysis.

4.5.1 Acceptance criteria

[Re-iterate the acceptance criteria for the assessment, this should match that defined in the protocol.]

The assay range will be measured over the individual dilutions which have a %CV ≤ 20 %

4.5.2 Assay range results

[Report the results (including any relevant tables of data, graphs, statistical analysis performed etc.) state how the assay performed against the acceptance criteria and if the assay characteristic is considered to have passed or failed against the acceptance criteria.]

Assay range was determined using all the transduced cell dilutions (Table 1) from four independent assay runs performed by a single operator over five days. Each result was run in triplicate and the reported results is the percentage CV of all twelve replicates (triplicate samples from each of four assay runs).

The results are reported as follows:

1. Percentage CV for TCR-X expression in each of the dilutions in the CD3 population (Table 17)
2. Percentage CV for TCR-X expression in each of the dilutions in the CD4 population (Table 18)
3. Percentage CV for TCR-X expression in each of the dilutions in the CD8 population (Table 19)

Table 17. Percentage CV for TCR-X expression in the CD3 population

	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Dilution 6	Dilution 7
% CV for TCR-X	5.8	12.7	10.5	7.6	6.0	12.5	13.8

Table 18. Percentage CV for TCR-X expression in the CD4 population

	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Dilution 6	Dilution 7
% CV for TCR-X	6.3	11.9	9.4	7.3	11.3	21.2	22.5

Table 19. Percentage CV for TCR-X expression in the CD8 population

	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Dilution 6	Dilution 7
% CV for TCR-X	15.9	17.0	18.2	18.9	21.0	34.0	31.6

Summary

1. In the CD3 population the assay range (CV ≤ 20%) covered all the dilutions of the transduced cells representing a dilution of the original material down to 1:64.
2. In the CD4 population the assay range covered five dilutions of the transduced cells, equivalent to a 1:16 dilutions of the original material.
3. In the CD8 population the assay range covered four dilutions of the transduced cells, equivalent to a 1:8 dilutions of the original material.

The assay range performed as expected with increasing loss of precision as the analysed cell number decreases in the sub-populations giving a range hierarchy CD3> CD4> CD8.

5. Conclusion

[Present the overall conclusion for the study, summarising those characteristics of the assay validation protocol that passed and those that failed. Provide an overall statement as to whether the assay is fit-for-purpose based on the outcome of the results.]

This study demonstrates that the flow cytometry assay panel passes the validation criteria for the measurement of TCR-X expression in the CD3, CD4, and CD8 cell populations following transduction with the TCR-X vector.

The results of the validation of TCR-X measurements are summarised in Table 20.

Table 20. TCR-X expression in CD3, 4 and 8 populations: validation results summary.

Assay Parameter	Cell Population	Acceptance criteria	Result	Pass / Fail
Linearity	CD3+	R2 $\geq$ 0.90	0.9945	**Pass**
	CD4+	R2 $\geq$ 0.90	0.9804	**Pass**
	CD8+	R2 $\geq$ 0.90	0.9857	**Pass**
Intra-assay Precision	CD3+	CV $\leq$ 20%	3.1%	**Pass**
	CD4+	CV $\leq$ 20%	5.5%	**Pass**
	CD8+	CV $\leq$ 20%	8.1%	**Pass**
Intra-assay / Intermediate Precision (Different days)	CD3+	CV $\leq$ 20%	5.8%	**Pass**
	CD4+	CV $\leq$ 20%	6.3%	**Pass**
	CD8+	CV $\leq$ 20%	15.9%	**Pass**
Intra-assay / Intermediate Precision (Different operators)	CD3+	CV $\leq$ 20%	8.3%	**Pass**
	CD4+	CV $\leq$ 20%	8.1%	**Pass**
	CD8+	CV $\leq$ 20%	14.3%	**Pass**
Specificity	CD3+	p Value $\leq$ 0.05	0.00001	**Pass**
	CD4+	p Value $\leq$ 0.05	0.00086	**Pass**
	CD8+	p Value $\leq$ 0.05	0.00118	**Pass**

This study demonstrates that the flow cytometry assay panel passes the validation criteria for the measurement of Ki67 expression in the CD3, CD4 and CD8 cell populations following transduction with the TCR-X vector.

The results of the validation of Ki67 measurements are summarised in Table 21.

Table 21. Ki67 validation results summary.

Assay Parameter	Cell Population	Acceptance criteria	Result	Pass / Fail
Intra-assay / Intermediate Precision (Different days)	CD3+	CV $\leq$ 20%	3.8%	**Pass**
	CD4+	CV $\leq$ 20%	13.2%	**Pass**
	CD8+	CV $\leq$ 20%	14.3%	**Pass**
Intra-assay / Intermediate Precision (Different operators)	CD3+	CV $\leq$ 20%	3.4%	**Pass**
	CD4+	CV $\leq$ 20%	11.7%	**Pass**
	CD8+	CV $\leq$ 20%	4.0%	**Pass**

This study demonstrates that the flow cytometry assay panel passes the validation criteria for intra-assay precision for the characterisation of the immune-phenotype in the CD3, CD4 and CD8 cell populations.

The assay also passes the validation criteria for the measurement of intermediate precision in the central memory and effector memory populations but does not meet the acceptance criteria for measurement of intermediate precision in the terminally differentiated and Naïve cell populations.

The results of the validation of immune-phenotype measurements are summarised in Table 22.

Table 22. Validation results summary with respect to immune phenotype.

Assay Parameter	Phenotype	Cell Population	Acceptance criteria	Result	Pass / Fail
Intra-assay / Intermediate Precision (Different days)	Naïve	CD3+	CV ≤ 20%	17.9%	Pass
		CD4+	CV ≤ 20%	18.5%	Pass
		CD8+	CV ≤ 20%	13.4%	Pass
	Terminally differentiated	CD3+	CV ≤ 20%	11.0%	Pass
		CD4+	CV ≤ 20%	15.8%	Pass
		CD8+	CV ≤ 20%	16.6%	Pass
	Central Memory	CD3+	CV ≤ 20%	16.5%	Pass
		CD4+	CV ≤ 20%	13.0%	Pass
		CD8+	CV ≤ 20%	16.7%	Pass
	Effector Memory	CD3+	CV ≤ 20%	7.7%	Pass
		CD4+	CV ≤ 20%	7.1%	Pass
		CD8+	CV ≤ 20%	8.9%	Pass
Intra-assay / Intermediate Precision (Different Operators)	Naïve	CD3+	CV ≤ 20%	48.0%	Fail
		CD4+	CV ≤ 20%	52.2%	Fail
		CD8+	CV ≤ 20%	48.1%	Fail
	Terminally differentiated	CD3+	CV ≤ 20%	40.1%	Fail
		CD4+	CV ≤ 20%	51.1%	Fail
		CD8+	CV ≤ 20%	38.4%	Fail
	Central Memory	CD3+	CV ≤ 20%	19.7%	Pass
		CD4+	CV ≤ 20%	16.0%	Pass
		CD8+	CV ≤ 20%	18.0%	Pass
	Effector Memory	CD3+	CV ≤ 20%	8.7%	Pass
		CD4+	CV ≤ 20%	8.8%	Pass
		CD8+	CV ≤ 20%	9.3%	Pass

6. Appendices - Gating strategies

[Include any relevant supplementary data to support that presented in the main body of the report e.g. this may include exemplars of the real gating strategies e.g. dot plots etc.]

Figures 10 -14 illustrate the gating strategies used during the validation studies.

Figure 10. Gating strategy for live cell selection

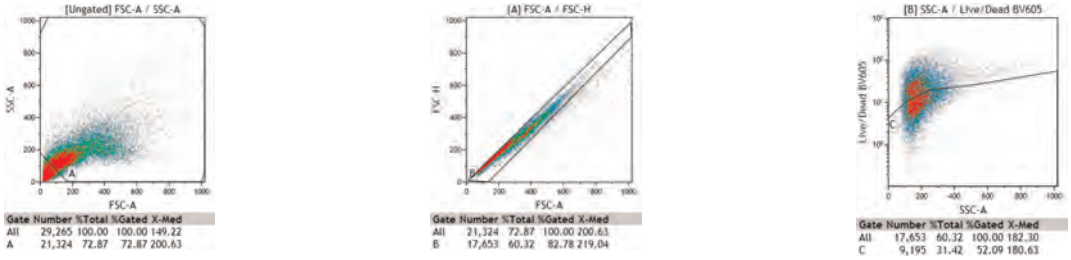
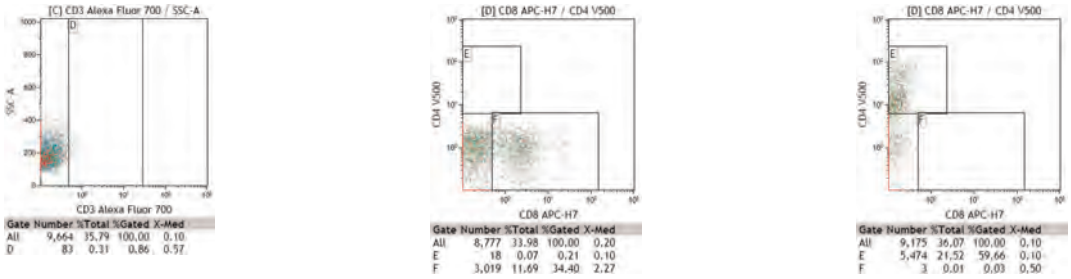


Figure 11. Gate setting based on FMO controls

CD3 FM0 **CD4 FM0** **CD8 FM0**



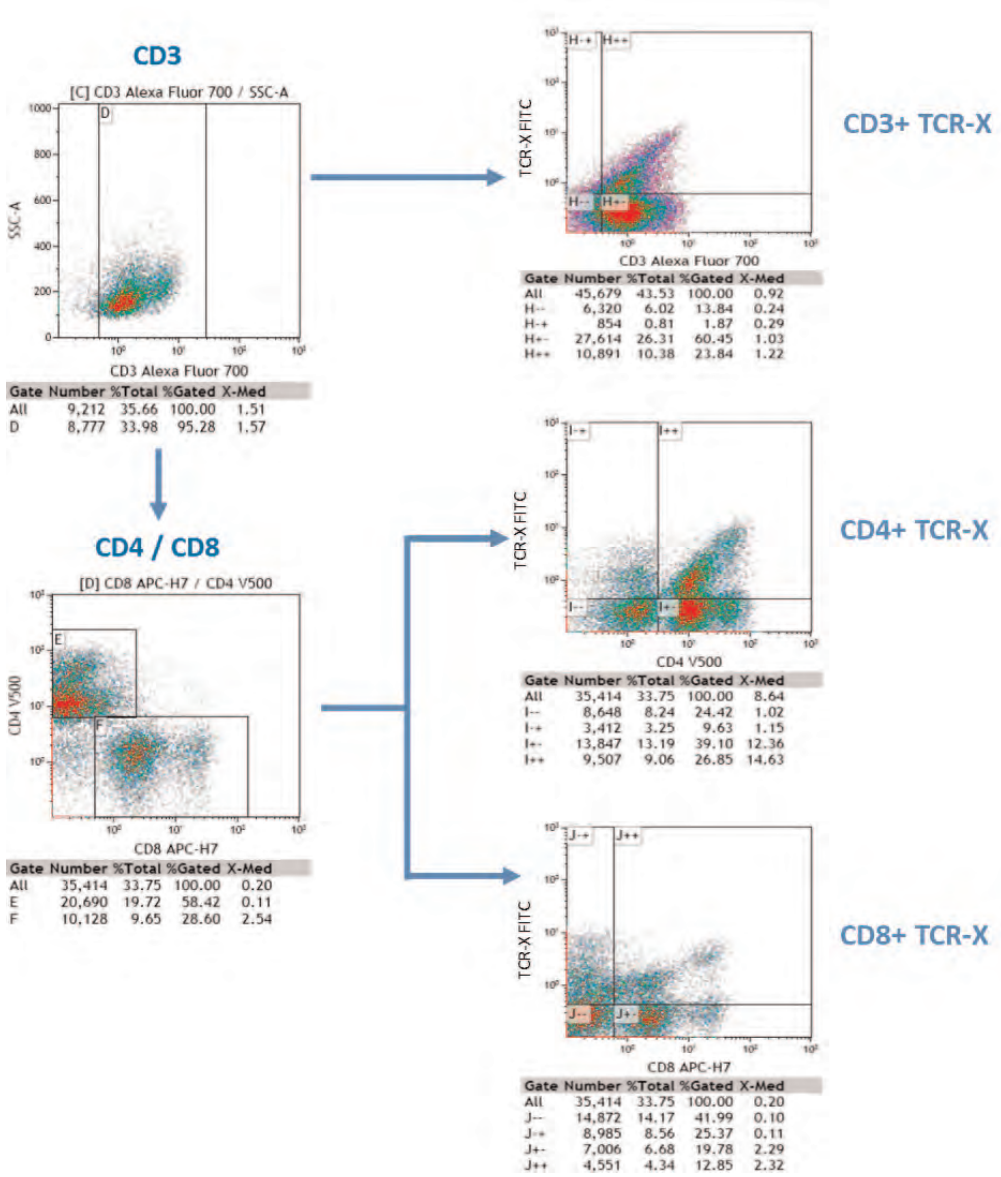


Figure 12. Measurement of TCR-X

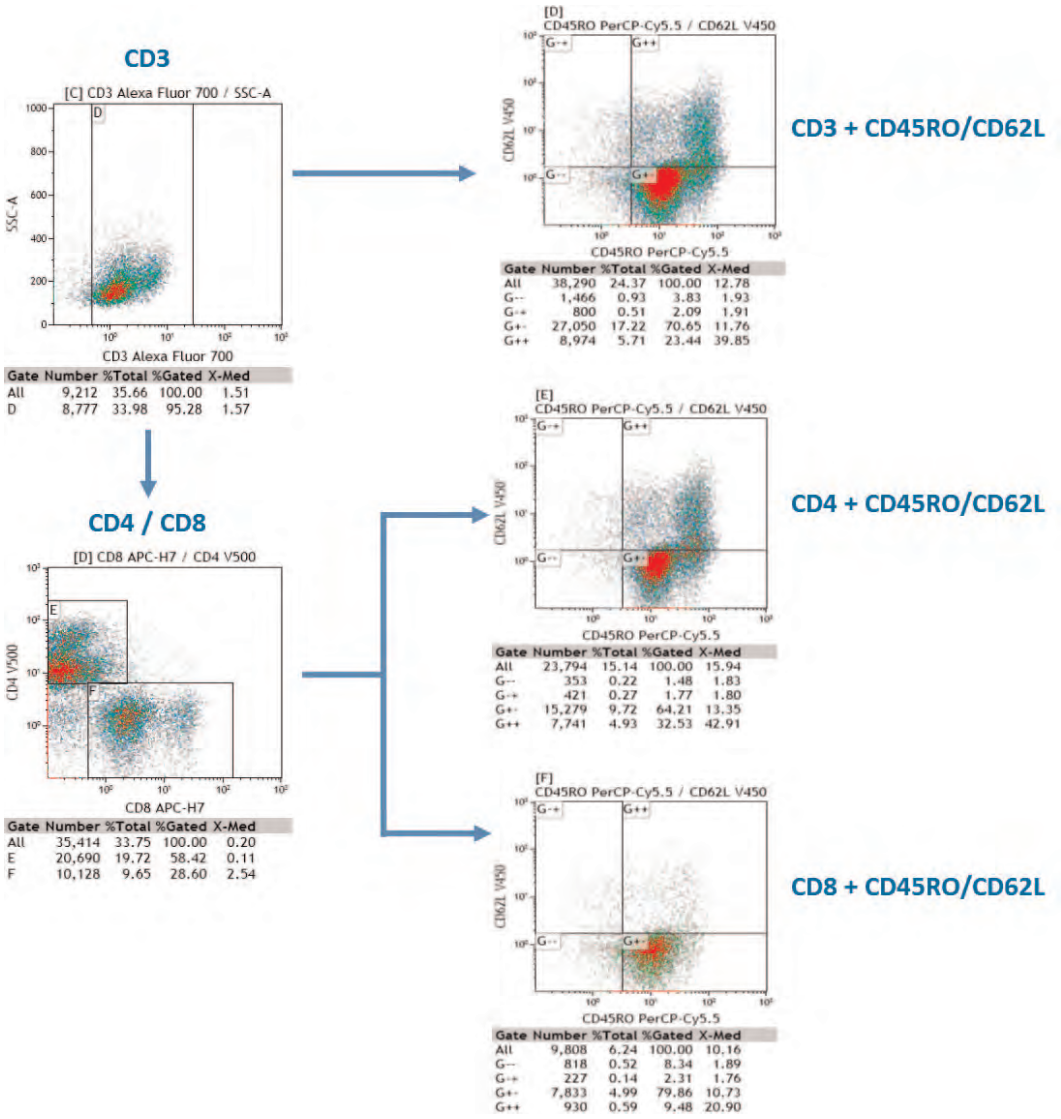


Figure 13. Immune-phenotype using CD45RO and CD62L

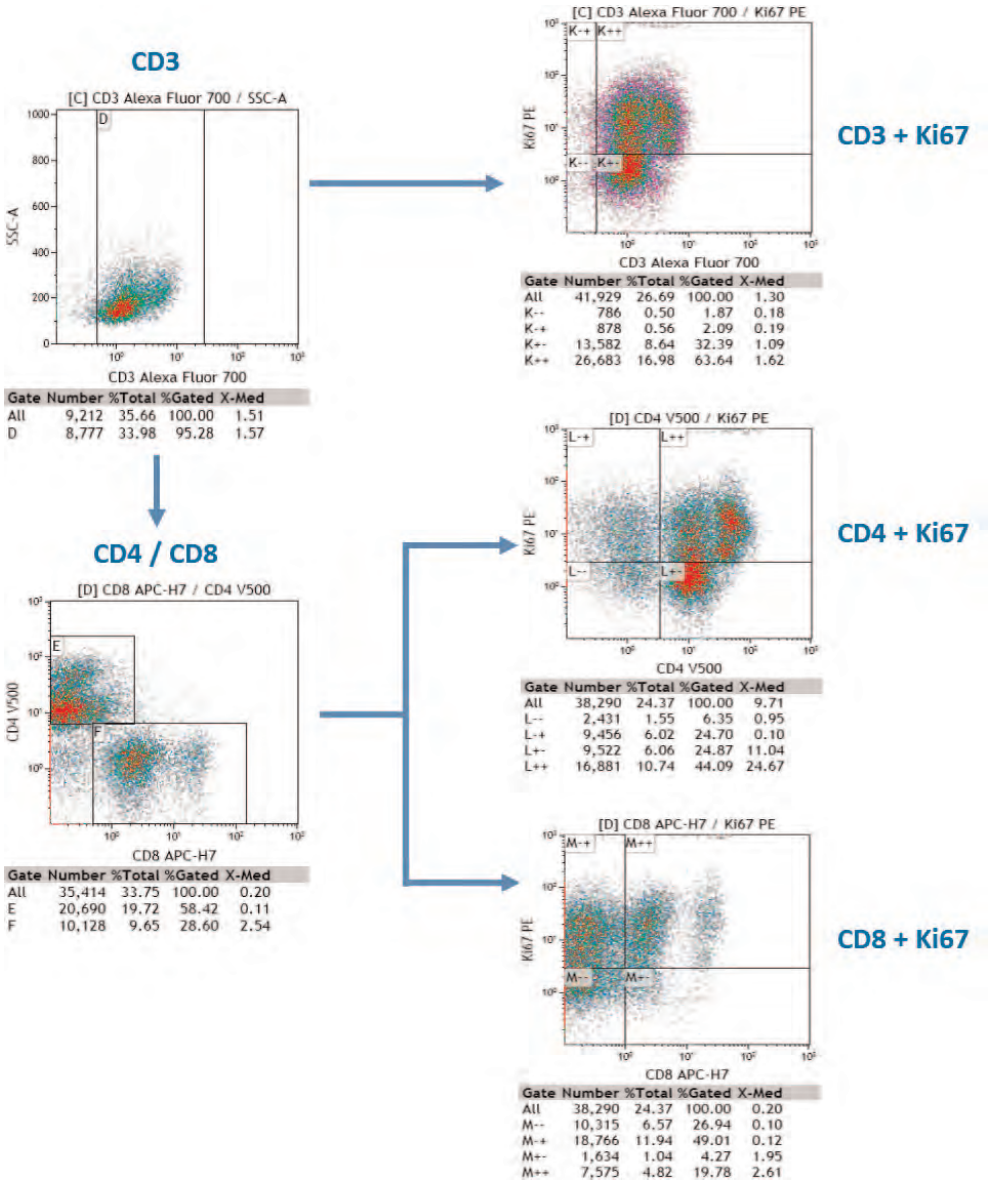


Figure 14. Measurement of Ki67