Quality Control Document:

Assay Validation Flow Cytometry Guidance

# Purpose

Analytical assays performed to Good Clinical Practice (GCP) standards must be properly validated prior to undertaking the analysis of clinical trial samples to ensure data integrity. The performance characteristics of the assay must be demonstrated to be suitable and reliable for the intended application through the use of specific laboratory investigations. In addition, acceptance criteria for each assay must be transparent and clearly defined.

This document provides a guideline for assay validation and gives examples of assay parameters which may be considered as part of the validation process for flow cytometry assays. This document is designed to be used for clinical trials of investigational medicinal products (CTIMPs) but could also be used for non-CTIMP trials and studies to support best practice. It is possible to create project specific sample kit management documents, which should contain the specifications outlined in the Laboratory Analysis SOP (UoB-CRL-SOP-004).

# Instructions

1. Remove this first instruction page.
2. Update the header to include the trial/study ID.
3. Update the footer; include a version date and retain the document reference information relating to this quality control document (QCD).

## Assay Validation Plan

1. Remove red instruction text.
2. Complete an assay validation plan for each analytical assay of clinical trial samples to be completed by the laboratory.
3. Ensure the assay validation plan is approved by an appropriate member of the research team (e.g., the Laboratory Academic Lead (LAL), the Chief Investigator (CI).
4. Send a copy of (each) validation plan via email to the Clinical Research Compliance Team (CRCT) (crct@contacts.bham.ac.uk) to be checked for compliance with Laboratory Analysis SOP (UoB-CRL-SOP-004).
5. File each approved validation plan in the ‘Assay Validation’ section of the Laboratory Master File (LMF); see the quality control document (QCD) Setting Up a Laboratory Master File (UoB-CRL-QCD-001). Archive with the other trial records upon closure, see Archiving SOP (UoB-ARC-SOP-001).

## Assay Validation Report

1. Carry out the assay validation as described in Assay Validation Plan.
2. Record the outcomes in the Assay Validation Report. Note - all assay validation reports must be approved by an appropriate member of the research team (e.g., the LAL, the CI) before the assay can be used to analyse clinical trial samples.
3. File each approved validation report in the ‘Assay Validation’ section of the LMF; see the QCD Setting Up a Laboratory Master File (UoB-CRL-QCD-001). Archive with the other trial records upon closure; see Archiving SOP (UoB-ARC-SOP-001).

# Related documents

* UoB-ARC-SOP-001 Archiving
* UoB-CRL-QCD-001 Setting Up a Laboratory Master File
* UoB-CRL-SOP-001 Laboratory Set Up and Management
* UoB-CRL-SOP-002 Laboratory Facilities
* UoB-CRL-SOP-003 Sample Management
* UoB-CRL-SOP-004 Laboratory Analysis
* UoB-CRL-SOP-005 Reportable Issues

UoB QMS documents can be found on the [CRCT website](https://www.birmingham.ac.uk/research/activity/mds/mds-rkto/governance/index.aspx). Internal work instructions can be obtained from the CRCT (mailto:crct@contacts.bham.ac.uk) and/or from the RGT (researchgovernance@contacts.bham.ac.uk).

Validation Plan

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| Assay Name |
| Flow Cytometry |
| Assay details |
| *Method Overview* |
| Assay Methodology |
| *List the trial-specific procedures involved, giving the code and title for each.* |
| Validation parameters being tested |
| *Describe how each of these parameters is being addressed during the validation. Justify any exclusion. It is accepted that not all performance parameters can be easily validated for some types of analytical assay e.g., some pharmacodynamic cell-based assays.*For validation, use the same biological matrix as the test samples and the same analytical process or method. For example, if peripheral blood mononuclear cells (PBMCs) will be isolated from blood and frozen prior to the study analysis, then PBMCs isolated in the same manner and frozen under the same conditions, should be used for the validation. However, any potential limitations of the chosen biological matrix also need to be identified. For example, the phenotypic profile or frequencies of some cell types may be altered following PBMC isolation and/or freezing and these should be explored. |
| Characterisation of controls |
| Where possible samples will be the same biological matrix as the test samples.The data used from replicate analysis of control samples and reference standards (where appropriate and/or available) should be used to obtain intra- and inter-assay data on precision and accuracy.A number of controls will be considered to ensure the validity of the data. * **Negative Control**: These are samples that do not express the antigens being tested. Negative controls can be irrelevant cells, knockout cells or untreated cells, where appropriate. An intrinsic negative control uses cells within the same sample that are known to be negative for the marker of interest. For example, checking for myeloid markers on lymphoid cells or vice versa in PBMC, bone marrow etc. This is ideal as the control is exposed to identical test conditions as the cells of interest.
* **Positive Control**: These are samples that express the antigens being tested. This can be the same sample type or commercially available stabilised or lyophilised cells. For rare or weakly expressed antigens, cells can be engineered to express those antigens or treated to induce expression. The positive control can also serve as a reference control (see below)
* **Reference Control**: A reference control is used to demonstrate consistency between experiments. A positive sample can be stained over a period of time to establish a reference range to reflect the inherent variability of the assay. Identical aliquots can then be stored and analysed with each test run. If the number of aliquots required for the duration of the study exceeds what is feasible from a single sample, then a reference range can be established for multiple samples, as and when they are needed, throughout the duration of the study. Any deviation from the reference range is indicative of potential inconsistencies in the assay procedure on that run.
* **Fluorescence-Minus-One (FMO) Control**: Here, a sample is stained with all the antibodies in the panel, except for one, excluding a different one each time. This is a gating control used to define positive and negative populations. This is important when antigen expression is not clearly bimodal. An FMO control also identifies any fluorochrome spill over into the empty channel from neighbouring channels.
* **Isotype Control**: An isotype control uses an antibody of the same isotype as the test antibody but is targeted to an irrelevant antigen. Historically an isotype control was the control to determine antibody specificity and set gates. However, its effectiveness is based on assumptions for which there are no clear answers and so it introduces another experimental variable. For this reason, it is no longer recognised as an adequate control for specificity or gating but can effectively be used to gauge cell ‘stickiness’.
* **Isoclonic Control**: This control determines whether the signal observed is specific antibody-antigen mediated binding or non-specific fluorochrome-mediated binding. It consists of a mixture of fluorochrome-conjugated antibody and an excess amount of the same unconjugated antibody in a competitive binding setup. Specific binding is outcompeted whilst non-specific binding is not.

All controls, where relevant, should be run during the validation phase of a Flow Cytometry panel. Thereafter, the positive/reference and negative controls are essential to each assay run, whilst the other controls can be included where necessary. For example, a FMO control used to guide gating for only those markers that are less well characterised or do not display a bimodal staining pattern. |
| Precision*Precision is the closeness of replicate determinations. This parameter can be further subdivided into intra-assay precision and inter-assay precision.* *Measure precision using a minimum of five determinations.*Precision helps identify the variability or spread of the data. Precision for rare populations is often poor and may require more replicates to identify. Precision experiments should use an identical sample type to the one used in the study. The assessment of intra- and inter-assay precision is mandatory. Depending on the set-up of the laboratory and research team there are other variables that may need to be considered, as detailed below.* **Intra-assay**: This determines variability **within** the assay. A minimum of five tubes of an identical sample, should be assayed identically. If an antibody mastermix is routinely used to stain all cells within an assay, then a mastermix should also be used here.
* **Inter-assay**: This determines variability **between** assays. A minimum of five sample aliquots need to be stored. These are then assayed identically at different times which can be days or months apart, ideally to mimic the study design where possible.
* **Inter-operator**: This determines variability between ‘operators’ i.e., the people conducting the assay. If only one operator is conducting the study analysis, then this is not applicable. However, if multiple operators are needed, their performance will need to be assessed. If big variation is observed, this can be addressed with extra training, clearer standard operating procedures, or adaptation of the method.
* **Inter-analyst**: This determines variability between data analysts. If only one analyst is conducting the data analysis or a fixed analysis template is created, then this is not applicable. Otherwise, potential variability should be assessed. See Sample Analysis in Accuracy below.
* **Instrument**: This determines inherent variation within the instrument during an assay run. It can form part of intra-assay precision, where a single tube is prepared and run on the Cytometer five consecutive times.
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| Accuracy*Accuracy is the closeness of the test results to the true value of the analyte.* Accuracy in Flow Cytometry is rather difficult to determine as the true value of the experimental output is unknown. This is compounded when analysis involves a rare cell population or a population with a previously unreported phenotype. However, careful consideration of the experiment design, starting from sample acquisition through to analysis, is essential for minimising experimental artefacts and ensuring the accuracy of the data. Every validation parameter described in this QCDcontributes to the accuracy of the assay and can be referred to here. Experimental factors that should be considered are detailed below.* **Sample Acquisition/Handling**: Validation of the pre-analytical phase is just as important as the assay itself. The type of needle used to collect blood and the anticoagulant in vacutainers can be selected to suit downstream applications. You may want to consider the effects of under-filled vacutainers, where the concentration of anticoagulant is higher.

Delays between collection and processing and storage temperature have been shown to be critical for immunological assays and so this process requires validation. See also Handling/Transport Stability in Stability below.* **Sample Processing**: It is worth considering the impact of sample processing on the populations of interest. PBMC isolation is commonly used as it excludes granulocytes (important for functional studies) and allows cryopreservation of cells for future use, longitudinal analysis, or shipping. There are many different methods for PBMC isolation with varying ease. For example, cell preparation tubes are quicker and require less skill than a conventional Ficoll gradient, and so may be better suited to ensure reproducibility between operators and/or study sites.

Red blood cell (RBC) lysis protocols may be utilised for smaller blood volumes and have added benefit of minimal sample processing, which may better preserve cell populations. However, immature red blood cells are resistant to lysis and this approach also means that samples must be analysed promptly and is incompatible with functional studies. Cryopreservation, as shown in numerous studies, can introduce a phenotypic and functional bias and its effects on the populations of interest may need to be considered. Any steps to mitigate such effects, for example by optimisation of the freezing process, can be considered here also. If cell sorting is required, thought needs to be given as to whether FACS or magnetic-bead sorting (positive or negative selection) is more appropriate for downstream applications. See also Processing Stability in Stability below.* **Staining Procedure**: Accuracy of the staining protocol can be measured in different ways. For example, cells of interest (such as purified, stimulated or tetramer positive) can be spiked into a pool of irrelevant cells in varying numbers and the sensitivity of the protocol in detecting changes in cell numbers can be assessed. Also assessing antibody staining singly first and then within the combined panel, can help identify any potential hindrance of antibody binding within the panel. Compensation controls, to correct for fluorescence spill over, must be included to ensure accuracy of the data. See also Specificity below.
* **Sample Analysis**: Gating of populations can be rather subjective. If multiple analysts are involved, establishing a fixed analysis template and/or setting clear instructions can help guide consistent gating. Descriptions of staining patterns, such as dim, moderate, bright can be used for clarity.

Gate exclusion of experimental artefacts such as cell doublets (FSC-A vs FSC-H plot) and dead cells (viability dye) can increase the accuracy of the generated data. Time plots can be used to exclude spurious events during the assay run. Also, post-acquisition compensation may be necessary, for example if compensation beads were used instead of stained cells. See also FMO Control in Characterisation of Controls above. |
| **Calibration (where appropriate)***Calibration is the relationship between the experimental value and the analytical concentration.*There are no metrological standards that can be used for fluorescence calibration. Calibration also applies to the Cytometer. It is essential to run performance checks each time the Cytometer is used, to monitor the optical alignment, fluidics, laser power and PMT voltages. Fluorescent beads for example, BD CS&T beads are commonly used to track performance and calibrate BD Cytometers. |
| Specificity*Specificity is the ability of an analytical method to differentiate the analyte in the presence of other constituents in the sample.*Whilst the manufacturer may provide specificity data, it is the end users responsibility to determine the specificity of an antibody in their hands. Several factors can aid the specific binding of an antibody. * **Antibody Selection:** An antibody may bind to a common epitope on different proteins. If that is believed to be the case, then a different antibody clone can be used. Choosing a monoclonal over a polyclonal antibody can also help to increase specificity.
* **Antibody Titration:** The use of an excess concentration of antibody, increases the chances of non-specific binding and so titration of the antibody can circumvent this.
* **Fc Receptor Blocking:** Antibodies may bind non-specifically to certain cell types through their Fc ends. This is a particular problem in myeloid cells which professionally bind antibodies through their Fc-receptors. The addition of an Fc-receptor blocker can help alleviate this.
* **Dead Cell Exclusion:** Dead cells have compromised membrane integrity and are inherently sticky. Gating out of dead cells with a viability dye, or more crudely, courtesy of their low FSC/SSC profiles can increase the accuracy of the data.

To determine the correct antigen binding of an antibody, gene knock-out cells, cell lines known to be negative for the antigen or intrinsic antigen negative cells in a mixed cell sample (such as PBMC or bone marrow etc; see Negative Control in Characterisation of Controls above) can be used. For more novel antigens, more complex biochemical approaches can also be used such as immunoprecipitation followed by electrophoresis and Mass Spectrometry or Western blotting.  |
| Stability*Stability is a function of the storage conditions of the analyte.**Stability experiments should address standards, control samples, test samples, and key test reagents.* *Stability experiments on test samples should reflect situations likely to be encountered during actual sample handling i.e., from being taken from the patient, sample transit, short and long term storage at the intended temperature, and also freeze-thaw cycles if appropriate.*Stability experiments should, as far as possible, reflect the conditions that reagents and samples will be subjected to during the study and will identify acceptable stability limits.For reagents and kits, it is sufficient to use the manufacturer’s recommendations for storage and expiration date. It is important however to evidence temperature monitoring for the duration of the study (see UoB-CRL-SOP-002 Laboratory Analysis for further information). When *in house* buffers/reagents are required, their stability should always be assessed, and an expiration date assigned.For samples, the following factors need to be considered and stability data compared back to the baseline sample: * **Handling/Transport Stability:** Cells inevitably start to deteriorate as soon as they are removed from the body. Transport to the lab (local, national or international), temperature and time to processing all need to be considered. If a sample requires shipping, how will that affect the populations of interest and will temperature monitoring or pre-processing be necessary? For posted samples, posting a sample back to the lab is a good way to assess stability. See also Sample Acquisition/Handling in Accuracy above.
* **Processed Stability:** Samples can be processed in many ways and the chosen method should be dictated by the downstream applications. PBMC isolation and cryopreservation is compatible for longer term storage, shipping, and functional assays. In the medium term, whole blood stabilisation products can be used to fix antigens for a couple of weeks, however these are only suited to phenotypic analysis. In the shorter term, RBC lysis can be used after a couple of days. See also Sample Processing in Accuracy above.
* **Storage Stability:** For RBC lysis protocols, the number of days that a blood collection tube can be left and at what temperature, before lysis and analysis, needs to be considered. If cryopreservation is used, deterioration with time and during the freeze/thaw process also needs to be assessed. Determination of long and short term stability, may impose time constraints and delay the start of a study. It may therefore be necessary to validate stability *in study*.
* **Post-staining Stability:** There may be times when it is not possible to run the samples immediately after staining and so post-staining stability can be considered. For example, how long can the cells be left for, at what temperature and should they be fixed or stored in a suitable buffer?
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| Acceptance criteria |
| *Precision - Variability for low, medium, and high concentrations should be <15% and at the limit of detection it should be <20%.* *Accuracy - Mean experimental values should be within +15% of the nominal value at the low, medium, and high concentrations and should not deviate more than +20% at the limit of detection.**Sensitivity - The lowest standard should be accepted as the limit of quantitation if the precision and accuracy are as defined above.**Specificity - should be >95%.**Acceptance criteria should be defined in the validation report.**The validation report should rationalise the acceptance of any reduced performance specifications.*  |
| Primary Data Storage |
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| Storage and management of reagents |
| *Key reagents, controls and standards should be managed and stored appropriately throughout. Try to ensure that the same lot or batch numbers of standards, control samples and key reagents are used for both the validation experiments and analyses throughout the trial. Substantial changes in any of these, or any important item of equipment, may mean that the method must be re-validated during the study.*  |
| References |
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| Appendices |
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| Prepared by: |
| Name: | Signature: |
| Function: |
| Date (dd-mmm-yyyy): |
| Approved by: |
| Name: | Signature: |
| Function: |
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