

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

Standard Operating Procedure

Training Requirements	
Initial/ Refresher Training	<ol style="list-style-type: none"> 1. All analysts performing Quanterix Multiplex ELISAs must read and acknowledge this SOP and all SOPs detailed in the related documents section in Q-Pulse. 2. All analysts performing Quanterix Multiplex ELISAs must be signed off in Q-Pulse as competent to perform. 3. Refresher training is required upon major revision of this SOP or if a trained Analyst has not conducted the method within 13 months.
Training on Revision	<ol style="list-style-type: none"> 1. All analysts performing Quanterix Multiplex ELISAs must read and acknowledge this new revision in Q-Pulse. No further training is required.

Author	Owner
Molly Glenister-Doyle	Holly Butterworth

A record of all approvers, revision history and active date is maintained within Q-Pulse and can be accessed from the document record.

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

1. List of Abbreviations

Table 1. Table of abbreviations.

CBC	Cancer Biomarker Centre
CHEMlog	Chemical Log
Concⁿ	Concentration
CSV	Comma Separated Value(s)
%CV	Coefficient of Variation
ELISA	Enzyme Linked Immunosorbent Assay
GCP	Good Clinical Practice
HRP	Horseradish Peroxidase
LIMS	Laboratory Information Management System
LLOQ	Lower Limit of Quantification
LOB	Limit of the Blank
LOD	Limit of Detection
PDF	Portable Document Format
PM	Project Manager
QC	Quality Control
%RE	Percent Recovery (actual concentration/expected concentration x 100)
SOP	Standard Operating Procedure
Std	Standard
ULOQ	Upper Limit of Quantification
VWR	VWR International Ltd (Supplier)

2. Introduction

This SOP describes the procedure for performing Quanterix SP-X ELISAs (*Formerly known as Aushon Ciraplex and referred to simply as multiplex in this document*).

(Enzyme Linked ImmunoSorbant Assay) using the Quanterix SP-X imaging system (*Formerly known as Aushon Cirascan*).

The immunoassay kit is a multiplex sandwich ELISA for the quantitative measurement of target proteins in samples. Each well of the 96-well microplate is pre-spotted with protein-specific antibodies and these antibodies capture specific proteins from the Standards and samples of interest. After unbound proteins are washed away, biotinylated detecting antibodies are added which bind to a secondary site on the target proteins. After the removal of excess detection antibody, streptavidin-horseradish peroxidase is added. HRP is an enzyme that reacts with a substrate to produce a luminescent signal that is detected by the Quanterix SP-X Imaging System. The intensity of the signal produced is directly proportional to the quantity of each protein in the Standard or sample of interest. The Cirasoft software is then used to

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

calculate the concentration of proteins of interest in the samples, using a standard curve of predetermined protein concentrations.

The multiplex ELISAs shown in Table 2 have been validated by CBC, and further details can be found in Section 22.

Table 2. List of analytes included in each multiplex ELISA covered by this SOP.

LIMS Multiplex Name	Number of Analytes	Analyte(s)
IMMUN_01	10	IFN γ , IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF α
IMMUN_02	2	GM-CSF, IL-15
IMMUN_03	1	IL-2R α
IMMUN_04	1	IL-5

3. Scope

3.1. This SOP applies to all analysts performing Quanterix Multiplex ELISA analysis.

4. Responsibilities

4.1. Analysts

4.1.1. It is the responsibility of all analysts performing Quanterix Multiplex ELISA analysis in the GCP laboratory to follow the procedure described in this SOP and report any issues directly to the process owner. If unavailable, analysts must contact their line manager.

4.2. Process Owner

The process owner is responsible for:

- 4.2.1. Ensuring the appropriate equipment documents, including this SOP, are in place and are reviewed appropriately according to SOP-1.
- 4.2.2. Resolving issues and addressing non-conformances related to the process.
- 4.2.3. Escalating issues to Senior Management if unable to resolve them.
- 4.2.4. Ensuring that the asset records are kept up to date and to facilitate appropriate training in the process.

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

4.3. Quality Assurance

4.3.1. Quality Assurance are responsible for the periodic audit of the processes within this SOP to assure they are being followed by GCP staff as requested by the process owner or head of Quality Assurance.

4.4. Senior Management

4.4.1. Senior Management is responsible for providing the resources to enable GCP compliance with this SOP.

5. Related documents

Table 3. Related Documents.

SOP-12	Laboratory Record Keeping
SOP-20	Management and Use of Reagents
SOP-21	Environmental Monitoring and Housekeeping
SOP-23	Sample Management
SOP-31	Use and Maintenance of GCP Pipettes
SOP-45	Use and Maintenance of Fridges and Freezers
SOP-46	Use and Maintenance of Temperature Monitoring Equipment
SOP-96	Use and Maintenance of the Quanterix SP-X Imaging System
SOP-115	Use and maintenance of the BioTek 50TS12 Microplate Washer
SOP-118	Use and Maintenance of Elga PURELAB Chorus 1 Complete
SOP-124	Use and Maintenance of Class II Microbiological Safety Cabinet
FRM-27	Working Concentrations of Laboratory Disinfectants
FRM-76	Quanterix Multiplex ELISA Pro forma
WKI-32	LIMS - Review, Release and Retest from Plate manager
WKI-36	LIMS – Quanterix SP-X
MISC-119	Cleaning, Waste Management and Spills
RPT-34	Validation of Aushon Ciraplex ELISA for Immune-related Analytes
RPT-36	Validation of the BioTek 50TS12 Microplate Washer
RSK-4	Production and Use of Distel
RSK-5	Ethanol
RSK-13	Blood and Plasma Handling
RSK-19	Use and Disposal of Sharp Objects
RSK-34	Quanterix Multiplex ELISA Risk Assessment
RSK-53	Risk Assessment for Working in a Containment Level 2 Laboratory
RSK-87	Use and Maintenance of the BioTek 50TS12 Microplate Washer
DOC3	Code of Practice for Working with Biological Agents and / or Material Which May Contain Them

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

5.1. Prior to completing the work described within this document all users must have read and acknowledged all SOPs and RSKs listed in the Related Documents section.

CRUK Manchester Institute
Cancer Biomarker Centre

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

6. Managing new shipments of ELISA kits

6.1. Upon collecting a new delivery of ELISA kits; the analyst receiving the kits is required to log them in as described in **SOP-20** and upload the kit insert/manufacturer's instructions provided to the CHEMlog record.

Note: the product code, lot number and expiry date of each individual kit component does not need to be recorded as these are covered by the kit lot number and expiry date. **Reagents and kits supplied by Quanterix may contain an expiry date in the American format MM/DD/YYYY.**

6.2. If this is not the first shipment, cross check the kit insert/manufacturer's instructions against this SOP (see section 18). If anything has changed inform the process owner so that the SOP can be updated. In the meantime, plan to conduct the work as per the kit insert/manufacturer's instructions but inform the Project Manager (PM) of the SOP deviations that will occur.

7. Preparing for an ELISA in LIMS

7.1. Create a plate plan, pick list and work list in LIMS

7.1.1. **Plate plan:** print and append to the project laboratory notebook. The plate plan must be used to set up the assay and to make note of any loading discrepancies or observations encountered e.g. haemolysis or clotting.

7.1.2. **Pick list:** use list to find the location of samples to be analysed.

7.1.3. **Work list:** upload work list to the Cirascan imager for sample analysis.

7.2. If LIMS is unavailable, a plate plan and work list cannot be created. In this case, manually create a plate plan on the Cirasoft software and upload to the Cirascan imager, print a copy of the plate plan and insert into the project laboratory notebook. **Data should be added to LIMS retrospectively when next available.**

7.3. **Note:** Further details can be found in **WKI-36**

CRUK Manchester Institute
Cancer Biomarker Centre

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

8. Materials

8.1. Materials provided within the Quanterix Multiplex ELISA Kits

- 8.1.1. 96-well plate pre-spotted with protein-specific antibodies
- 8.1.2. Lyophilised Recombinant Standards *aka Calibrators* (1 vial/protein)
- 8.1.3. Sample diluent*
- 8.1.4. Standard diluent (excluded from some kits, where sample diluent is used in its place)
- 8.1.5. Biotinylated Antibody Reagent
- 8.1.6. Streptavidin-HRP Reagent
- 8.1.7. SuperSignal® Stable Peroxide Solution
- 8.1.8. SuperSignal® Luminol/Enhancer Solution
- 8.1.9. Wash Buffer (25x)
- 8.1.10. Microclimate® Lid

***Note:** multiple types of sample diluent may be provided within a kit to be used with different types of sample. **Please be aware that assay validation may have been carried out on only one sample type using one diluent type (see section 22). This must be checked for each assay.**

8.2. Additional Materials Required

- 8.2.1. Vortex 1.5mL Eppendorf tubes (*e.g.* autoclaved, from Lab Services)
- 8.2.2. Disposable reagent reservoirs
- 8.2.3. Grade A measuring cylinder (borosilicate, 2L/1L capacity)
- 8.2.4. Disposable filter pipette tips (*e.g.* StarLab TipOne Filter Tips)
- 8.2.5. 96 well deep well plate (*e.g.* Nunc™ #260251)
- 8.2.6. Flat-bottom 96-well plate (*e.g.* Corning® Costar #3590)
- 8.2.7. UltraPure water ($\geq 18.0\text{M}\Omega\cdot\text{cm}$) (*e.g.* bottles supplied by VWR or dispensed from the Elga PURELAB Chorus 1 Complete)
- 8.2.8. Absorbent paper *e.g.* Blue Roll

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

9. Equipment

- 9.1. Quanterix SP-X Imaging System (*Formerly known as the Aushon Cirascan Imaging System*)
- 9.2. Laboratory computer MAC2062 (connected to the Quanterix SP-X Imaging System)
- 9.3. Plate Shaker (*i.e.* EQUIP-029 or equivalent orbital plate shaker)
- 9.4. Automated Plate Washer (*i.e.* EQUIP-512)
- 9.5. Manual pipettes (*i.e.* Gilson Pipetman P20, P100, P200, P1000)
- 9.6. Electronic pipettes (*i.e.* Starlab Single channel 200 μ L (E200), 8 channel 300 μ L(8-Ch 300))
- 9.7. Radio controlled digital clock
- 9.8. Temperature monitoring equipment (*i.e.* temperature data logger or calibrated thermometer)

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

10. Assay Procedure

- 10.1. Follow this method in conjunction with the appropriate kit insert and **FRM-76**. Record details of the kit insert (*i.e.* booklet) in **FRM-76**.
- 10.2. Some parts of this method are specific to a kit insert and are subject to change depending on the batch *e.g.* reconstitution of recombinant standards. These will be **highlighted in the text (in orange) and details recorded in FRM-76.**
- 10.3. The acceptable temperature range for this assay is 20-25° C. Check that the laboratory is within this temperature range before proceeding with the assay using a temperature data logger or equivalent placed on the laboratory bench. Record the equipment number of the temperature logger on FRM-76
- 10.4. Any deviation from the incubation times stated in sections 10.14, 10.16, 10.18 and 10.20 must be recorded in the comments section, and flagged to the PM.
- 10.5. ***IMPORTANT: when washing the plate using the automated plate washer, ensure that the volume of liquid in the waste bottle does not go above the level specified on the side of the bottle. See SOP-115 for more details.***

10.6.	Prepare the Plate Washer
	Before using the plate washer perform the DAY_RINSE maintenance detailed in SOP-115 and record in FRM-76
10.7.	Equilibrate Reagents
	Bring all kit reagents to room temperature (20-25°C) for at least 15 minutes

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

10.8.	Make Wash Buffer
	<ul style="list-style-type: none"> • Dilute 25X Wash Buffer to 1X with UltraPure Water in a plate washer bottle and invert to mix. Record volumes in FRM-76. <ul style="list-style-type: none"> ○ <i>Note: if precipitate can be seen in the 25X wash buffer place the wash buffer in a warm water bath (20-30°C) for up to one hour to re-dissolve crystallised salts. Some precipitate may remain however the wash buffer can still be used.</i> • Attach the wash buffer to the plate washer and run Quick>Prime (system) using 60mL of buffer.

10.9.	Prepare Samples
	<ul style="list-style-type: none"> • Thaw samples at room temperature (20-25°C) on the laboratory bench. A roller mixer may be used for this step. • Once at room temperature, centrifuge the samples for 5 minutes @ 2000g if detecting soluble analytes (do not centrifuge if detecting insoluble analytes). <p style="text-align: center;"><i>Repeat freeze thaw cycles are NOT recommended. Samples should be used within 30-120 minutes of removal from storage and should be returned to freezer storage as soon as possible.</i></p> <p style="text-align: center;"><i>Sample conditions, such as Haemolysis or clotting, must be noted in the appropriate laboratory notebook at time of use and added to LIMS so this can be reported against the results (see SOP-23 for more details).</i></p> • Dilute samples as described in Table 4.

Table 4. Sample Dilution Information.

Multiplex Name	Dilution Factor	Diluent Type
IMMUN_01	1:2	Sample Diluent
IMMUN_02	1:2	Sample Diluent
IMMUN_03	1:10	Sample Diluent
IMMUN_04	1:2	Sample Diluent

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

10.10.	Reconstitute Standards and Prepare Standard Curves									
	<ul style="list-style-type: none"> Reconstitute lyophilised standards with appropriate diluent as described in Table 5 using the volume of diluent specified on the vial label or kit insert and record the volumes used and time created on FRM-76. <p style="text-align: center;">Table 5. Standard Dilution Information.</p> <table border="1" style="margin-left: auto; margin-right: auto;"> <thead> <tr> <th>Multiplex Name</th> <th>Diluent Type</th> </tr> </thead> <tbody> <tr> <td>IMMUN_01</td> <td>Sample Diluent</td> </tr> <tr> <td>IMMUN_02</td> <td>Sample Diluent</td> </tr> <tr> <td>IMMUN_03</td> <td>Sample Diluent</td> </tr> <tr> <td>IMMUN_04</td> <td>Sample Diluent</td> </tr> </tbody> </table> <ul style="list-style-type: none"> Mix on the roller mixer for at least 15 minutes and use within 1 hour. Prepare the standard curve (see section 19) using the diluent specified in Table 5. 	Multiplex Name	Diluent Type	IMMUN_01	Sample Diluent	IMMUN_02	Sample Diluent	IMMUN_03	Sample Diluent	IMMUN_04
Multiplex Name	Diluent Type									
IMMUN_01	Sample Diluent									
IMMUN_02	Sample Diluent									
IMMUN_03	Sample Diluent									
IMMUN_04	Sample Diluent									
10.11.	Prepare Quality Controls (QCs)									
	<ul style="list-style-type: none"> QCs are produced from the re-constituted standards provided by the manufacturer. Prepare high and low QCs as detailed in Appendix IV (see section 21) using the same diluent that is used to prepare the standard curve (see Table 5). <i>If QC values are not currently defined or if you are using alternative concentrations, record the QC concentration in FRM-76.</i> 									
10.12.	Prepare Microclime® Lid and Plate									
	<ul style="list-style-type: none"> Prepare the Microclime® lid (see section 20). Wash the plate prior to use using the ‘CIRAPLEX’ protocol on the BioTek 50TS12 Microplate washer. Flick the remaining wash buffer into the sink and blot the plate firmly on blue roll. Position A1 may be marked with a white marker pen or a small section of white tape to ensure the plate is in the correct orientation prior to loading. 									

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

10.13.	Add Standards, QCs and Samples to the Plate
	<ul style="list-style-type: none"> • Add 50µL of standards, QCs and samples to the plate in duplicate according to the plate plan. • <i>Note #1: high and low QCs are run at the start, middle and end of the plate in duplicate.</i> <ul style="list-style-type: none"> ○ High QCs: wells A3, A4, D7, D8, G11 & G12 ○ Low QCs: wells B3, B4, E7, E8, H11 & H12 ○ One set of duplicate high and/or low QC samples may be omitted from the plate if needed to allow the analysis of all samples from one patient profile, this must be recorded in FRM-76. • <i>Note #2: standards and QCs must be vortexed before adding to the plate (do not vortex samples before adding to plate)</i>
10.14.	Incubate the Plate for 2 hours and then Wash the Plate
	<ul style="list-style-type: none"> • Incubate the plate for 2 hours at room temperature (20-25°C) on the plate shaker set between 400 and 600 RPM. • After 2 hours wash the plate using the 'CIRAPLEX' protocol on the BioTek 50TS12 Microplate washer. • Flick the remaining wash buffer into the sink and blot the plate firmly on blue roll.
10.15.	Add Biotinylated Antibody Reagent
	Add 50µL of Biotinylated Antibody Reagent to all wells.
10.16.	Incubate the Plate for 30 minutes and then Wash the Plate
	<ul style="list-style-type: none"> • Incubate the plate for 30 minutes at room temperature (20-25°C) on the plate shaker set between 400 and 600 RPM. • After 30 minutes wash the plate using the 'CIRAPLEX' protocol on the BioTek 50TS12 Microplate washer. • Flick the remaining wash buffer into the sink and blot the plate firmly on blue roll.
10.17.	Add Streptavidin-HRP Reagent
	Add 50µL of Streptavidin-HRP Reagent to all wells.

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

10.18.	Incubate the Plate for 30 minutes and then Wash the Plate
	<ul style="list-style-type: none"> • Incubate the plate for 30 minutes at room temperature (20-25°C) on the plate shaker set between 400 and 600RPM. • After 30 minutes wash the plate using the ‘CIRAPLEX’ protocol on the BioTek 50TS12 Microplate washer. • Flick the remaining wash buffer into the sink and blot the plate firmly on blue roll.

10.19.	Prepare SuperSignal Substrate
	Combine equal volumes of Stable Peroxide and SuperSignal Luminol enhancer (e.g. add 4mL of of Stable Peroxide to 4 mL of SuperSignal Luminol enhancer) in a 15mL falcon tube and invert gently to mix.

10.20.	Add SuperSignal Substrate and Read the Plate
	<ul style="list-style-type: none"> • Add 50µL of SuperSignal Substrate to all wells. • Read the plate on the Quanterix SP-X Imaging System (see SOP-96). <p><i>The plate should be read within 2-4 minutes of substrate addition (optimal). The plate must be read within 10 minutes. Plates read after 10 minutes will need to be flagged as deviating from this SOP in LIMS. Ensure that well A1 is in the top left hand corner when loading the plate onto the imager. For further details see SOP-96.</i></p> <ul style="list-style-type: none"> • Record the camera temperature and raw data file name(s) in FRM-76.

11. Audit Trail Report

- 11.1. Open the ‘SPX Audit Trail Viewer’ software located on the laboratory computer desktop.
- 11.2. Go to **File > Open Analysis File** and open the appropriate .kit file
- 11.3. This will then display the Audit Trail (**Logs > Audit Trail**) or the Imager Event Log (**Logs > Imager Event Log**).
- 11.4. Export the Audit Trail and Imager Event Log by clicking **File > Export Audit Trail to PDF** or **File > Export Imager Log to PDF**, respectively.
- 11.5. Store these in the in the following location
 \\vapp2\GCLP\4.ELISA\16.Quanterix _Audit Trails and Imager Event Logs.

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

12. Data Analysis

- 12.1. If any outlying points require exclusion from the standard curve within the Cirasoft software according to the acceptance criteria stated in section **17.1**, comprehensive details should be recorded on **FRM-76**. An approval signature is required from the process owner or a member of senior management to authorise the assay. This will need to be reported against the results in LIMS.

13. Data Export and Storage

- 13.1. **Data processed in LIMS** (*further details can be found in **WKI-36***)
- 13.1.1. Export all files to the following location prior to analysis
[\\vapp2\GCLP\ Temporary LIMS data].
- 13.1.2. Following analysis the data should be classified as accepted or rejected and the files moved accordingly to the locations detailed in **WKI-36**.
- 13.2. **Data processed outside of LIMS**
- 13.2.1. Export all files to \\vapp2\GCLP\Temporary Data.
- 13.2.2. Plate layouts should be filed in \\vapp2\GCLP\ projects\ CEPXXX (where XXX is the project number).
- 13.3. If the plate has been read multiple times (for example if the plate was read upside down the first time) details must be recorded in **FRM-76** and all data must be saved to a secure location.

14. Complete LIMS Test

*Further details can be found in **WKI-36***

- 14.1. Populate experimental information.
- 14.2. Complete first review.
- 14.3. Add any test comments (*e.g. if there was insufficient sample or there was a temperature excursion*).

15. First and Second Review

- 15.1. The first review should be completed by the **analyst who carried out the procedure**.

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

- 15.2. The second review should be completed by an **independent reviewer who is competent to QC check this procedure.**
- 15.3. Both first and second reviewers must check the following:
- 15.3.1. Check that the paperwork has been completed appropriately (see **SOP-12** for details).
- 15.3.2. Check that incubation times and temperatures are within specification (see section 10.4 for details). **Note:** if they are not in specification then this should be recorded in the comments section and transcribed into LIMS (if applicable) as a deviation from **SOP-60**, and a non-conformance raised.
- 15.3.3. Check that all raw (KIT File) and derived (LIMS CSV File, Excel Report and PDF Report) data files have been stored in the appropriate secure location(s).
- 15.3.4. Check that the correct plate layout is attached to **FRM-76**.
- 15.3.5. Check that any sample conditions noted during analysis e.g. haemolysis or clotting have been updated in LIMS.
- 15.3.6. The second reviewer must also complete the following:
- 15.3.6.1. Check that any points excluded from analysis (highlighted in red in the PDF report stored in the appropriate secure location) are detailed in **FRM-76** and that they have been signed off by the process owner or a member of senior management. Any additional excluded values should be raised as a non-conformance and will need to be investigated by interrogating the audit trail report as described in section 11.
- 15.3.6.2. Check that the user ID stated on the PDF report matches the analyst stated on **FRM-76**.
- 15.3.6.3. Review and release data as described in **WKI-32**.

16. QC Range Setting

- 16.1. QC ranges automatically default to 20% of the expected concentration prior to validation and sample analysis.

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

- 16.2. QCs will be monitored continually during sample analysis to determine assay variability and if deemed appropriate QC ranges may be adjusted to accommodate the variability of the assay.
- 16.3. In such cases it may be necessary to increase or decrease the QC range. *For example, if the QCs are routinely falling within 10% of the expected concentration you may wish to reduce the QC range or if the QCs are regularly 21% of the expected concentration you may wish to increase the QC range to account for the inherent variability of the assay.*
- 16.4. QC ranges cannot be increased to more than 50% of the expected concentration. *This would be project specific, where it would depend on the scientific question you are asking e.g. if you are looking for large changes a larger QC range may be acceptable and if you are looking for small changes then a high QC range would not be acceptable.*
- 16.5. QC ranges can only be adjusted after analysis of at least 5 assay plates and discussion with the ELISA SME and members of management. Suitable justification will be given and changes detailed in a new revision of this document and a note to file where appropriate.

17. Assay Acceptance Criteria

Note: if any assay fails the standard curve or QC acceptance criteria then data must to be reported to the sponsor with a flag detailing the assay failure.

17.1. Calibration Curve Acceptance Criteria

Table 6. Calibration Curve Acceptance Criteria

R²	The standard curve must have an R² value >0.985 to pass.
%CV	The %CV between standard duplicates that are within the quantification range of the assay (see section 23) must be <15%, or <20% for the standard on or above the LLOQ, or on or below the ULOQ.
%RE	The spike recovery (%RE) of standards that are within the quantification range of the assay (see section 23) must be 80-120% of the nominal concentration value.

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

17.1.1. Editing of the Calibration Curve

- 17.1.1.1. Masking is defined as removal of a calibrator point from the standard curve regression while the calibrator value remains available in the system.
- 17.1.1.2. The standard curve consists of seven calibrator standards and one blank calibrator standard which is used as an anchor point when fitting the curve.
- 17.1.1.3. Only one outlying calibrator standard may be masked from the calibration curve (single or duplicate) to allow the calibration curve to pass the acceptance criteria stated in Section 17.1.
- 17.1.1.4. In the event of a standard with a %CV outside of the acceptance criteria (Table 6) a single or duplicate standard may only be masked if it is outside of 80-120% of the expected concentration.
- 17.1.1.5. In the event of a calibrator with a %RE outside of the acceptance criteria (Table 6) this calibrator (single or duplicate) may be masked.
- 17.1.1.6. The blank calibrator standard (single or duplicate) may be masked if it is interfering with the fit of the standard curve. This does not contribute to the allowance stated in Section 17.1.1.3 and does not need to be signed off as detailed in 17.1.1.8, however should still be recorded on FRM-76.
- 17.1.1.7. More calibrator standards may be masked from the calibration curve (single only) than stated in Section 17.1.1.3, for example if there are multiple calibrator standards with high %CV values. However, the calibration curve must still be reported as a failure.
- 17.1.1.8. An approval signature is required from the Process Owner or Project Manager to authorise any editing of the calibration curve. This signature must be recorded on **FRM-76** and the page signed off by the analyst completing the work. Any curve editing must be reported against the results in LIMS.

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

17.1.1.9. If the outlier removed is at the ULOQ or LLOQ calibrator standard, then the quantification range must be reduced when reporting the data to the sponsor.

CRUK Manchester Institute
Cancer Biomarker Centre

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

17.2. QC Acceptance Criteria

17.2.1. A QC fails if the %CV of replicates is >15% and/or the QC value is not within the QC limits specified section 21.

17.2.2. *Note: if QC limits are not specified in section 21, then QC values must be within 20% of the expected value.*

Table 7: QC Acceptance Criteria

Number of duplicate QC	Number of Failures	Assay Pass / Fail	Action
3 High & low	No failure	Pass	None
	1 duplicate <i>(i.e. 1 high <u>or</u> 1 low QC failure)</i>	Pass	None
	2 duplicates in different concentration ranges <i>(i.e. 1 high <u>and</u> 1 low QC failure)</i>	Pass	None
	2 or more duplicate failures in the same concentration range <i>(i.e. 2 or more high or low QC failures)</i>	Fail	Assay Repeated

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

17.2.3. If one QC has been omitted from the plate, as detailed in section 10.13, then use the following acceptance criteria (Table 8):

Table 8: QC Acceptance Criteria with five QCs

Number of duplicate QC	Number of Failures	Assay Pass / Fail	Action
3 High & 2 Low	No failure	Pass	None
	1 High duplicate	Pass	None
	1 Low duplicate	Fail	Assay Repeated
	2 High duplicates	Fail	Assay Repeated
2 High & 3 Low	No failure	Pass	None
	1 Low duplicate	Pass	None
	1 High duplicate	Fail	Assay Repeated
	2 Low duplicates	Fail	Assay Repeated

17.3. Sample Acceptance Criteria

17.3.1. Samples must have a CV <30% if they are within the quantification range of the assay (see section 23).

17.3.2. If a sample fails this acceptance criterion, the sample must be reported as a CV failure and repeated only once.

17.3.3. A %CV sample failure does not invalidate the whole assay.

17.3.4. If a sample has a %CV failure and is below the LLOQ specified in section 23 then it is a decision to be made by the project manager whether it is necessary to repeat the analysis. If repeated, both results must be reported to the sponsor.

**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

18. Appendix I (Managing new shipments of ELISA kits)

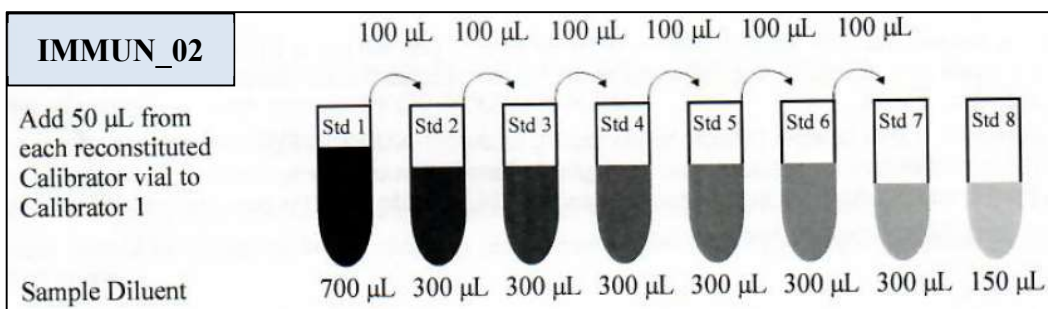
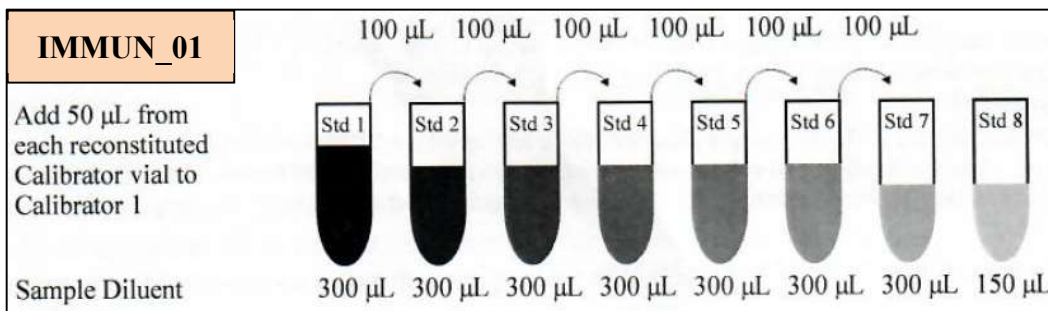
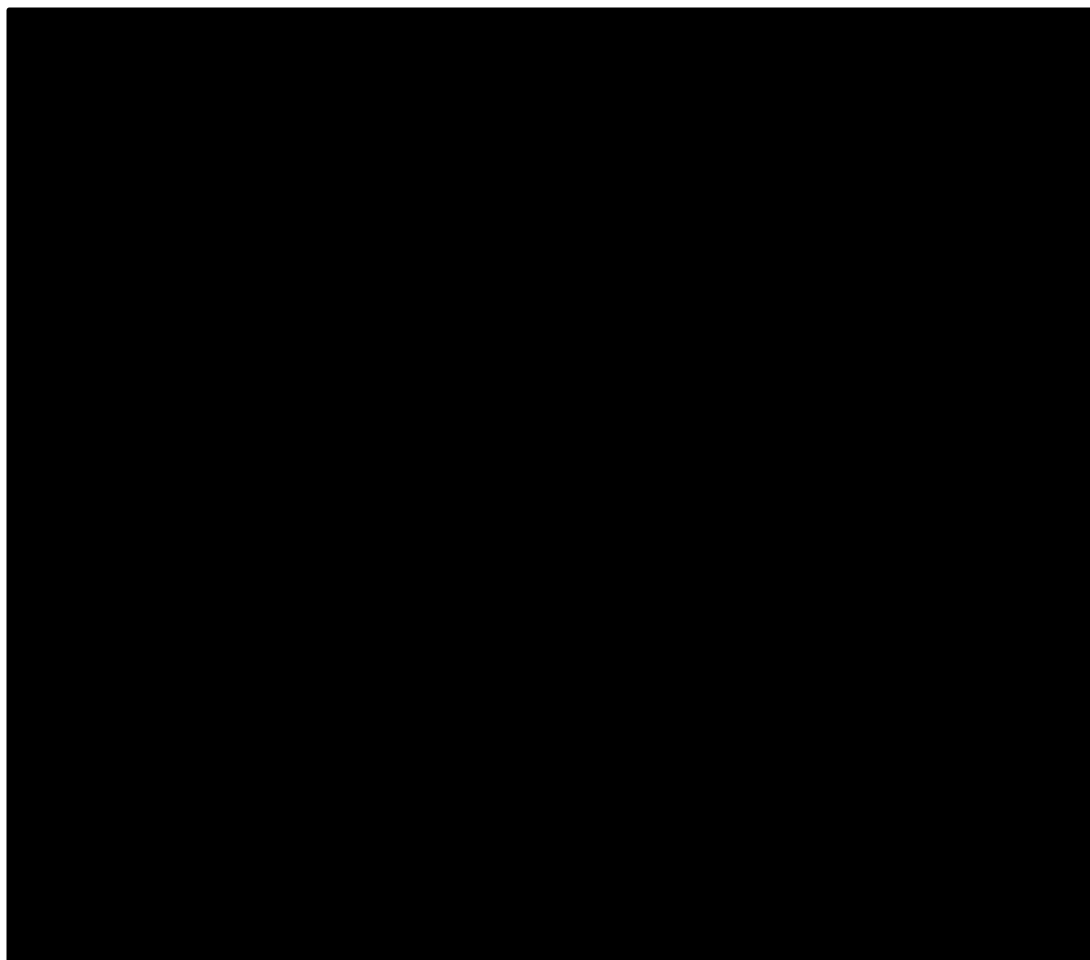
On receiving a new batch of ELISA kits, the kit inserts must be checked against this SOP to ensure the following sections have not been changed:

- 18.1. **Sample dilution factor** (see section 10.9)
- 18.2. **Sample diluent type** (see section 10.9)
- 18.3. **Standard diluent type** (see section 10.10)
- 18.4. **Standard curve preparation** (see section 19)
- 18.5. **Incubation times** (see sections 10.14, 10.16 and 10.18)

CRUK Manchester Institute
Cancer Biomarker Centre

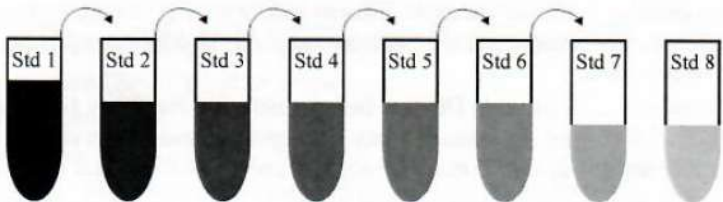
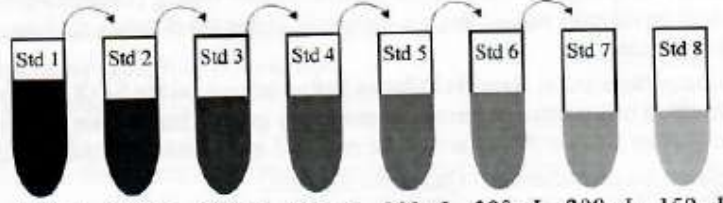
Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

19. Appendix II (Standard Curve Preparation)



**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

IMMUN_03	300 μ L, 100 μ L, 100 μ L, 100 μ L, 100 μ L, 100 μ L
Add 50 μ L from the reconstituted Calibrator vial to Calibrator 1	
Sample Diluent	750 μ L, 300 μ L, 300 μ L, 300 μ L, 300 μ L, 300 μ L, 100 μ L, 150 μ L
IMMUN_04	100 μ L, 100 μ L, 100 μ L, 100 μ L, 100 μ L, 100 μ L
Add 50 μ L from the reconstituted Calibrator vial to Calibrator 1	
Sample Diluent	750 μ L, 300 μ L, 300 μ L, 300 μ L, 300 μ L, 300 μ L, 300 μ L, 150 μ L

20. Appendix III (Preparation of the Microclime® Lid)

- 20.1. Remove Microclime lid from packing material and position so that the filling trough and corners are facing up.
- 20.2. Using a P1000 pipette, slowly and carefully dispense 4 lots of 1mL UltraPure water into the filling trough on the top of the long edge of the lid.
- 20.3. Repeat this procedure for the filling trough on the bottom of the long edge of the lid so that a total of 8mL has been added to the lid.
- 20.4. When properly filled, there will be a gap in the middle of the felt that is not wetted.



**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

21. Appendix IV (Preparation of QCs and QC Acceptance Criteria)

Multiplex Name	Analyte	QC Standard			High QC					Low QC				
		Volume of reconstituted protein (µL)	Volume of diluent (µL)	Final protein concentration (pg/mL)	Volume of QC Standard (µL)	Volume of diluent (µL)	Final protein concentration (pg/mL)	Lower Limit (pg/mL)	Upper Limit (pg/mL)	Volume of QC Standard (µL)	Volume of diluent (µL)	Final protein concentration (pg/mL)	Lower Limit (pg/mL)	Upper Limit (pg/mL)
IMMUN_01	hIFNg	50	300	50	250	750	12.5	10	15	50	950	2.5	2	3
	hIL1a	50		1800			450	360	540			90	72	108
	hIL1b	50		200			50	40	60			10	8	12
	hIL2	50		200			50	40	60			10	8	12
	hIL4	50		200			50	40	60			10	8	12
	hIL6	50		114			28.5	22.8	34.2			5.7	4.56	6.84
	hIL8	50		400			100	80	120			20	16	24
	hIL10	50		200			50	40	60			10	8	12
	hIL12p70	50		1200			300	240	360			60	48	72
	hTNFa	50		400			100	80	120			20	16	24
IMMUN_02	hGMCSF	50	700	6000	250	750	1500	1200	1800	50	950	300	240	360
	hIL15	50		800			200	160	240			40	32	48
IMMUN_03	hIL2Ra	50	750	800	250	750	200	160	240	50	950	40	32	48
IMMUN_04	hIL5	50	750	200	100	300	50	40	60	20	380	10	8	12



**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

22. Appendix V (Validation Information)

Validation Parameters													
✓ = parameter assessed, ✗ = parameter not assessed													
Multiplex Name	Plan (PLN-X)	Report (RPT-X)	Sample Type (s) Validated	Freeze Thaw Stability	Sample Stability (-80°C)	LLOQ and ULOQ	LOB	LOD	Parallelism	Dilution Linearity	Inter- and intra-assay precision	Accuracy	Hook Effect
IMMUN 01	70	34	P	✗	✓	✓	✓	✗	✗	✓	✓	✓	✓
IMMUN 02	70	34	P	✗	✓	✓	✓	✗	✗	✓	✓	✓	✓
IMMUN 03	70	34	P	✗	✓	✓	✓	✗	✗	✓	✓	✓	✓
IMMUN 04	70	34	P	✗	✓	✓	✓	✗	✗	✓	✓	✓	✓

P, Plasma, S, Serum.



**CRUK Manchester Institute
Cancer Biomarker Centre**

Document Number	SOP-60
Title	Quanterix Multiplex ELISA SOP

23. Appendix VI (Assay Quantification Ranges)

Multiplex Name	Analyte	Report	Report Version	LLOQ		ULOQ	
				Conc ⁿ (pg/mL)	Equivalent Standard	Conc ⁿ (pg/mL)	Equivalent Standard
IMMUN_01	hIFNg	RPT-34	1	0.195	Std 5	58	15% above Std 1
	hIL1a		1	1.758	Std 6	2070	15% above Std 1
	hIL1b		1	0.195	Std 6	230	15% above Std 1
	hIL2		1	0.195	Std 6	230	15% above Std 1
	hIL4		1	0.195	Std 6	230	15% above Std 1
	hIL6		1	0.111	Std 6	131	15% above Std 1
	hIL8		1	1.563	Std5	460	15% above Std 1
	hIL10		1	0.195	Std 6	230	15% above Std 1
	hIL12p70		1	4.688	Std 5	1200	Std 1
	hTNFa		1	1.563	Std 5	460	15% above Std 1
IMMUN_02	hGMCSF	RPT-34	1	5.859	Std 6	6000	Std 1
	hIL15		1	12.500	Std 4	920	15% above Std 1
IMMUN_03	hIL2Ra	RPT-34	1	6.25	Standard 6	920	15% above Std 1
IMMUN_04	hIL5	RPT-34	1	0.391	Between Std 5 & 6	50	Std 2