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PhD IN BIOMEDICAL SCIENCES AND ONCOLOGY
XIX CYCLE

THESIS TITLE:

Drug development process: from R&D to BQC.

Biomarkers determination in clinical studies: method development & validation

Viral clearance: viral titration method development & validation

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2. Drug development process

Drug development is a very long and complex process that bring a new pharmaceutical drug to the market. It takes around 14 years to be completed and it is very expensive for pharma companies.

The process mainly consists in five different phases: discovery and development, pre-clinical research, clinical research, submission for approval to Health Authorities and, finally, post-market safety monitoring.

Research for a new drug begins in the laboratory with discovery. At this stage of the process, thousands of compounds may be potential candidates to be developed as a medical treatment. After early testing, however, only a small number of them look promising and call for further study. Once researchers identify a promising compound for development, they conduct specific experiments to gather information on ADME (Absorption, Distribution, Metabolism and Excretion), mechanism of action, dosage, side effects, etc…

After the discovery part, drugs undergo to a long series of studies divided into Pre-clinical research, with studies *in vitro* and *in vivo* studies, and Clinical research which is conducted in humans. These studies are performed to understand the properties of the active substance, to answer basic questions about safety and toxicokinetics of the molecules on complex living organisms, and to quantify the relationship between the possible risks and benefits due to its assumption. Before testing a drug in human, researchers must find out whether it has the potential to cause serious harm, also called toxicity. Firstly, studies are performed *in vitro* in order to understand the characteristic of chemical molecules. Only when it is found in the laboratory that the molecule has potential therapeutic effects, it is possible to go to *in vivo* testing in animals.

In the clinical phase drugs are tested on human to make sure they are safe and effective. Clinical trials follow a typical series from early, small-scale, Phase I studies to late-stage, large scale, Phase III studies. An additional step (Phase IV) aims to monitor the safety after the commercialization of the product. During Phase I studies, researchers test a new

drug in healthy volunteers for studying safety and to select the dosage to be used. In Phase II studies, researchers administer the drug to a group of patients with the disease or condition for which the drug is being developed in order to test the efficacy and the possible side effects. Researchers design Phase III studies to demonstrate whether or not a product offers a treatment benefit to a specific population confirming efficacy and monitoring adverse reactions.

Phase IV trials are carried out once the drug or device has been approved by FDA during the Post-Market Safety Monitoring to verify the product safety and efficacy after the drug commercialization.

The latest phase of drug development process is the approval (1).

Health Authorities examine all of the submitted data related to the drug or device and make a decision to approve or not to approve it in the specific Country of interest.

After lunch on the market, Health Authorities continuous monitors drugs and devices safety once products are being used by the public.

Schematic drug development process representation is reported in *Figure 1*.

Stage 1
Drug discovery

10,000 compounds

Stage 2
Pre-clinical development

250 compounds

Stage 3
Clinical development

5 compounds

Phases

0	Effect on body
I	Safety in humans
II	Effectiveness at treating diseases
III	Larger scale safety and effectiveness
IV	Long term safety

Regulatory approval

1 compound
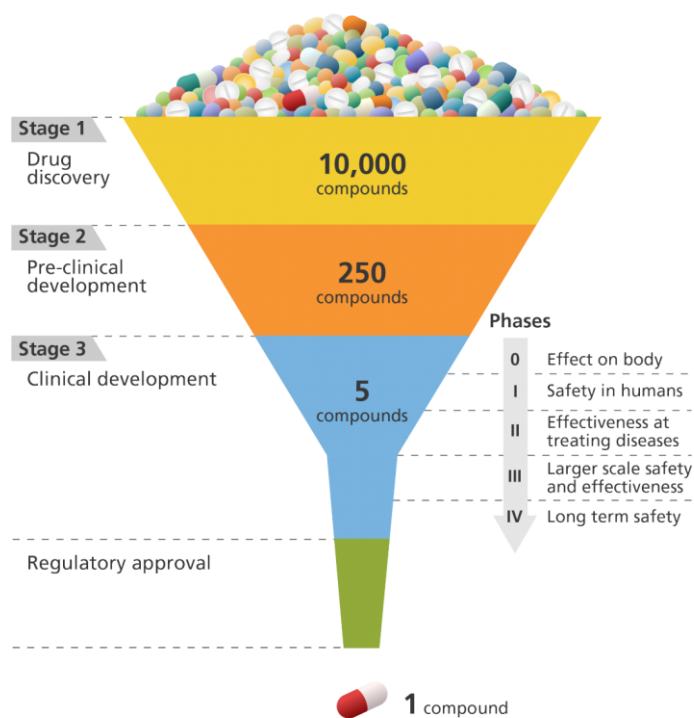
Figure 1: Drug development process phases.

During my PhD I was mainly involved in the clinical research, from phase I to phase III. The first part of the PhD was aimed at develop and validate methods for biomarker determination in clinical studies. In particular, I supported the clinical development of a molecule which has now been approved as cancer treatment in multiple countries. The second part of the PhD was focus on viral titration method development and validation for viral clearance studies from clinical phase I to phase III.

PART I

Biomarkers determination in clinical studies: method development & validation

3. Biomarkers

3.1. Biomarker definition

Biomarker or biological marker is define as *"a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic response to a therapeutic intervention"* (2).

Surrogate endpoint: a biomarker that is intended to substitute for a clinical endpoint. A surrogate endpoint, also referred to as subset of biomarkers, is expected to predict clinical benefit, harm, or lack of benefit based on scientific evidence (2).
A clinical investigator uses epidemiological, therapeutic, pathophysiological, or other scientific evidence to select a surrogate endpoint

Clinical endpoint: a characteristic or variable that reflects how a patient feels or functions, or how long a patient survive.

3.2. Types of biomarkers

Biomarkers can be classified in many different ways, even if a univocal classification is currently not available. One of the commonly used classification is based on the sequence of events from exposure to disease with different applications and is reported below (2,3,4):

- DIAGNOSTIC, used to detect or confirm presence of a disease or condition of interest or to identify individuals with a subtype of the disease.
- DISEASE STAGING, used to classify the extent of disease.
- PROGNOSTIC, indicator of disease
- PREDICTIVE, used to identify individuals who are more likely than similar individuals without the biomarker to experience a favorable or unfavorable effect from exposure to a medical product or an environmental agent.

- STRATIFICATION, used for selecting patient populations more or less responsive to the drug.

In addition, biomarkers can also be classified depending on the clinical phase in which are used (5):

- EXPLORATORY biomarkers in the early phase.
- PROBABLE VALID biomarker in the middle phases.
- KNOWN VALID biomarker in the advanced phase.

Finally, biomarkers can be grouped on the basis of their biochemical nature, in particular:

- PROTEIN biomarkers.
- GENOMIC biomarkers.
- METABONOMIC biomarkers.

The first part of my PhD project has been focusing on protein biomarkers to support clinical trials.

For their versatile characteristics, biomarkers can have many different applications in several fields.

3.3.   Biomarker analytical method development and validation process: parameters tested and correlated regulatory aspects

The role of biomarkers in drug discovery and development has gained precedence over the years. Currently biomarkers are widely used in drug discovery and development, from target identification and validation to clinical application (5). For example, in the initial investigations of therapeutic candidates in humans, biomarkers can provide a basis for the selection of leads compounds for phase III clinical trials.

As biomarkers become integrated into drug development process and clinical trials, quality assurance and, in particular, assay validation become essential with the need to

establish standardized guidelines for analytic methods used in biomarker measurements. Therefore, all the activities that involve biomarker quantification to support clinical studies, should be performed in accordance with international guidelines in order to assure data reliability and robustness.

Despite general absence of official guidelines for the validation of laboratory biomarker assays (6), researchers involved in biomarkers quantification refer to other important guidelines: the International Conference of Harmonization *(ICH) E6 on Good Clinical Practices (GCP)* for Europe, Japan and United States (1996) (7, 8, 9), the *Guideline on bioanalytical method validation* (10), and the *reflection paper on guidance for laboratories that perform the analysis or evaluation of clinical trial samples* (11), made by European Medicine Agency (EMA, respectively 2011 and 2010) and the Food and Drug Administration (FDA) *Guidance for Industry on Bioanalytical Method Validation*. (12, 13), It is also available an updated Food and Drug Administration (FDA) draft guidance (14) with more specific indication on method validation of ligand binding assays used to measure biomarkers in clinical samples. These guidelines does not fully met the needs of drug development and application of biomarker assays.

Moreover, the relevant research paper *"fit for purpose method development and validation for successful biomarker measurement"* (6) was used as reference point for biomarker validation to overcome the lack of regulatory guidelines. The key component of the "fit for purpose" approach is the notion that the assay validation should be tailored to meet the intended purpose of the biomarker study, with a level of rigor commensurate with the intended use of the data.

The *ICH E6 on Good Clinical Practices (GCP)* guideline mentioned above describes the quality standard that should be used during clinical studies. In particular it refers to the *GCP* standard which is *an international ethical and scientific quality standard for designing, conducting, recording and reporting trials that involve the participation of human subjects. Compliance with this standard provides public assurance that the rights, safety, and well-being of trial subjects are protected, consistent with the principle*

that have their origin in the Declaration of Helsinki, and that the clinical trial data are credible. (7,8,9)

Moreover, this guidance on GCP and the reflection paper listed above, provide information on Sponsor and investigators organization and documents to be provided. (7,8,9,11)

3.4. Biomarker analytical method development and validation process

The biomarker analytical method development and validation process are composed of: assay set-up and optimization, pre-validation and clinical validation (5, 15).

Analytic method validation is the crucial part of a biomarker development process. During this process the performance characteristics, and the optimal conditions that will generate the reproducibility and accuracy of the assay are assessed.

Validation should also demonstrate that a method is reliable for the intended application. The complexity of method validation increases as the biomarker data are used for increasingly advanced clinical or otherwise business clinical making decision. For example, a biomarker under exploratory development in an early phase clinical trial would be less rigorously validated than an already well-qualified biomarker in the same trial. Exploratory biomarker data would be used for less critical decision than data describing a well-qualified biomarker (6).

Before going into the analytical validation, method set-up and optimization must occur. Method set-up includes selection of the most suitable reagents (e.g. antibodies or kit) matrix minimal required dilution identification, selection of the calibrators for the standard curves (16). In this phase control samples (validation samples) are also selected to assess the reliability of the assay, such as reference standard for quantitative (e.g. standard for quantification in serum/plasma) analyses. Control samples may be commercially obtained or experimentally prepared. In any case, the origin of the control must be specified: Certificate of Analyses (CoA), bibliographic references or information from databases (e.g. National Center for Biotechnology Information, NCBI) should always be attached to the study documentation. During the set-up, the operator

records the single steps of the analysis in a dedicated Research Record Book (RRB). Moreover, reference to any publications will be specified and attached to the study folder.

At the end of the set-up phase, at least a draft biomarker method summarizing the materials, methods and procedures used for the analysis should be prepared.

After method set-up is complete, optimisation of the method performance is assessed and, if necessary, test conditions could be improved such as, for example, incubation time and temperature and dynamic range of the calibration curve. The aim of this phase is to define the details of the analytical method and interpretation of results.

During the optimization phase, as well as calibrator standard controls, it is advisable to use samples representing the biological matrices of the samples to be analysed.

At the end of the optimization phase, the biomarker method must be consolidated, summarizing the materials, equipment and procedures used for the analysis. The biomarker method should also report a specific section for data analysis.

Before entering into the validation phase, a pre-validation of the assay is conducted, in order to test the performance of the method, define and/or confirm the assessment to be done and the acceptance criteria to be used in the validation phase.

The pre-validation phase should simulate the actual validation and take into account all the potential variables of the method in order to be ready to deal with any unexpected events. Additional variables that may affect the method performances should be considered when possible (e.g. sample preparation at clinical sites; when applicable verify the accurate QC sample preparation).

At the end of this stage, a finalized biomarker method should be released and a validation plan should be available. It must include all the parameters to be tested and the acceptance criteria to be applied.

Finally, the last and most critical phase is the assay validation which should asses the performance of the method and confirm the data obtained from the previous phases. In

order to be more close to the reality, it is advisable to use samples of the actual matrix to be analysed in a clinical study, whenever these are available.

Once the validation is positively completed, the method can be used to analyse samples coming from clinical trials.

The validation phase should be documented by writing a plan and a report. Any exceptions or changes to the plan should be reported and justified in the experimental folder of the study and in the study report

According to Regulatory Agency guidelines and recommendations of the scientific community (6-14), the minimum parameters that must be assessed in order to validate a biomarker quantitative assay are: dynamic range, accuracy, precision, ruggedness and stability.

The acceptance criteria for each parameter are set according to the context in which the method will be used and are reported in the validation protocol. In addition, the acceptance criteria for each parameter could be slightly different in case of single or multiplex assay.

In any case, if the acceptance criteria of the calibration curve are not met, all the experiments done should be rejected.

3.4.1. Dynamic range (of the calibration curve)

The calibration curve is generally made up of at least 6 Calibration Standard, CS (at least in duplicate) distributed within the range of quantification (dynamic range), including the Lower Limit of Quantification (LLOQ) and Upper Limit of Quantification (ULOQ).

The calibration curve established during development should be confirmed in a minimum of 3 independent runs, those in which accuracy and precision are usually assessed.

The run is considered valid if the standard curve acceptance criteria are met.

The Calibration Standard should be prepared fresh every day.

Usually the back-calculated concentration of each CS should be within 20% of its nominal concentration to be valid, except for LLOQ (30%).

3.4.2. Accuracy (recovery) and Precision

Accuracy represents the closeness of mean results obtained after analyses by the method to the true value (nominal concentration) of the analyte.

Precision is a quantitative measure (usually expressed as standard deviation and coefficient of variation: CV %) of the random variation between a series of measurements from multiple sampling of the same homogenous sample under the prescribed condition. Precision may be considered at two levels: repeatability and reproducibility.

In summary, accuracy and precision of an analytical method are parameters that define systematic error (average BIAS) and the degree of variation following repeated measurements of the same homogenous sample. Accuracy and precision are confirmed during validation of the method using samples prepared at a known concentration (Validation Samples, VS) in the same biologic matrix (e.g. serum or plasma) as the samples to be analysed during the clinical studies. The VS are prepared by adding a known quantity of analyte, usually the assay calibration standard stock, to the appropriate matrix. If an external calibration standard is not available it can be replaced by actual samples with an appropriate concentration of the analyte of interest. Considering the levels of analyte to be determined in the specific matrix, three different concentrations of VS are usually prepared, when possible:

- ❖ VS- Low: about 3 times the LLOQ concentration;
- ❖ VS-Medium: a concentration near the central (geometrical and not arithmetical) point of the calibration curve;
- ❖ VS-High: a concentration about 75% of the ULOQ.

When feasible, also the VS at the lowest concentration that can be quantified with sufficient accuracy and precision by the analytical method (LLOQ) is prepared. When the VS have been prepared they are divided into aliquots and frozen; some of them are also used for stability testing. At least three independent replicates are analysed for each VS, with a minimum of three analytical runs carried out on at least three different days. "Intra" and "inter" assay variation should be calculated. Usually the mean concentration

of each VS should be within 20% of their nominal concentration (30% for LLOQ tested in 5 independent replicates).

3.4.3. Robustness or ruggedness

Measures the capability of the assay to remain unaffected by small but deliberate changes in method parameters and provides an indication of assay reliability during normal experimental (run) conditions. Different instruments, operators, length of the analytical run, analysis days, incubation times or varying environmental factors should not have a significant impact on the assay. While changing in the critical parameters such as temperature or duration of incubation as well as batch of reagents and kit used is evaluated during optimisation phase, ruggedness by a second operator is usually evaluated during validation exercise. The Validation Samples are tested by a second operator in at least one analytical run.

Usually the back-calculated concentration of each validation sample should be within 20 % of its nominal concentration for both operators for run to be valid. The ruggedness should be calculated as the ratio in percentage between the mean accuracy for each validation sample processed by a second operator and the average of mean accuracy for each validation sample processed by the two operators. This value should be in the range 80-120%.

3.4.4. Stability (e.g. short and long-term stability)

Stability is evaluated as chemical stability of an analyte in a given matrix under specific conditions for given time intervals.

Stability testing is aimed at mimicking as far as possible sample storage and processing conditions. Stability of the analyte in the studied matrix should be assessed using the VS (Low-Medium-High), in order to be able to cover the whole quantification range. Stability should be assessed using standard calibration curves prepared on the day of analysis and with three samples (replicates) for each of the VS. The nominal concentration should be used to calculate stability. It is recommended to test the

sample(s) to be used for long-term stability to check that accuracy at t=0 is between90% and ≤110% of the nominal value to avoid wrong data interpretation during the stability studies.

Stability is usually evaluated using the VS as below reported:

- ❖ after at least 3 freeze/thaw cycles;
- ❖ after storage at room temperature for 2 and 4 hrs (short-term stability or bench-top stability) to cover the time needed to dilute the real samples;
- ❖ After storage at -20°C and/or -80°C to cover the entire storage period before testing of the real samples. This time frame normally corresponds to at least 3 year.
- ❖ Stability is usually fulfilled when the back-calculated concentration of each validation sample is within 20% of its nominal concentration.

As describe in the paragraphs above, the validation phase should asses the performance of the method. The validation may include further parameters depending on the use of the biomarker, the type of biomarker, the technology used and the information shown on the certification provided by the kit manufacturer.

Some additional parameters can be assessed during validation:

3.4.5. Lower limit of quantification of the method (LLOQ)

LLOQ is the lowest concentrations of analyte that have been demonstrated to be measurable with acceptable levels of bias, precision and total error (with sufficient accuracy and precision).

When the samples to be analysed do not contained detectable levels of the endogenous analyte, both accuracy and precision can be assessed also at the LLOQ level. On the contrary, when endogenous analytes are present at quantifiable concentrations in the study samples, a "real" study sample, or commercially available sample, could be selected at a suitable concentration. In addition, a pool of samples could also be prepared at a suitable concentration. Serial dilutions of the samples, or of the pool, (at least three

independent replicates for each dilution) are prepared to evaluate precision in the quantification of the concentration close to the selected LLOQ. For each dilution should be calculated the CV% of the results. The LLOQ is usually the last dilution showing a CV% ≤ 30.

If the endogenous levels of analyte are undetectable in the study samples, a spiked sample can be produced at the LLOQ concentration. The sample is than tested at least at five independent replicates and precision and accuracy are evaluated.

The CV% of the results and the mean accuracy should be calculated. LLOQ is usually validated if the back-calculated concentration is within 20% of its nominal concentration and the CV% ≤ 30.

3.4.6. Selectivity

Selectivity is the ability of the method to determine the analyte unequivocally in a complex matrix (e.g. serum, plasma) in the presence of components that may be expected to be present in the sample.

Selectivity should be evaluated using the most relevant compounds and matrices to anticipate all potential interferences in the assay. Matrices from patients often contain components (e.g. rheumatoid factor, soluble receptors, heterophilic interferences from autoimmune disorders, lipemic and haemolysed samples) that may not be present in the control matrix. Therefore, whenever possible, it is strongly recommended to repeat selectivity testing as soon as matrix from patients is available. To evaluate selectivity of the method at least twelve different individual samples of an appropriate matrix (e.g. healthy volunteers) are tested unspiked and spiked at a nominal concentration equal to the VS-Medium. The method con be considered selective if the back-calculated concentration of at least 2/3 of the tested samples is within 20% of VS-Medium nominal concentration. In case the unspiked samples contain measurable levels of analyte, this can be subtracted to the spiked one before evaluating accuracy.

3.4.7. Dilution linearity

Is the ability of the method to quantify the analyte of interest when present at a concentration above the range of quantification (AULOQ, Above Upper Limit Of Quantification) with good accuracy and precision. A real samples, or spiked sample at the highest concentration that can be obtained is normally used. The sample is serially diluted until its concentration falls within the method quantification range. At least three independent replicates of the same sample should be analysed. The method can be considered useful to analyse high concentrated samples if the CV% of the measured concentration for each dilution sequence is ≤ 30.0. In the same experiment the hook effect can be evaluated. The hook effect is based on the saturation curve of antibody with antigen. Primarily, the hook effect depends on analyte concentration and implies the presence of huge excess of analyte which saturates all binding sites on antibody. This results in falsely decreased value of the measured analyte which could even lie in the reference interval.

3.4.8. Integrity of dilution

Is the demonstration that the concentration of the analyte is a linear function of the dilution, which does not occur when the analyte forms complexes with matrix components. Integrity of dilution must be tested on an actual sample. The sample is spiked and then serially diluted to a suitable concentration so as to obtain at least two points in the range of the curve. Three independent replicates for each point are tested and the CV% of the back calculated concentration and the accuracy of each diluted sample versus the nominal concentration are calculated. Integrity of dilution is usually met if the values CV% is ≤ 30 and the accuracy is between 80 and 120%.

3.4.9. Parallelism

It is a performance characteristic that can only be evaluated during in-study validation and must be performed based on the nature and scientific knowledge of the analyte. It is conceptually similar to the dilution linearity assessment except that it is assessed with

multiple dilutions of study samples in order to check this performance on real samples. Parallelism must be assessed using at least 6 high concentration samples (from a given study) serially diluted to at least three concentrations within the quantification range. The precision between samples in a dilution series must not exceed 30% (CV% of the overall mean concentration from the dilutions).

Acceptance criteria for all the above described parameters must be clearly specified in the validation protocol. They can vary on the base of the analyte of interest (e.g. if endogenous levels are present) and on the base of the assay (e.g. single or multiplex). If the validation results do not meet the acceptance criteria specified, the reason must be identified and, if needed, the method should be re-optimize appropriately. Any change to the analytical method should be appropriately detailed and described in a method revision and a new version must be released. Any foreseen changes occurred during validation study conduction should be duly documented by specific amendments. On the contrary, unforeseen changes may occur should be duly documented as a deviation and reported in the final report. A dedicate validation study folder is prepared to collect all the documentation related to the validation exercise.

When the validation is completed, the biomarker method can be used for clinical study samples testing.

4. Immunoassay-based technologies used for biomarker quantification.

Many different technologies are commonly used for protein biomarker quantification. This is due to the biomarkers heterogeneity and their several applications.

Among the available technologies, conventional ELISA, Luminex, AlphaLISA, MesoScaleDiscovery and Erenna Singulex were compared during the first part of the PhD.

These technologies share a common basic principle: all are immunoassays-based thus utilize the ability of an antibody to recognize its specific antigen (analyte of interest).

4.1. Immunoassays

Immunoassays rely on the ability of an antibody (Ab) to recognize and bind a specific macromolecule in a complex mixture (e.g. biological matrix). This ability can be used to detect or quantify the recognized molecule, the so-called antigen (Ag) or analyte, or vice versa, the antigen can be used to capture and allow quantification of a specific antibody. The binding event is associated to the generation of a measurable signal, which is usually compared to a signal generated by a reference sample at a known concentration.

Immunoassays involve chemically linked or conjugated antibodies or antigens with some kind of detectable label that are able to generate the signal. A large number of labels exist in modern immunoassays, and they allow for detection through different means. Many labels are detectable because they either emit radiation, produce a color change in a particular solution, fluoresce under light, or because they can be induced to emit light.

The first immunoassays was developed by Rosalyn Sussman Yalow and Solomon Berson in the 1950s (17). Yalow obtained the Nobel Prize for medicine and physiology for her work on immunoassays in 1977. The first immunoassays were all based on the labelling of antibodies with radioactive iodine (RadioImmuno Assays: RIA).

Immunoassays became considerably simpler to perform and more popular when techniques for enzymes chemically linked to antibodies were demonstrated in the late 1960s (Enzyme Immuno Assays: EIA or Enzyme-Linked ImmonoSorbent Assay: ELISA) (18). In 1983 Professor Anthony Campbell at Cardiff University replaced radioactive iodine used in immunoassay with an acridinium ester that makes its own light. This was the bases of chemiluminescence. This type of immunoassay is now used worldwide to measure a wide range of proteins, pathogens and other molecules in blood samples. Enzymes used in ELISAs include horseradish peroxidase (HRP), alkaline phosphatase (AP) or glucose oxidase. These enzymes allow for detection often because they produce an observable color change in the presence of certain reagents. In some cases these enzymes are exposed to reagents which cause them to produce light or chemiluminescence. Moreover, fluorogenic reporters like phycoerythrin are used in a number of modern immunoassays (e.g. Luminex technology). Some other labels work on an electrochemiluminescent principle, in which the label emits detectable light in response to electric current (e.g. Mesoscale technology).

ELISA is the most widely used detection platform for the quantification of analytes in biological samples, in particular for biomarkers quantification.

Unfortunately, ELISA technique suffers of some disadvantages such as a low throughput due to the difficulty in automatize, higher volume of samples must be used, only single-plex measurement can be done. For these reasons, other technologies have been evaluated.

4.2. Colorimetric measurements (Spectramax)

Enzymatic/colorimetric immunoassay (ELISA) is the "classic" and most widely technology used since years.

A spectrofotometer is used to reveal a colorimetric signal . Briefly, a capture antibody able to recognise the antigen of interest is coated on a microtiterplate (normal or functionalised plates depending on the capture reagent) and the uknown samples are incubated into the plate to allow the specific binding. After a wash step to remove

unbound antigen, a detection antibody cojugated with HRP (Horse Radish Peroxidase) able to recognise the immunocomplex, is added to the microplate. After a second wash step the HRP substrate is added and the signal (absorbance) is generated after a 450 nm wavelength is applied. in . The signal measured by thespectrofotometer is directly proportional to the concentration of the analyte of interest in the samples.

Different spectrofotometers are availble on the market, in *Figure 2* the Spectramax (Molecular Device) instrument, used during my PhD, is showed. It allows absorbance measurement in the all range of UV-VIS, having a monocromator which enables wavelength selection.

Many different commercially available pre-casted kits could be used.

An ELISA assay requires from 4 to 5 hours to be completed and the sample volume range is from 50 to 150 uL.

Figure 2. Spectramax 190 microplate reader

4.3. Luminex technology

Fairly recently a new technology that offers the benefits of the ELISA, but also enables the added value of higher throughput, increased flexibility, reduced sample volume, multiplexing analyses and low cost with the same workflow as an ELISA, has emerged: the Luminex® technology (19).

The assay format is based on the immunoassay principle such as for conventional ELISA, however, instead of having the capture antibody coated on a solid surface (such as microtiter plate), it is coated on a magnetic and/or polysterene microspheres called

beads, improving the coating surface. This explains why it is also known as bead-based assay. All of the beads are of the same size: 5.6 um in diameter. This small size of microspheres allows them to be easily suspended in liquid solution.

The suspended beads allow for assay flexibility in a single-plex or multiplex format, and for testing simultaneously a large number of analytes (up to 100, see *Figure 3*). This technique involves 100 distinctly colored bead sets created by the use of two fluorescent dyes at distinct ratios.
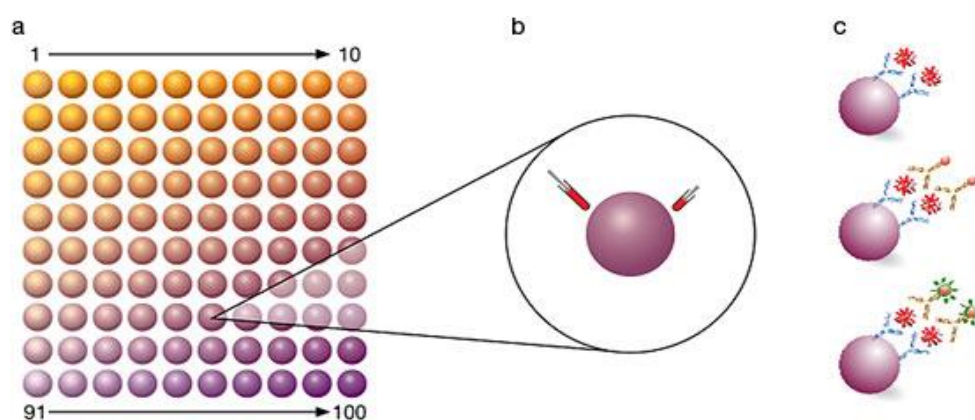
Figure 3. Multiplex immunoassay technology. Beads are colored internally with two different fluorescent dyes (red and infrared). Different concentrations of red and infrared dyes are used to generate up to 100 distinct bead regions. Each bead region is conjugated to a specific target analyte (a) followed by binding with a biotinylated detection antibody (b) and a reporter dye, streptavidin-conjugated phycoerythrin (c).

Luminex technology enables multiplex immunoassays in which one antibody to a specific analyte is attached to a set of beads with the same color, and the second antibody against the analyte is attached to a fluorescent reporter dye label. The use of different colored beads enables the simultaneous multiplex detection of many other analytes in the same sample. A dual detection (red and infrared to classify the beads and green to quantify the analyte of interest) flow cytometer is used to sort out the different assays by bead colors in one channel and determine the analyte concentration by measuring the reporter dye fluorescence in another channel (green). In particular, during data acquisition, the contents of each microplate well are drawn into the array reader and

precision fluidics align the beads in single file through a flow cell where lasers excite the beads individually: the red classification laser excites the dyes in each bead, identifying its spectral address, whereas the green reporter laser excites the reporter molecule associated with the bead, which allows quantitation of the captured analyte. High-speed digital signal processors and software record the fluorescent signals simultaneously for each bead, translating the signals into data for each bead-based assay (see *Figure 4*).

Flow Cytometry–Based Analysis — Traditional xMAP Technology (Bio-Plex 200, Bio-Plex 3D Systems)
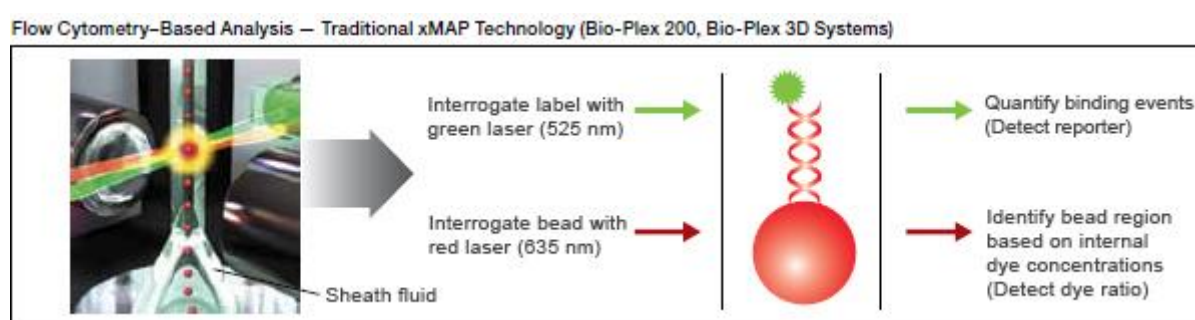
Figure 4. *Data acquisition and reduction. Dyed beads are pushed through a detection chamber in a single file or magnetically immobilized. The red classification laser (635 nm) interrogates the internal dyes to identify bead regions. The green reporter laser (532 nm) interrogates the fluorescent reporter to measure analyte concentration.*

The assay consists in three main steps: the first step is the incubation of samples with magnetic beads coated with the specific capture antibody against the analyte of interest, then, after a wash, the beads are incubated with a secondary detection antibody biotin-conjugated and finally, after a second wash, streptavin is added to the microplate to allow signal amplification. The plate is finally analyzed by the instrument. The steps are pictured in *Figure 5*.

The assay takes a maximum of two hours and a half to be completed and the time of analysis is about one hour for a 96 wells microtiter plate.
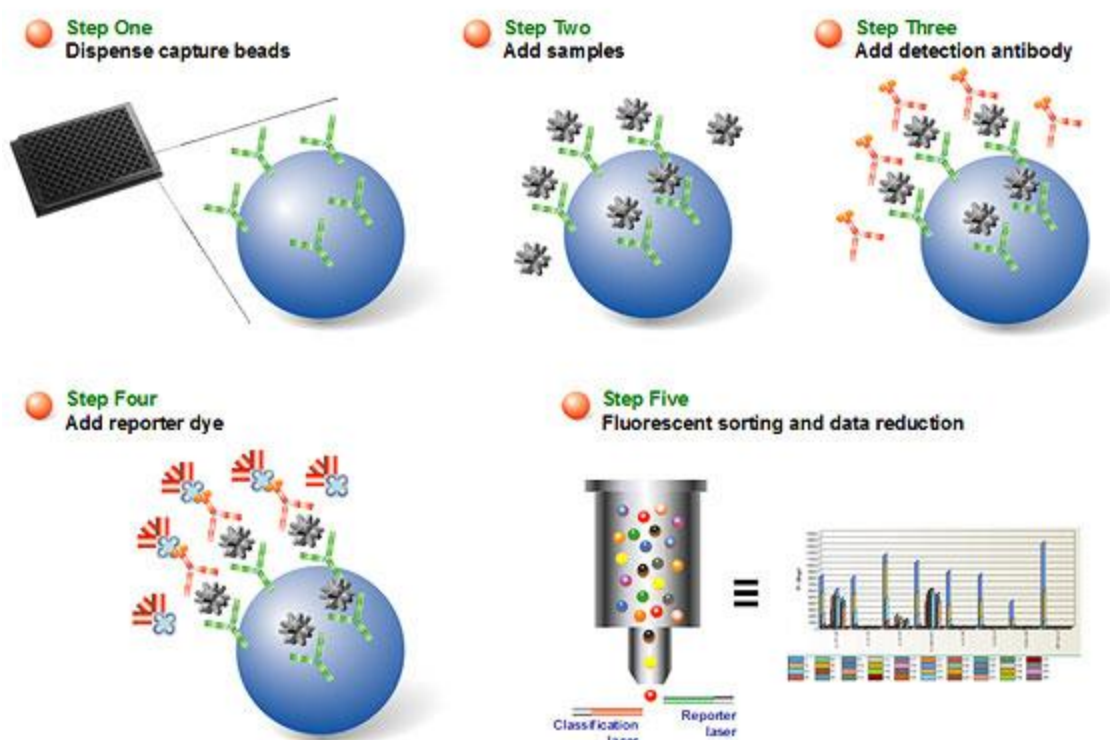
Figure 5. Schematic representation of an immunoassay sandwich-based assay workflow using luminex technology.

Two types of Luminex instruments are available: Luminex 100 and 200 (*Figure 6*). The first flow cytometer designed specifically for multiplexed microbeads analysis was released by Luminex Corporation in the late 1990s. It was called Luminex 100 system. The dual-laser Luminex-100 instrument has a three colour fluorescence signal-detection system. Two colours are dedicated to microsphere classification (red and infrared); the third colour (green) is used for measurement of the reporter fluorescence intensity.

In the 2005, the new Luminex 200 was launched. The system is the combination of two main components: the analyser, a flow cytometry-based instrument which integrates key xMAP detection components, such as lasers, optics, fluidics, and high-speed digital signal processors and the Bio-Plex Manager® software (Bio-Rad), which is designed for protocol-based data acquisition with robust data regression analysis. The software is 21 CFR part 11 compliant.

Figure 6. *Luminex200® system*

The main advantages of Luminex technology, if compared to conventional ELISA, are: the reduction time of analysis (less than 3 hours), a smaller sample volume is required to conduct the analyses and the non-specific binding to the beads is reduced. The possibility to reduce the sample volume is very important when particular type of samples (such as cerebrospinal fluid or samples from pediatric patients) needs to be analysed.

4.4. AlphaLISA immunoassay technology

AlphaLISA is a bead-based assay technology working in a microplate format, which can be used for single and multiplex analyses of protein biomarkers.

The assay was developed by Ullmann in the '90s for the purpose to design a simple and more robust alternative to classic ELISA for the quantification of biomarkers in a high throughput screening format (20). This method was initially called Luminescent Oxygen Channelling Immunoassay (LOCI). Subsequently, Perkin Elmer acquired the technology and changed its name in AlphaLISA. The acronym "Alpha" stands for Amplified Luminescent Proximity Homogeneous Assay. It is a homogeneous assay where no washing steps are necessary.
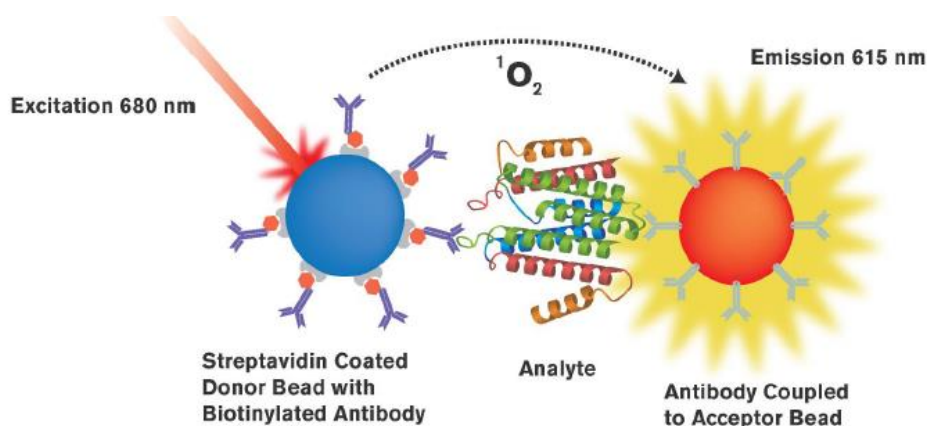
Figure 7. Principle of AlphaLISA technology. A biotinylated antibody to the analyte binds to the streptavidin coated donor beads and a second antibody to the analyte is directly conjugated to AlphaLISA acceptor beads. In the presence of the analyte, the two beads come into close proximity. The excitation of the donor beads at 680 nm generates singlet oxygen molecules that trigger a series of chemical reactions in the acceptor beads resulting in a sharp peak of light emission at 615 nm.

Two type of beads are used: donor and acceptor beads. Both have a diameter of 250 nm, are latex-based and coated with a layer of hydrogel and each of them contains a different proprietary mixture of chemicals. Donor beads contain a photosensitizer, phthalocyanine, which converts ambient oxygen to an excited and reactive form of O_2, singlet oxygen, upon illumination at 680 nm while acceptor beads contain Europium.

Briefly, as reported in *Figure 7*, the analyte of interest binds to the capture reagent which, in turn, is able to bind the donor beads (for example through the streptavidin-biotin binding), leading to an energy transfer from the donor beads to the acceptor beads coated with the detection reagents. The close proximity of the two beads produces a luminescent signal, detected by the equipment (e.g. Enspire from Perkin Elmer).

Within its 4 µsec half-life, singlet oxygen can diffuse approximately 200 nm in solution. If the analyte of interest is present in the sample and the immunocomplex is formed, the donor and acceptor beads are at the correct distance to allow exchange of singlet oxygen, resulting in light production at 615 nm. If the donor bead is not in proximity of an acceptor bead, the singlet oxygen falls to ground state and no signal is produced.

Pre-casted kits to measure the concentration of many biomarkers and different type of pre-coated beads (e.g. Protein A, protein G, protein L and anti-species beads) to allow customization of a specific assay are commercially available.

One of the advantages of this technology is the duration of the assay: after 1 hour incubation of samples with specific antibody-coated acceptor beads, streptavidin-coated beads are added to the microplate and incubated for 30 minutes or 1 hour (depending on the protocol used) and, finally the plate is analyzed by the instrument. The procedure is described in *Figure 8*.

Add analyte to microplate.

Add biotinylated anti-analyte antibody and anti-analyte antibody-conjugated acceptor beads. Incubate for 60 minutes.

Add streptavidin-coated donor beads. Incubate for 30 to 60 minutes. The interaction between bead-bound molecules and analyte brings the beads into proximity.

Excite the donor beads with red light.

Singlet oxygen generated by donor beads excites the acceptor beads, which emit light proportional to the level of interaction.

Figure 8. AlphaLISA protocol. AlphaLISA assays are conducted using a simple mix and read protocol that substantially reduces assay development and hands-on time, while improving throughput and ease to automation.

Enspire Multimode plate reader produced by Perkin Elmer (*Figure 9*) is the equipment used in the laboratory for the AlphaLISA immunoassay signal generation and detection. Enspire allow to excite the donor beads present in each microplate well at a wavelength of 680 nm and to capture the signal generated from the acceptor beads present in each sample. The results are reported as counts by the software and are interpolated to reference standards to calculate the analyte concentration in the samples.

Figure 9. Enspire® Multimode plate reader

Over the AlphaLISA main advantage of no-wash steps that save analyses time (less than 2 hours), there are other advantages of using AlphaLISA to measure biomarkers level in biological samples. Firstly, AlphaLISA assays are highly robust when using sample volumes as low as 1 or 5 µl (depending on the protocol chosen) in a total assay volume of 10 µl. This allow to use limited quantity of precious samples. Secondly, assays can be performed in 96 or 384 well microplates, increasing assay throughput.

Finally, matrices interferences are kept out because of the emission of the acceptor beads at 615 nm, matrices compounds do not emit at that wavelength, reducing background effects and allowing analytes quantification in complexed samples such as serum and plasma samples.

It is important to highlight that the reduction of sample volume used to conduct an AlphaLISA as well as the fact that no washing steps are applied, do not impact on the sensitivity of the assay. The high sensitivity achieved using AlphaLisa is mostly due to the nature of the assay: the flow of a singlet oxygen produced upon donor beads irradiation induces remarkable signal amplification in nearby acceptor beads. Moreover, the high antibody density on beads creates an avidity phenomenon, increasing sensitivity.

The main disadvantage of the AlphaLISA is the assay format as a single-plex, is not possible to multiplexing different analytes in the same microplate well.

4.5. Erenna Singulex technology platform

More recently another bead-based technology came out: the Erenna Singulex system. The Erenna Singulex immunoassay system utilized a Ligand Binding Assay (LBA) procedure followed by capillary flow analysis on an instrument that performs Single Molecule Counting (SMC) of fluorescent detector molecules (21, 22).

The combination of a traditional immunoassay workflow with patented SMC™ technology enables precise measurement of molecules (e.g. low-abundance biomarkers) at levels previously undetectable, capturing concentrations down to the femtogram/mL level and allowing the monitoring of changes in, extremely low levels of disease biomarkers such as cytokines.

The SMC assay workflow is reported in the *Figure 10*: a primary antibody conjugated to magnetic beads captures the analyte of interest. The unbound analyte than is removed by a wash step and a secondary antibody labelled with Alexa Fluor® Dyes is added to the solution. After a second wash, the immunocomplex between the analyte and the secondary antibody is disrupted with a specific elution buffer and the eluate is transferred to a 384 wells microplate for the analysis using the Erenna® instrument.
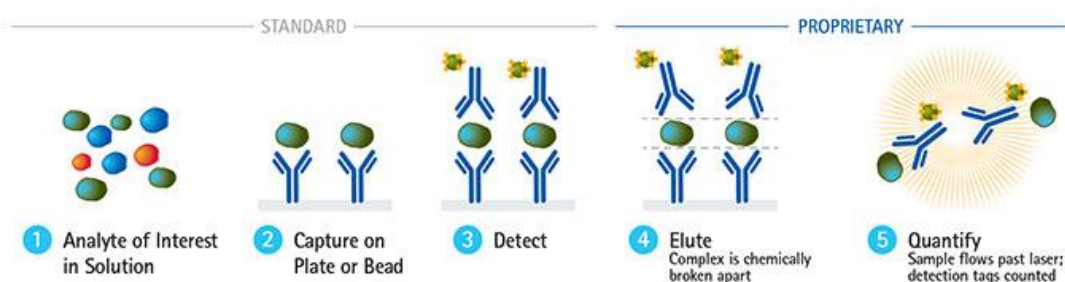
Figure 10. SMC™ Assay Workflow. During the capture and detection steps, specific antibodies translate each biomarker into a signal. During the elution step, fluorescent dye-labeled detection antibodies are released from the immune complexes. The eluate is then drawn into the Erenna® System capillary tube, which contains a very small interrogation space that is illuminated by a laser. Single fluorescently labeled molecules are detected as they generate intense flashes of light when passing through the interrogation space. Detected signals with peak intensity above the threshold of background fluorescence are counted as digital events.

Erenna® instrumentis composed of a fluidic system, a confocal microscopy (laser to excitate and Sgx Link® software for signal acquisition and elaboration (see *Figure 11*).
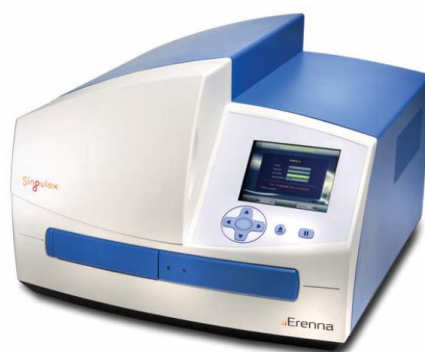
Figure 11. Erenna instrument with Singulex technology

It records three different signal generated from the labelled secondary antibody for each well: Detected Event (DE), Event Photons (EP) and Total Photons (TP). DE are detected events with signal intensity higher than the background, which is automatically calculated by the instrument. DE signal is proportional to low concentration of the analyte of interest in the sample. EP represents the number of photons recorded during a DE event. This value is proportional to the middle concentration of analyte in the sample. Finally, TP represents all the photons recorded during the sample acquisition time, higher and lower than background. This signal is proportional to high concentration of the analyte in the sample. The software generates three different standard curves corresponding to each signal type and a fourth one that represents the weighted sum of the other thre. In particular, the lower part of the curve is represented by the data obtained from the DE, the middle part from the EP and, the higher party from the TP.

The main advantage of this technology is the detection at very low level (fg/mL), very important for low abundant biomarkers. However the technology is very sensitive to background interference such as powder, different operators and the steps are not so easy to be performed. Moreover the samples need to be filtered before being analyzed, this

means that a high sample volume is required. Finally, the instrument requires at least one minute for well for a total of about 6 hours for a 384 wells plate.

4.6.   Mesoscale discovery (MSD) technology

Meso Scale Discovery® (MSD) company develops, manufactures, and markets biological assays for the quantification of different molecules, including biomarkers. MSD's products are based on MULTI-ARRAY® technology, a proprietary combination of patterned arrays and electrochemiluminescence detection that results in high sensitivity and fast assay with a broader dynamic range (23).

MSD multi-array technology is applied to microplates as single-spot (single assay) or multi-spot with patterned spot assays in each well (multiplex assay, 10 different spots: in each spot capture antibody against different analytes are immobilized, see *Figure 12*). This enables precise quantitation of multiple analytes simultaneously in a single sample, increasing throughput and requiring less time and effort than other assay platforms described above (e.g Luminex, Singulex). MSD multi-array microplates are available in 96-, 384-, and 1536-well formats with standard or high-binding surfaces.

Figure 12. Multiplex microarray plate

The principle of the assay is based on sandwich immunoassays. The plates are pre-coated with capture antibodies specific for the analyte of interest, in each independent and well-defined spot, as shown in *Figure 13*.
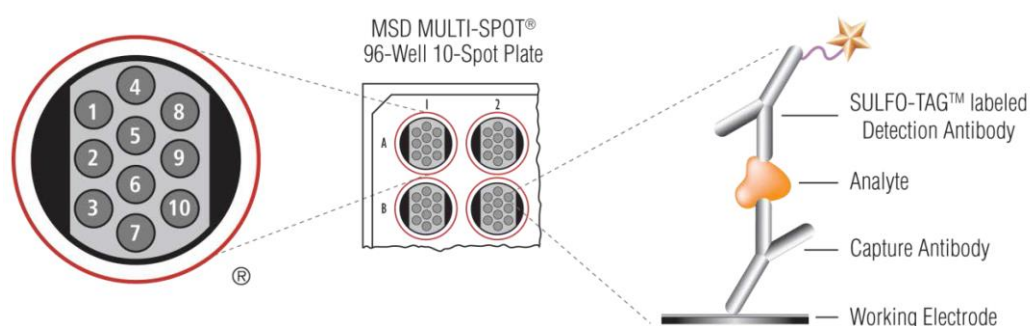
Figure 13. Multiplex plate spot diagram showing placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.

The samples, containing the analyte(s) of interest, are added to the microplate in order to allow the binding of analyte(s) in the samples to capture antibodies immobilized on the working electrode surface (made by high binding carbon electrodes that allows for easy attachment of biological reagents, 10X greater binding capacity than polystyrene) at the bottom of multi-array microplates. After the incubation period, a wash step is performed and a solution containing detection antibodies conjugated with electrochemiluminescent labels (MSD SULFO-TAG™) is put into the array microplate over the course of another incubation periods. The immune-sandwich is completed with the recruitment of the detection antibodies by the analyte which in turn is bound to the capture antibody. After another wash step, an MSD buffer (read buffer) is added to the plate. This creates the appropriate chemical environment for electrochemiluminescence (ECL). Finally, the plate is loaded into an MSD instrument where electricity, applied to

the plate electrodes, causes the detection antibody labels (sulfo-tag) to emit light. The instrument measures the intensity of emitted light, which is proportional to the amount of analyte present in the sample, and provides a quantitative measure of each analyte. The process is described in *Figure 14*.
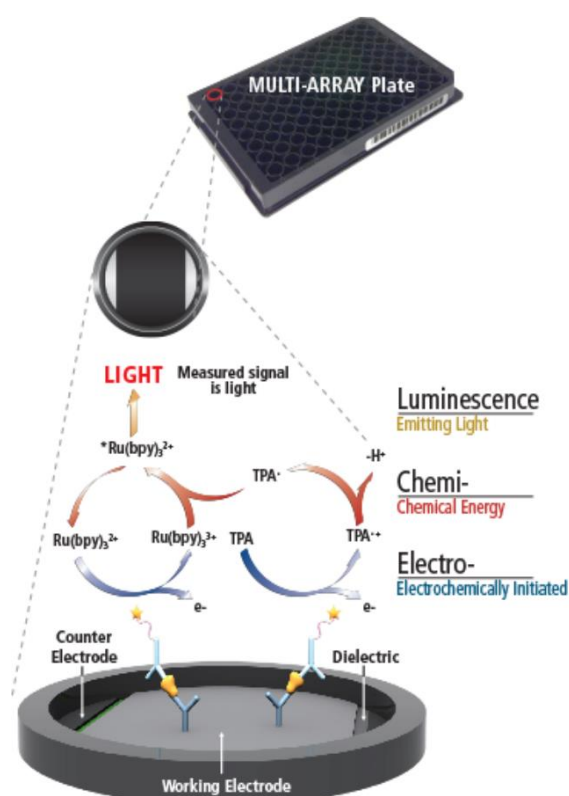
Figure 14. Electrochemiluminescent signal generation: at the multi array plate bottom is present a working electrode containing detection antibody conjugated with a sulfo-tag. When electricity is applied to the plate, sulfo-tag labels react with light emission that is detected by the MSD instrument.

Pre-casted kits are commercially available. Alternatively, it is possible to require MSD® to provide for custom panel kits with a combination of antibodies against different cytokines of interest in each spot. Moreover, differently tagged reagents for the assay development are obtainable. Finally, the plates can be purchased uncoated or coated with proteins such as streptavidin or avidin, with anti-species antibodies such as goat anti-

mouse or goat anti-rabbit, or with antibodies against specific analytes. Custom coatings and surface treatments are also available.

.MSD multi-array technology is detected with proprietary equipments. Among them, the Meso QuickPlex SQ 120 instrument (*Figure 15*) was used in the laboratory during the PhD. It is a multiplexing imager that provide high sensitivity and dynamic range (up to 5 logs). The plates could be read quickly and continuously with simple protocols using Discovery Workbench assay analysis software. This instruments reads multi-array plates one well at a time. One entire plate is read in about 3 minutes and the multiplexing does not affect plate read time.

Figure 15. MSD Quickplex SQ120 instrument

The main advantages of this technology is the combination of fast assay processing and read times and the ability to perform multiple, simultaneous tests on a single sample allow to increase the number of assay performed in a day and save sample volume (low sample volume requirements: 30-50 µl) with minimal consumption of reagents. In particular, electrochemiluminescence provide high sensitivity with high precision and low background, broad dynamic range and great flexibility. High sensitivity is obtained through multiple excitation cycles that can amplify signals to enhance light levels. The

wide dynamic range of this detection systems means high and low expression levels can be measured without multiple sample dilutions. The stimulation method (electricity) is decoupled from the signal (light) allowing only labels near the electrode surface to be detected, resulting in low background. Moreover, MSD is easy to use: immunoassay method is similar to conventional ELISA, but quicker and with great flexibility: labels are stable, non-radioactive, and conjugated to biological molecules. Moreover, multi-array technology allow to conserves valuable samples by allowing multiple results from very low sample volumes and enables the measurement of native levels of biomarkers in normal and diseased samples without multiple dilutions.

Finally, another advantage which allow to save time during the analysis is the fact that the instrument does not need calibration or maintenance and between-read cleaning instead of e.g. Luminex technology.

5. Aim of the study

The aim of the first part of my PhD was to explore different immunoassay technologies to be used to optimize and validate methods for protein biomarker quantification in clinical samples fulfilling very critical requirement such as sensitivity in the real matrix. All the technologies available in the lab (AlphaLISA, Singulex, Luminex, MSD) were evaluated for this purpose. The validation of the best selected technology was not completed due to group reorganization.

The second part of the PhD was focused on a method development for obtaining purified virus stock characterized by high titer and low concentration of impurities (e.g. cell protein). In particular, the work was done with Mo.A.MuLV virus.

Furtheremore, validation of Mo.A-MuLV (Moloney Amphotropic Murine Leukemia Virus) virus titration and impurity quantification according to GMP (Good Manufacturing Practice) principles and international guidelines (e.g. ICH Q2 (R1) were performed.

6. Materials and methods

6.1.   Alpha technology (Kit Cat. Number AL208C, PerkinElmer)

Kit content:

- AlphaLISA Anti-TNFα Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2, 50 μL, 5 mg/mL concentrated (1 brown tube, white cap);

- Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Proclin-300, pH 7.4, 200 μL, 5 mg/mL concentrated (1 brown tube, black cap);

- Biotinylated Antibody Anti-TNFα stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4, 50 μL, 500 nM concentrated (1 tube, black cap);

- AlphaLISA human TNFα (0.1 μg), lyophilized analyte (1 tube, clear cap);

- AlphaLISA Immunoassay Buffer (10X), 10 mL, (1 small bottle).

In addition, white ½ area plate-96 (cat #6005560) are required to perform the tests.

6.1.1.   Assay procedure

All tubes (including lyophilized analyte) were centrifuged before use to improve recovery of content (2000 g, 10-15 sec). All reagents were resuspended by vortexing before use.

Milli-Q® grade H_2O (18 MΩ•cm) was used to dilute 10X AlphaLISA Immunoassay Buffer and to reconstitute the lyophilized analyte.

The standard curve was prepared in FBS (Fetal Bovine Serum) and the samples were diluted at least 2-fold with FBS before testing.

Standards were assayed in triplicate.

6.1.2.   Reagents preparation

Reagents and samples were prepared as reported below:

- 1X **AlphaLISA Immunoassay Buffer** was prepared by adding 300 µL of 10X AlphaLISA Immunoassay Buffer to 2700 µL of Milli-Q® grade H_2O.

- **Lyophilized human TNFα analyte** was reconstituted in 100 µL Milli-Q® grade H_2O and standard dilutions were prepared as follow:

Tube	Volume of human TNFα (µL)	Volume of FBS (µL)	human TNFα concentration in standard curve (pg/mL in 5 µL)
A (STD 1)	10 µL of reconstituted human TNFα	45	100 000
B (STD 2)	30 µL of tube A	70	30 000
C (STD 3)	30 µL of tube B	60	10 000
D (STD 4)	30 µL of tube C	70	3 000
E (STD 5)	30 µL of tube D	60	1 000
F (STD 6)	30 µL of tube E	70	300
G (STD 7)	30 µL of tube F	60	100
H (STD 8)	30 µL of tube G	70	30
I (STD 9)	30 µL of tube H	60	10
J (STD 10)	30 µL of tube I	70	3
K (STD 11)	30 µL of tube J	60	1
L (STD 12)	30 µL of tube K	70	0.3
M (background) STD 0	0	50	0

Table 1. AlphaLISA standard curve preparation.

Standards were diluted in FBS. Standard curve was assessed in triplicate.

- 5X **AlphaLISA Anti-TNFα Acceptor beads** (50 µg/mL) were prepared by adding 6 µL of 5 mg/mL AlphaLISA Anti-TNFα Acceptor beads to 594 µL of 1X AlphaLISA Immunoassay Buffer.

- 5X **Biotinylated Antibody Anti-TNFα** (5 nM) was prepared by adding 6 µL of 500 nM Biotinylated Antibody Anti-TNFα to 594 µL of 1X AlphaLISA Immunoassay Buffer.

- 2X **Streptavidin (SA) Donor beads** (80 µg/mL): the beads were kept under subdued laboratory lighting and prepared by adding 24 µL of 5 mg/mL SA-Donor beads to 1476 µL of 1X AlphaLISA Immunoassay Buffer.

6.1.3. Assay protocol

The assay was performed according to the high sensitivity protocol as reported below:

1) 5 μL of each analyte standard dilution or 5 μL of sample were added to a 96-well microplate.

2) 10 μL of 5X Anti-Analyte Acceptor beads (10 μg/mL final) were added to the plate.

3) The plate was incubated 30 minutes at 22˚± 2 °C in a precision cooled incubator.

4) 10 μL of 5X Biotinylated Antibody Anti-analyte (1 nM final) were added to the plate.

5) The plate was incubated for 60 minutes at 22˚± 2 °C in a precision cooled incubator.

6) 25 μL of 2X SA-Donor beads (40 μg/mL final) were added to the plate.

7) The plate was incubated for 30 minutes at 22˚± 2 °C in the dark in a precision cooled incubator.

8) The plate was finally read using EnSpire-Alpha Reader. The standard protocol was used without any modification (Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 8 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).

Data analyses were performed with Soft Max Pro GxP software.

6.2. Singulex technology (kit Cat number 03-0088-00, Millipore):

Kit content:

- TNFα Coated Beads, component part number 02-0598-00
- Standard Diluent, component part number 02-0225-01
- TNFα Detection Antibody, component part number 02-0597-00
- Assay Buffer, component part number 02-0306-00
- Human TNFα standard, component part number 02-0280-00
- Erenna® Human TNFα Immunoassay Kit Instructions, component part number 05-0453-01
- 10X Wash Buffer, component part number 02-0001-06
- Elution Buffer B, component part number 02-0211-02
- Buffer D, component part number 02-0359-00

All reagents were stored at 2-8 °C except for human TNFα standard.

Additional reagents:

- Erenna® 10X Systems Buffer (1 L), component part number 02-0111-00
- Erenna® 10X Wash Buffer (1 L), component part number 02-0514-00
- Acroprep ™ 96-well filter plates, PALL, cat n° 5041
- 96-Well Clear V-Bottom Polypropylene Deep Well Plate, Axygen®, cat n° P-96-450V-C)

6.2.1. Assay procedure

The following reagents were warmed to room temperature prior to use: Standard Diluent, Assay Buffer, Coated Beads, Elution Buffer B, Buffer D, Detection Antibody and 10X Wash Buffer.

The Detection Antibody was stored away from light until ready to use.

1X Wash Buffer (from 10X Wash Buffer) was prepared as follows: 30 mL of 10X Wash Buffer were added to 270 mL of deionized water. The solution obtained was mixed thoroughly by gentle inversion.

TNFα Coated Beads (coated microparticles) were mixed on a spin rotator, for 10 minutes until all MPs were completely resuspended.

Standards and quality controls were assayed in triplicate while unknown samples were analyzed in duplicate.

6.2.2. Reagents Preparation

6.2.2.1. Standard curve preparation

The TNFα Standard Analyte vial was spin down in a mini-centrifuge prior to opening and the Working Stock solution, was prepared at 50 pg/mL in a 1 mL final volume with standard diluent.

The standard curve was prepared by serial diluting 1:2 the Working Stock from well 2 to well 11 of a dilution plate, to achieve a curve from 50 pg/mL to 0.049 pg/mL as described below and reported in the *Figure 16*:

1) 500 μL Standard Diluent was added to wells 2 through 12 of a 12-channel reservoir dilution plate.

2) 1000 μL of the 50 pg/mL Analyte Working Stock were added from standard preparation into well 1.

3) Well 1 was thoroughly mixed and then 500 μL from well 1 was transferred into well 2. Serial dilutions were continued from well 2 stopping at well 11. A fresh tip was used with each transfer.

Well 12 not contain any standard analyte and was run as zero standard. The standards were run in triplicate.

1	2	3	4	5	6	7	8	9	10	11	12
50	25	12.50	6.25	3.13	1.56	0.78	0.39	0.195	0.098	0.049	0
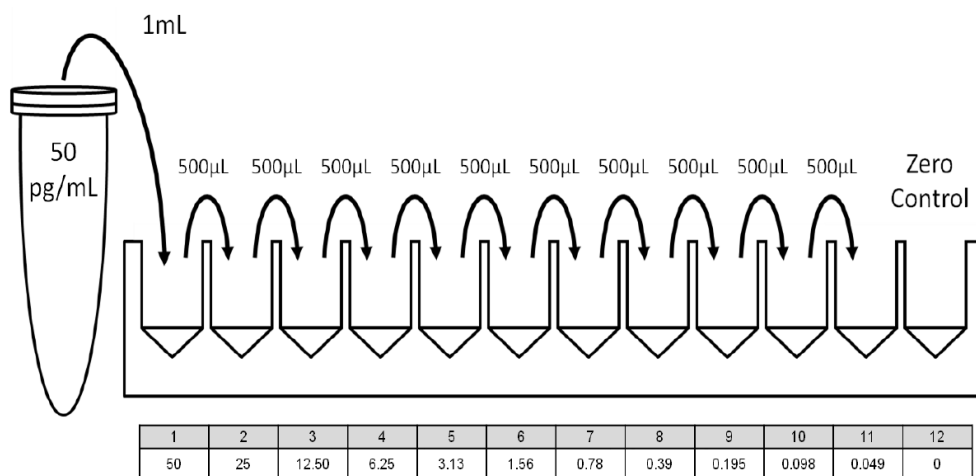
Figure 16. Standard curve preparation scheme

6.2.2.2. Quality controls Preparation

Lyophilized calibrator was reconstituted with 500 µl of Standard diluent obtaining a standard stock solution (SSS) used to prepare Quality controls as in the table below:

Starting solution	Starting solution (µL)	Standard diluent (µL)	Pooled human serum	Final solution
SSS	10	990	/	WSA
WSA	10	990	/	WSB
WSA	10	490	/	WSC
WSC	25	/	475	QC 1
WSB	10	170	/	WSD
WSD	25	/	475	QC 2
WSD	12	28	/	WSE
WSB	25	/	475	QC 3

Table 2. Singulex Quality controls preparation.

For quality controls concentration refer to the table below:

Quality controls	Concentration (pg/mL)
QC 1	37.0
QC 2	1.0
QC 3	0.30

Table 3. Quality Controls concentration

6.2.2.3. Sample Preparation

Samples were prepared by the following method:

A filter plate with prefilter (Pall PN: 5041) was used on top of a 96-well receptacle plate: 200 µL of each sample were placed into the filter plate well and briefly centrifuged to spin down the liquid for ≥ 10 minutes at 1,100 x g.

6.2.2.4. Selectivity samples preparation

TNF-α Standard stock was reconstituted with 1 mL of Standard Diluent to produce a standard stock solution in diluent (SSS) at 3.7 mg/mL.

SSS will be diluted at a concentration of 20.6 pg/mL using the dilution reported in the table below:

Starting Solution	Spiked volume (µL)	Indvidual Human Serum (µL)	Concentration pg/mL	Final solution
SSS	10	990	37000	WSA
WSA	10	990	370	WSB
WSB	10	170	20.6	WSD

Table 4. Working solution preparation for selectivity samples.

Each individual sample was run un-spiked and spiked with a concentration equal to 1.0 pg/mL as described below:

Starting Solution	Spiked volume (µL)	Indvidual Human Serum (µL)	Concentration pg/mL
WSD	10	190	1.0

Table 5. Selectivity samples preparation.

6.2.3. Assay protocol

1) Target Capture

- 100µL per well of Standards or Samples was added to Plate 1 (96-well PolyPropylene).

- Microparticles (MPs) was mixed by gentle inversion until all MPs were completely resuspended.

- Immediately before adding to the assay plate, 10.5 mL of Assay Buffer were added to the vial of TNFα Coated Bead and the solution was mixed by gentle inversion.

- 100 µL per well of the TNFα Coated Beads were pipetted into Plate 1 and the plate was covered with an Axyseal plate cover.

- The plate was incubated for 1 hour at $22 \pm 2°C$ on a horizontal orbital shaker set at 800 ± 50 rpm.

- Approximately 10 minutes prior to the end of Target Capture incubation, the TNFα Detection Antibody was prepared: 10µL of the Detection Antibody was added to 90 µL of Assay Buffer to make a 1:10 dilution. Then 30µL of the diluted Detection Antibody was added to 2970 µl of Assay Buffer.

2) Post-Capture Wash

Post-capture wash was performed with a Bio Plex Pro II wash station (program PCW-v1).

3) Detection

- 20 µL per well of TNFα Detection Antibody were added to Plate 1 and the plate was covered with an Axyseal plate cover.
- The plate was incubated for 30 minutes at $22 \pm 2°C$ on a horizontal orbital shaker set at 800 ± 50 rpm.

4) Pre-TransferWash

Pre-transfer wash was performed with a Bio Plex Pro II wash station (program PTW-v1).

5) Plate Transfer

Plate transfer was performed with a microplate processor precision XS diluitor, BioTek (program Erenna 96-96).

6) Final Aspiration

Final aspiration was performed with a Bio Plex Pro II wash station.

7) Elution

- 10 µL of Elution Buffer B per well were added to plate 2 and the plate was covered with an AxySeal plate cover.
- The plate was incubated for 10 minutes at $22 \pm 2°C$ on a horizontal orbital shaker set at 800 ± 50 rpm.
- 10 µL per well of Buffer D was added to assay Plate 3 (384-well polypropylene plate, Nunc, PN 264573) using a 12-channel manual P20.
- Plate 2 was placed on bar magnet bed, AxySeal plate cover was removed, and MPs was left to form a tight pellet for 2 min.
- Eluate was transferred from Plate 2 to Plate 3 by columns, avoiding the pelleted MPs.
- Plate 3 was covered with a Universal Plate Cover and centrifuged 5 minutes at $22 \pm 2°C$, approximately 1,100 x g.

- Plate 3 was covered with a Heat Sealing Foil using an heat sealer.

8) Run on Erenna®Immunoassay System

Assay Plate 3 was finally loaded onto the Erenna® Immunoassay System.

Data analyses were performed with Sgx link™ software.

6.3. MSD technology (Kit Cat. Number K15049D, Mesoscale discovery)

Proinflammatory panel 1 human kit was used.

Kit content:

- **Proinflammatory Panel 1 (human) Calibrator Blend** (catalog # C0049-2, 5vial): recombinant human proteins in diluent, buffered and lyophilized. Individual analyte concentration is provided in the lot specific certificate of analysis. The calibrator should be stored at 2–8°C.

- **Diluent 2** (catalog # R51BB-3, 1 bottle containing 40 mL): Diluent for samples and calibrator; contains serum, blockers, and preservatives.

- **Diluent 3** (catalog # R51BA-5, 1 bottle containing 25 mL): Diluent for detection antibody; contains protein, blockers, and preservatives.

Diluent 2 and diluent 3 should be stored at ≤ -10°C

- **Read Buffer T** (4X) (catalog # R92TC-3, 1 bottle containing 50 mL): Buffer to catalyze the electro-chemiluminescence reaction. Read buffer should be stored at room temperature.

Additional reagents:

- **Cytokine Panel 1 (human) Control 1, Cytokine Panel 1 (human) Control 2 and Cytokine Panel 1 (human) Control 3** (catalog # C4050-1, 5 vials each): Multi-analyte controls in a non-human matrix, buffered, lyophilized, and spiked with recombinant human analytes. The concentration of the controls is provided in the lot-specific COA. Controls should be stored at room temperature.

- **Wash Buffer** (20X) (catalog # R61AA-1, 1 bottle containing 100 mL): 20-fold concentrated phosphate buffered solution with surfactant.

- **Plate Seals**: Adhesive seals for sealing plates during incubations.

6.3.1. Assay procedure

All reagents were brought to room temperature.

Upon first thaw, Diluent 2 and Diluent 3 were aliquot into suitable volumes before refreezing.

Standards and quality controls were assayed in duplicate while unknown samples were analyzed as a single point.

6.3.2. Reagents preparation

6.3.2.1. Calibrator dilutions preparation (standard curve)

MSD supplies a multi-analyte lyophilized calibrator that yields the recommended highest calibrator concentration (369 pg/mL) when reconstituted in 1,000 μL of Diluent 2.

Calibrator solutions plus a zero calibrator were prepared as below:

- The highest calibrator (Calibrator 1) was prepared by adding 1,000 μL of Diluent 2 to the lyophilized calibrator vial. After reconstituting, was inverted at least 3 times. The reconstituted solution was equilibrated at room temperature for 15-30 minutes and then vortexed briefly using short pulses.

- The next calibrator was prepared by transferring 100 μL of the highest calibrator to 300 μL of Diluent 2. The solution was well mixed by vortexing. 4-fold serial dilutions were repeated 5 additional times to generate 7 calibrators (STD 1 – STD 7) as reported in *Figure 17*.

- Diluent 2 was used as the zero calibrator (STD 0).
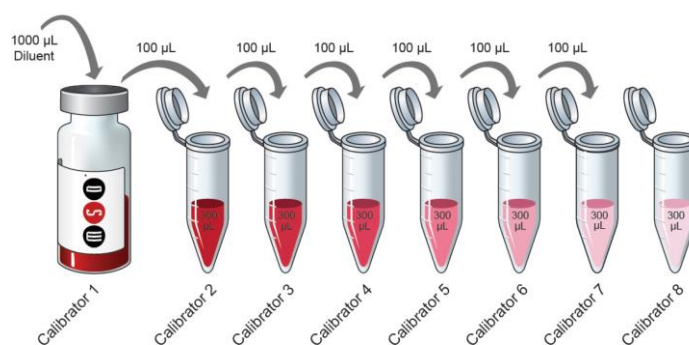
Figure 17. *Dilution schema for Calibrator Standards preparation.*

Standards concentration are reported in the table below:

Sample ID	Concentration (pg/mL)
STD 1	369.00
STD 2	92.25
STD 3	23.06
STD 4	5.77
STD 5	1.44
STD 6	0.36
STD 7	0.09
STD 0	0

Table 6. Calibrator Standards concentration.

6.3.2.2.Quality controls preparation

Lyophilized calibrator was reconstituted with 1 mL of Diluent 2 obtaining a standard stock solution (SSS) used to prepare Quality controls as in the table below:

Starting solution	Starting solution (µL)	Diluent 2 (µL)	Pooled human serum	Final solution
SSS	10	/	290	QC 2
SSS	10	220	/	WSA
WSA	10	/	290	QC 3
WSA	20	40	/	WSB
WSB	10	/	290	LLOQ

Table 7. *Quality Controls preparation.*

For quality controls concentration refer to the table below:

Quality controls	Concentration (pg/mL)
QC 2	12.30
QC 3	0.53
LLOQ	0.18

Table 8. Quality Controls concentration.

6.3.2.3. Samples preparation

Samples were 2-fold diluted with Diluent 2 by adding 60 µL of sample to 60 µL of Diluent 2.

6.3.2.4. Selectivity samples preparation

TNF-α Standard stock was reconstituted with 1 mL of Diluent 2 to produce a standard stock solution in diluent (SSS-D) at 369 pg/mL.

SSS will be diluted at a concentration of 16 pg/mL using Diluent 2 (e.g. 10 µL + 220 µL Diluent 2) to produce WSA.

Starting Solution	Spiked volume (µL)	Indvidual Human Serum (µL)	Concentration pg/mL
WSA	10	290	0.53

Table 9. Selectivity samples preparation.

6.3.2.5. Detection Antibody Solution preparation

Each detection antibody was provided as a 50X stock solution. The working solution is 1X. The detection antibody solution was prepared immediately prior to use by combining 60 µL of sulfo-tag Anti-human TNF-α Antibody to 2,400 µL of Diluent 3.

6.3.2.6. Wash Buffer preparation

100 mL of Wash Buffer as a 20X stock solution are provided in the V-PLEX kit. The working solution is 1X. For one plate were combined 15 mL of wash buffer (20X) with 285 mL of MilliQ grade water.

6.3.2.7. Read Buffer T preparation

Read Buffer T is provided as a 4X stock solution. The working solution is 2X.

For one plate, 10 mL of Read Buffer T (4X) were added to 10 mL of MilliQ® grade water.

6.3.3. Assay Protocol

Reagents were prepared as instructed before beginning the assay protocol.

1) **STEP 1: Wash and Add Sample**

   - The plate was washed 3 times with at least 150 μL/well of Wash Buffer.
   - 50 μL of prepared samples, calibrators, or controls were added per well. The plate was sealed with an adhesive plate seal and incubated at room temperature with shaking for 2 hours.

2) **STEP 2: Wash and Add Detection Antibody Solution**

   - The plate was washed 3 times with at least 150 μL/well of Wash Buffer.
   - 25 μL of detection antibody solution were added to each well. The plate was sealed with an adhesive plate seal and incubated at room temperature with shaking for 2 hours.

3) **STEP 3: Wash and Read**

   - The plate was washed 3 times with at least 150 μL/well of Wash Buffer.
   - 150 μL of 2X Read Buffer T were added to each well. The plate was analyzed on an MSD instrument. Incubation in Read Buffer T is not required before reading the plate.

Data analyses were performed with Discovery Workbench software.

6.4. Luminex technology: R&D systems kit (cat. number LHSCM000)

Magnetic Luminex® Performance Assay Human High Sensitivity Cytokine Base Kit A contains:

- **Human HS Cytokine Panel A Standard Cocktail 1**: 2 vials of recombinant human cytokines in a buffered protein base with preservatives; lyophilized.
- **Microparticle Diluent**: 6 mL of a buffered protein base with blue dye and preservatives.
- **Biotin Antibody Diluent 2:** 5.5 mL of a buffered protein base with preservatives.
- **Calibrator Diluent RD6-40:** 21 mL of buffered protein base with preservatives. *May contain a precipitate. Mix well before and during use.*
- **Streptavidin-PE**: 5.5 mL of a 1X streptavidin-phycoerythrin conjugate with preservatives.
- **Wash Buffer Concentrate**: 21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow after time.*
- **Microplate**: 1 flat-bottomed 96-well microplate used as a vessel for the assay
- **Mixing Bottles**: 2 empty 8 mL bottles used for mixing Microparticles with Microparticle Diluent.
- **Plate Sealer**: 6 adhesive foil strips.
- **Standard Value Card**: 1 card listing Standard Cocktail reconstitution volume and working standard concentration.

Additional reagents:

- Magnetic Luminex® Performance Assay Human TNF-alpha High Sensitivity Kit (R&D systems Cat.Number LHSCM210) that contains:
  - **TNF-alpha Magnetic Microparticle Concentrate:** 0.075 mL of a 50X concentrated microparticle stock solution with preservatives.

    o **TNF-alpha Biotin-Ab Concentrate:** 0.075 mL of a 100X concentrated biotin antibody stock solution with preservatives.

6.4.1. Assay procedure

Calibrator Diluent RD6-40 and Microparticle Diluent were brought to room temperature while the remains reagents were brought to room temperature 5 minutes before make dilutions.

Standards and quality controls are assayed in duplicate while unknown samples are analyzed as a single point.

6.4.2. Reagents preparation

6.4.2.1.Standard Cocktail Solution preparation

Human HS Cytokine Panel A Standard Cocktail was reconstituted with 900 µl of Calibrator Diluent RD6-40. The standard cocktail solution was sit for a minimum of 15 minutes with gentle agitation prior to making dilutions and then the standard was transferred to appropriately labelled polypropylene tube.

500 µL of reconstituted Standard were pipetted into a Standard 1 tube and 300 µL of Calibrator Diluent RD6-40 into the remaining tubes. Standard 1 was used to produce a 4-fold dilution series (as in the *Figure 18*). Each tube was mixed thoroughly before the next transfer. Standard 1 serves as the high standard. Calibrator Diluent RD6-40 serves as the blank (STD 0).
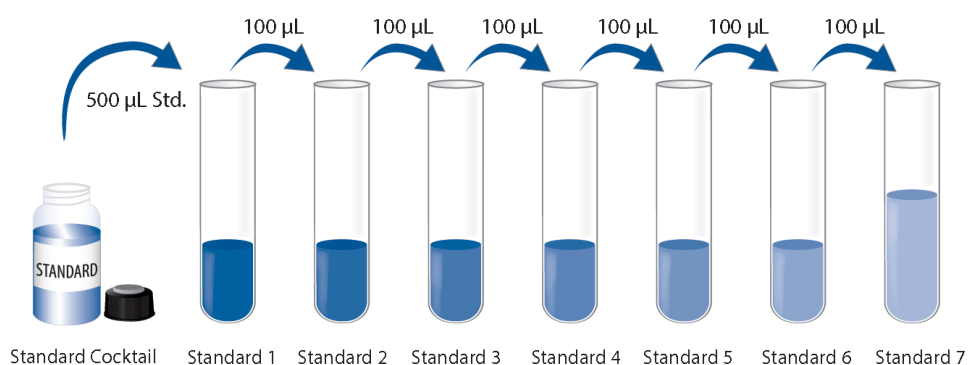
Figure 18. *Standard curve dilutions preparation scheme.*

Calibration Standard Solution concentrations are reported in the table below:

Calibration standard solutions	TNF-alpha (pg/mL)
STD 1	3550.00
STD 2	887.50
STD 3	221.88
STD 4	55.47
STD 5	13.87
STD 6	3.47
STD 7	0.87

Table 10. *Calibration standards concentration.*

6.4.2.2. Quality Control preparation

Quality controls must be prepared starting from the Standard Cocktail Solution in pooled human serum as described in the table below:

Starting Solution	Spiked Volume (µl)	Calibrator Diluent RD6-40 (µl)	Pooled Human Serum (µl)	Quality Controls working solution
Standard Cocktail Solution	10	-	190	QC1
Standard Cocktail Solution	10	50	-	WSA
WSA	10	-	190	QC2
Standard Cocktail Solution	10	290	-	WSB
WSB	10	-	190	QC3

Table 11. *Quality Controls preparation.*

Quality Controls and corresponding blank (QC blank) were 1:2 diluted in Calibrator Diluent RD6-40 prior to analysis (125 µl of QCs + 125 µl of Calibrator Diluent RD6-40).

For QCs concentration of each cytokine refer to the table below:

Quality Control	TNF-alpha (pg/mL)
QC1	177.50
QC2	29.58
QC3	5.92

Table 12. Quality Controls concentration.

6.4.2.3. Unknown samples preparation

Unknown samples were 1:2 diluted in Calibrator Diluent RD6-40 prior to analysis (70 µl of sample + 70 µl of Calibrator Diluent RD6-40).

6.4.2.4. Wash Buffer preparation

20 mL of Wash Buffer Concentrate were mixed with 480 mL of MilliQ water to prepare 500 mL of Wash Buffer.

6.4.2.5. Diluted Microparticle cocktail preparation

The Microparticle Concentrate vial was vortexed for 30 seconds to resuspend the microparticles, taking precaution not to invert the vial. The Microparticle Concentrate was diluted in the mixing bottle provided.

Refers to the table below as an example of volumes used:

Number of Well Used	TNF-alpha Microparticle Concentrate (μL)	Microparticle Diluent (μL)	Total Volume (μL)
96	50	2450	2500
72	40	1960	2000
48	25	1225	1250
24	20	980	1000

Table 13. Microparticle preparation.

Note: microparticles were protected from light during handling.

6.4.2.6. Diluted Biotin Antibody Cocktail preparation

The Biotin Antibody Concentrate vial was gently vortexed and diluted in Biotin Antibody Diluent 2 with gently mixing.

Refers to the table below as an example of volumes used:

Number of Well Used	TNF-alpha Biotin Antibody Concentrate (μL)	Biotin Antibody Diluent 2 (μL)	Total Volume (μL)
96	50	4950	5000
72	35	3465	3500
48	25	2475	2500
24	20	1980	2000

Table 14. Antibody preparation.

6.4.3. Assay Protocol

1) All reagents, calibration standard solution, QCs, QC blank and unknown samples were prepared as described in the previous sections.

2) The diluted microparticle cocktail was resuspended by vortexing. 25 µl of the microparticle cocktail were added to each well.

3) Add 100 µl of Calibration Standard, QCs, QC blank or unknown samples were added to the appropriate well. The assay plate were prepared within 15 minutes and covered with a foil plate sealer. The plate was incubated for 3 hours at $22 \pm 2°C$ on a horizontal orbital microplate shaker set at 800 ± 50 rpm.

4) At the end of the incubation, the plate was washed three times with the Bio-Plex Pro II Wash Station (program MAG 3X).

5) 50 µl of diluted Biotin-Antibody Cocktail were added to each well. The plate was covered with a new foil plate sealer and it was incubated for 1 hour at $22 \pm 2°C$ on a horizontal orbital microplate shaker set at 800 ± 50 rpm.

6) At the end of the incubation, a wash was performed for three time with the Bio-Plex Pro II Wash Station (program MAG 3X).

7) 50 µl of Streptavidin-PE were added to each well. The plate was covered with a new foil plate sealer and it was incubated for 30 minutes at $22 \pm 2°C$ on a horizontal orbital microplate shaker set at 800 ± 50 rpm.

8) At the end of the incubation, a wash was performed for three time with the Bio-Plex Pro II Wash Station (program MAG 3X).

9) The microparticles were resuspended by adding 100 µl of Wash Buffer to each well. The plate was ncubated for 2 minutes at $22 \pm 2°C$ on a horizontal orbital microplate shaker set at 800 ± 50 rpm.

10) The plate was read within 90 minutes using the Luminex analyser (instrument settings: TNF-alpha microparticle region 12; 50 beads per region; Low RP1 Target; DD gates values between 5000 and 25000; STD curve in 5PL equation fitting)

Data analyses were performed with Bio-plex Manager™ software.

6.5.   Bio-rad kit evaluation (cat. number 17000851)

The Bio-plex Pro™ Human Th17 Cytokine Assay kit components are:

- Assay Buffer (50 mL);
- Wash Buffer (200 mL);
- Standard Diluent HB (10 mL);
- Sample Diluent HB (8 mL);
- Detection Antibody Diluent (5 mL);
- Streptavidin-PE (100x, 1 tube);
- 96-well flat-bottom plate;
- Sealing tapes

In addition, Bio-Plex Pro™ Human Th17 Standard (cat n° 171DA0501) and Bio-Plex Pro Human Cytokine TNF-α Set (coupled magnetic beads and detection antibody cat n° 171B5026M) are required.

6.5.1. Assay procedure

6.5.2.   Reagents preparation

- Quality Controls were prepared in pooled human serum at the beginning of the study as ready-to-use single aliquots and used throughout the study to monitor assay performance.
- QCs and unknown samples were tested after 1:4 dilution with Sample Diluent HB.
- Standard curve was prepared in matrix (pooled healthy human serum) 25%. To obtain matrix 25% pooled healthy human serum was 1:4 diluted in Sample Diluent HB (300 µL pooled serum + 900 µL Sample Diluent). It was freshly prepared for each analytical run.

- Standards and quality controls are assayed in duplicate while unknown samples are analyzed as a single point.

- Coupled Magnetic Beads stock, Detection Antibodies stock and Streptavidin-PE are provided as a concentrate and were diluted prior to use. Coupled Magnetic Beads stock is 10X concentrate and was diluted e.g. for 96 well plate use 575 µL of 10X stock concentrate + 5,175 µL of Assay buffer to obtain the beads working solution. Detection Antibodies stock is provided as a concentrate (10x) and was diluted prior to use (e.g. for 96 well plate use 300 µL of 10X stock concentrate + 2,700 µL of Assay buffer) to obtain the detection Antibody working solution.

- Streptavidin-PE working solution is provided as a concentrate (100x) and was prepared by adding e.g. for 96 well plate use 60 µL of 100X stock concentrate + 5,940 µL of Assay buffer.

6.5.2.1. Calibration Standards preparation

The lyophilized Standard Stock was reconstituted with 781 µL of pooled healthy human serum (Standard Stock Solution at 9,451.0 pg/mL using lot 5041945).

The vial was then gently vortexed for about 5 seconds and incubated on ice for 30 min.

Standard X (STD-X) at 4,764 pg/mL was prepared by adding 124.0 µL of Standard Stock Solution to 122.0 µL of pooled healthy human serum.

Standard 1 at a concentration of 1,191.0 pg/mL (STD-1) was prepared by adding 100.0 µL of STD-X to 300.0 µL of Sample Diluent HB.

Calibration standards were freshly for each run as described in the following table:

Starting Solution	Spike Volume (μL)	Matrix 25% (μL)	Calibration standard ID	Final standard concentration (pg/mL)
---	---	---	STD-1	1,191.0
Standard 1 (STD-1)	70	140	STD-2	397.0
Standard 2 (STD-2)	70	140	STD-3	132.3
Standard 3 (STD-3)	70	140	STD-4	44. 1
Standard 4 (STD-4)	70	140	STD-5	14.7
Standard 5 (STD-5)	70	140	STD-6	4.9
Standard 6 (STD-6)	70	140	STD-7	1.6
Standard 7 (STD-7)	70	140	STD-8	0.5
-	-	140	Blank (B)	-

Table 15. Calibration standards preparation and concentration.

6.5.2.2. Quality Controls preparation

Quality Controls were prepared at the beginning of the study as single ready-to-use aliquots and used throughout the study to monitor assay performance.

Quality Controls were prepared starting from Standard Stock Solution in pooled healthy human serum as described in the table below:

Starting Solution	Spike Volume (μL)	Pooled Human Serum (μL)	Quality Control
Standard Stock Solution [9,451.0 pg/mL]	496	488	WSA
WSA	510	170	QC 1
WSA	15	690	QC 2
WSA	10	190	WSB
WSB	25	720	QC 3

Table 16. Quality controls preparation.

QCs were 1:4 diluted before analyses using Sample Diluent HB (e.g. 30 µl + 90 Sample Diluent HB µl).

For QCs concentration of each cytokine refer to the table below:

Quality Control	TNF-alpha (pg/mL)
QC1	3572.9
QC2	101.4
QC3	8.0

Table 17. Quality controls concentration.

6.5.2.3. Selectivity samples preparation

TNF-α Standard stock were reconstituted with 781 µL of Standard Diluent HB to produce a standard stock solution in diluent (SSS-D) at 9,451.0 pg/mL.

SSS-D were diluted at a concentration of 2,028 pg/mL using Standard Diluent (100 µL + 366 µL Standard Diluent) to produce WSA-D.

Each individual sample were run un-spiked and spiked with a concentration equal to 101.4 pg/mL as described below:

Starting Solution	Spiked volume (µL)	Indvidual Human Serum (µL)	Concentration pg/mL
WSA-D	5	95	101.4

Table 18. Selectivity samples preparation.

Selectivity samples (Ind-SS) were diluted 1:4 before analyses using Sample Diluent HB (30 µl + 90 µl Sample Diluent HB).

6.5.2.4. Unknown Samples preparation

Unknown samples were 1:4 diluted before analyses using Sample Diluent HB (e.g. 20 µl + 60 Sample Diluent HB µl).

6.5.3. Assay Protocol

1) The beads working solution was vortexed for 30 seconds immediately prior to use in assay.

2) 50 µL of the diluted Beads solution were added into each well.

3) The plate was washed twice with the BioPlex PRO II wash station (command MAG 2X).

4) The diluted standards, Blank, quality controls and unknown samples were gently vortexed for at least 3 sec.

5) 50 µL of each calibration standard, Blank, QCs and unknown samples were added to the appropriate wells and the plate was covered with the sealing tape and protected from light with aluminum foil.

6) The plate was incubated for 1 hour at $+22\pm2°C$ on an orbital shaker, the shaker speed was slowly increased up to 850 ± 50 rpm.

7) In the meanwhile, the Detection Antibody working solution was prepared as described above.

8) At the end of the incubation, the plate was washed three times with the BioPlex PRO II wash station (command MAG 3X).

9) 25 µL of Detection Antibody working solution to each well and the plate was covered with the sealing tape and protected from light with aluminum foil.

10) The plate was incubated for 30 minutes at $+22\pm2°C$ on an orbital shaker. The shaker speed was slowly increased up to 850 ± 50 rpm.

11) In the meanwhile, streptavidin-PE working solution was prepared as described above.

12) At the end of the incubation, the plate was washed three times with the BioPlex PRO II wash station (command MAG 3X).

13) 50 µL of Streptavidin-PE working solution were added to each well and the plate was covered with the sealing tape and protected from light with aluminum foil.

14) The plate was incubated for 10 minutes at $+22\pm2°C$ on an orbital shaker. The shaker speed was slowly increased up to 850 ± 50 rpm.

15) The Luminex 200™ instrument was prepared during this incubation step according to the relevant Working Instruction of the equipment.

16) At the end of the incubation, the plate was washed three times with the BioPlex PRO II wash station (command MAG 3X) and kept shielded from light.

17) 125 µL of assay buffer were added to each well and the entire plate was covered with the plate sealing tape. The plate was shaked on an orbital shaker (500 ± 50 rpm) for 30 seconds to resuspend the beads.

18) The plate was uncovered and then inserted into the XY platform of the Luminex 200™ instrument and the samples were analysed.

19) The plate was read within 90 minutes using the Luminex analyzer.

Data analyses were performed with Bio-Plex Manager™ software.

6.6. Plate Format

A typical plate scheme is reported below: Calibration standard solutions, blank and quality controls are usually analysed in duplicate, while unknown samples as single point. Plate scheme could vary depending on the technology, the standards number and the replicates numbers.

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD-1		Blank (B)		sample	sample	sample	sample	sample	sample	sample	sample
B	STD-2		QC 1		sample	sample	sample	sample	sample	sample	sample	sample
C	STD-3		QC 2		sample	sample	sample	sample	sample	sample	sample	sample
D	STD-4		QC 3		sample	sample	sample	sample	sample	sample	sample	sample
E	STD-5		Sample diluent HB		sample	sample	sample	sample	sample	sample	sample	sample
F	STD-6		sample	sample	sample	sample	sample	sample	sample	sample	sample	sample
G	STD-7		sample	sample	sample	sample	sample	sample	sample	sample	sample	sample
H	STD-8		sample	sample	sample	sample	sample	sample	sample	sample	sample	sample

Table 19. Plate format scheme.

7. Results

7.1. TNFα quantification using AlphaLISA technology

All the results were carry out using the SoftMax Pro GxP and Excel software.

7.1.1. Calibration standard curve

Standard curve was prepared as described in *section 6.1.2.*. Three replicates for each calibrator standard were analyzed. The ACC% of each replicate for each standard point was calculated according to the following formula:

$$ACC\% = \frac{ObsConc}{ExpConc} \times 100$$

The results are reported in the table below:

Calibrator Standard	Exp Conc (pg/mL)	Raw Values	Signal / Background (S/B)	Obs Conc (pg/mL)	ACC%
STD 0	0	528	na	na	na
		517		na	na
		392		na	na
STD 1	100000	274339	586.0	90424.7	90.4
		274948		91252.6	91.3
		292733		123137	123.1
STD 2	30000	175211	366.0	30157.3	100.5
		173262		29601.6	98.7
		177428		30802.5	102.7
STD 3	10000	72844	150.1	9949.7	99.5
		70213		9577.3	95.8
		72621		9918	99.2
STD 4	3000	20910	45.1	3069.8	102.3
		22064		3220.0	107.3
		21875		3195.5	106.5
STD 5	1000	6788	14.2	1136.1	113.6
		6945		1159.6	116.0
		6638		1113.6	111.4
STD 6	300	2020	4.4	355.9	118.6
		2229		394.9	131.6*
		2079		367	122.3
STD 7	100	848	2.7	111.1	111.1
		882		119.4	119.4
		814		102.8	102.8
STD 8	30	490	1.1	7.8	26.1*
		270		na	na
		270		na	na
STD 9	10	266	0.5	na	na
		198		na	na
		136		na	na
STD 10	3	183	0.4	na	na
		201		na	na
		35		na	na
STD 11	1	159	0.2	na	na
		115		na	na
		-185		na	na

Table 20. Calibration standard curve results obtained with AlphaLISA technology. The calibration curves used to calculate analyte concentrations were established by fitting the signals from the calibrators to a 4-parameter logistic. Data shown from one test. The test was repeated several times obtaining the same results.

The standard curve was analysed several times in the same condition to evaluate the reproducibility and the method range. The high sensitive protocol, as indicated by the supplier, was used. However, in all the experiments done the sensitivity appeared to be not higher than 100 pg/mL (the Signal to background for this calibrator standard was 2.7).

Considering the low performance of the kit in terms of accuracy and reproducibility and the limitation in the reagents available in the kit which did not allow to modify the procedure, it was decided to hold on the evaluation of the AlphaLISA technology and to move to the evaluation of other technologies.

7.2. TNFα quantification using Singulex technology

All the results were carry out using the Sgx Link software. The data were analyzed and elaborated using Excel.

7.2.1. Calibration standard curve

Standard curve was prepared as described in *section 6.2.2.1.*. Three replicates for each calibrator standards were analyzed. Analyte concentration were obtained from three different signals generated from the labelled secondary antibody: Detected Event (DE), Event Photons (EP) and Total Photons (TP). The software merge the three signals using a proprietary algorithm and calculated the concentration of the standards.

The results are reported in the tables below:

Calibrator Standard	Exp Conc (pg/mL)	Obs Conc (pg/mL)	70.0 ≤ ACC% ≥ 130.0
STD 0	0	na	na
		na	na
		na	na
STD 1	50.0	53.111	106.2
		50.560	101.1
		SAT	Na
STD 2	25.0	30.340	121.4
		22.053	88.2
		30.334	121.3
STD 3	12.5	12.337	98.7
		11.032	88.3
		10.866	86.9
STD 4	6.25	6.817	109.1
		6.477	103.6
		6.592	105.5
STD 5	3.13	3.391	108.3
		2.799	99.4
		2.850	91.1
STD 6	1.56	1.630	104.5
		1.523	97.6
		2.421	155.2*
STD 7	0.78	0.798	102.3
		0.771	98.8
		0.633	81.2
STD 8	0.39	0.437	112.1
		0.398	102.1
		0.491	125.9
STD 9	0.20	0.214	107
		0.194	97.0
		0.173	86.5
STD 10	0.10	0.134	134.0*
		0.094	94.0
		0.116	116.0
STD 11	0.05	0.044	88.0
		0.060	120.0
		0.038	76.0

Table 21. Calibration standard curve results using Singulex technology. The calibration curves used to calculate analyte concentrations were established by fitting the signals from the calibrators to a 4-parameter logistic. (*) out of acceptance criteria. SAT: signal saturation. na: not applicable.

y = 599.56x + 41.21 R^2 = 0.9977   y = 137914.15x + -84161.41 R^2 = 0.9935
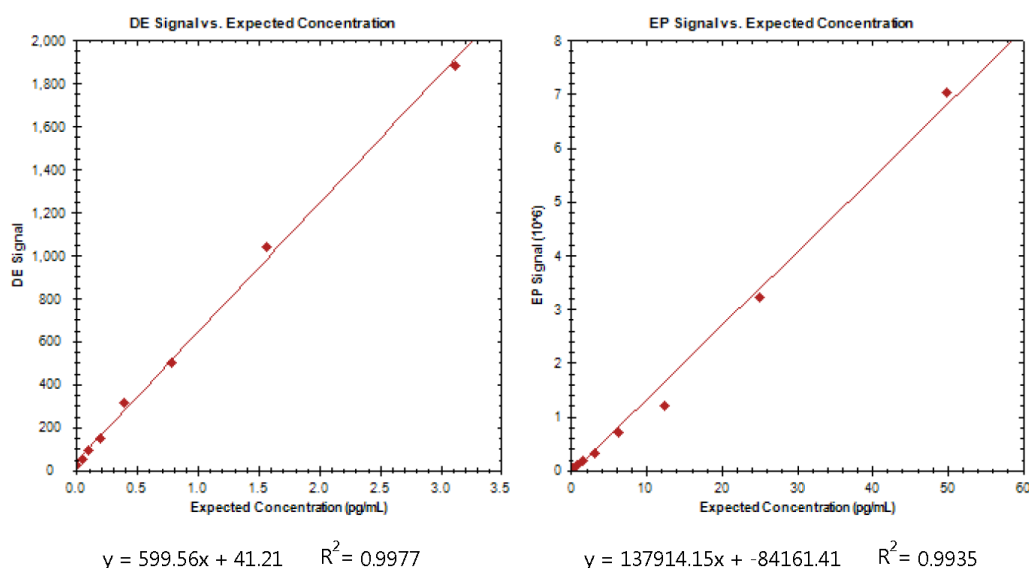
Figure 19. *Example of standard curve generated from the software. The two plots represent respectively the DE and the EP signals versus the expected concentration of the standard points (data from test 2).*

As showed in *Figure 19*, the calibration curve was repeated three times in the same experimental condition confirming a good accuracy and reproducibility.

7.2.2. Individual serum samples concentration

Twenty human serum samples were tested to evaluate the endogenous level of the biomarker of interest in the same analytical run. The samples were tested in duplicate. The CV% between the two replicates were calculated as below:

$$CV\% = \frac{Standard\ Deviation}{Mean\ Value} \times 100$$

 The results are reported in the tables below. For some samples, the CV% resulted to be higher than 30%, indicating a possible issue with method precision. The basal levels of the biomarkers of interest were between 0.071 to 2.534 pg/mL.

Sample Id	Conc. (pg/mL)	Average conc.	Conc. CV%	Sample Id	Conc. (pg/mL)	Average conc.	Conc. CV%
01 M	0.627	0.797	NR	01 F	1.074	1.027	6.4
	0.967				0.980		
02 M	1.182	0.688	NR	02 F	0.252	0.256	2.1
	0.194				0.260		
03 M	0.048	0.214	NR	04 F	2.171	2.383	12.6
	0.379				2.594		
05 M	0.773	0.804	5.5	05 F	0.326	0.343	6.8
	0.835				0.359		
07 M	0.244	0.231	8.4	06 F	0.168	0.197	NR
	0.217				0.225		
08 M	0.464	0.488	6.8	07 F	0.748	0.810	10.8
	0.512				0.872		
09 M	0.183	0.213	19.9	08 F	0.335	0.406	NR
	0.243				0.477		
10 M	0.083	0.071	NR	09 F	0.250	0.272	11.5
	0.059				0.294		
11 M	0.462	0.527	17.4	11 F	2.279	2.534	14.2
	0.592				2.788		
13 M	0.071	0.085	NR	12 F	0.505	0.526	5.5
	0.099				0.546		

Table 22. TNFα concentration in serum samples using Singulex technology.
Acceptance criteria: CV%≤30.0. NR: not reportable (CV out of acceptance criteria).

7.2.3. Accuracy

Quality controls were prepared as described in section 6.2.2.2..

Three independent replicates of each quality control were tested in two different analytical sessions. Accuracy % were calculated as described below:

$$ACC\% = \frac{ObsConc - BLK\ Conc}{ExpConc} \times 100 \qquad ACC\%\ ADD = \frac{ObsConc + BLK\ Conc}{ExpConc} \times 100$$

The results are reported in the tables below.

Sample ID	Obs Conc (pg/mL)	Average conc (pg/mL)	CV%	Exp conc (pg/mL)	Obs - BLK (pg/mL)	ACC%	Exp conc +BLK (pg/mL)	ACC% ADD
BLK	1.009	0.966	13.0	/	/	/	/	/
	0.824							
	1.064							
QC1	48.294	47.721	1.0	37	47.328	127.9*	37.966	127.2*
	47.411				46.445	125.5*		124.9*
	47.459				46.493	125.7*		125.0*
QC2	2.326	2.224	6.8	1	1.360	136.0*	1.966	118.3
	2.051				1.085	108.5		104.3
	2.296				1.330	133.0*		116.8
QC3	1.226	1.243	4.1	0.3	0.259	86.5	1.266	96.8
	1.203				0.237	79.1*		95.0
	1.300				0.334	111.2		102.7

Table 23. TNFα quality controls accuracy using Singulex technology.
Acceptance critera: ACC% between 70.0 and 130.0. (*) out of acceptance criteria. Data from test 01.

Sample ID	Obs Conc (pg/mL)	Average conc (pg/mL)	CV%	Exp conc (pg/mL)	Obs - BLK (pg/mL)	ACC%	Exp conc +BLK (pg/mL)	ACC% ADD
BLK	1.254	1.059	23.4	/	/	/	/	/
	0.780							
	1.142							
QC1	48.537	46.637	5.6	37	47.479	128.3	38.059	127.5*
	43.680				42.621	115.2		114.8
	47.694				46.636	126.0		125.3*
QC2	1.793	1.927	6.6	1	0.734	73.4	2.059	87.1
	1.940				0.881	88.1		94.2
	2.048				0.990	99.0		99.5
QC3	1.201	1.141	4.6	0.3	0.142	47.4*	1.359	88.4
	1.121				0.062	20.8*		82.5
	1.102				0.043	14.4*		81.1

Table 24. TNFα quality controls accuracy using Singulex technology.
Acceptance critera: ACC% between 70.0 and 130.0. (*) out of acceptance criteria. Data from test 02.

In both experiments the method resulted to be accurate in the quantification of known samples when the nominal concentration of the spiked samples plus the endogenous one was used as theoretical concentration to calculate the ACC%. The ACC% was indeed between 81.1 to 127.5.

7.2.4. Integrity of dilution

Integrity of dilution were tested using a pool of twenty human serum samples. The pool were tested pure and serially diluted 1:2 in standard diluent three times. Two replicates of each dilution point were tested. The ACC of each sample and the precision (CV%) of each back calculated concentration were calculated.

$$ACC\% = \frac{(Conc \ x \ dil \ fact) \ dil \ 1:2 \ or \ dil \ 1:4 \ or \ dil \ 1:8}{Average \ conc \ TQ} \ X \ 100$$

The results are reported in the tables below:

Sample ID	Conc (pg/mL)	Dil fact	Conc * Dil fact	Average Conc	CV%	ACC%	Total CV%
Pool TQ	0.850	1	0.850	0.883	5.2	/	
	0.915		0.915			/	
Pool dil 1:2	0.483	2	0.966	1.040	10.1	109.4	
	0.557		1.114			126.2	8.2
Pool dil 1:4	0.202	4	0.806	0.905	15.4	91.3	
	0.251		1.003			113.6	
Pool dil 1:8	0.132	8	1.059	1.053	0.9	120.0	
	0.131		1.047			118.5	

***Table 25.** Integrity of dilution results using Singulex technology. Acceptance criteria: ACC% between 70.0 and 130.0 and CV% ≤ 30.0. Data from test 02.*

The assessment was repeated twice showing a good performance in sample quantification since the ACC% of each diluted sample was between 91.3 and 120.0 and the overall CV lower than 30%.

7.2.5. Selectivity

Twelve individual healthy human serum samples (6 females and 6 males, HybriDomus) were analysed to verify any possible interferences with the matrix. Each individual sample was run un-spiked and spiked with a concentration equal to 1.0 pg/mL. Selectivity samples were prepared as described in *section 6.2.2.3.*.

The ACC% ADD for each Ind-SS were calculated according to the formula reported in *the paragraph 6.2.3.*. The results are reported in the tables below:

Sample ID	Endogenous Conc	Conc (pg/mL)	Exp conc (pg/mL)	Exp conc +BLK (pg/mL)	ACC% ADD
01 M SS	0.442	1.529		1.442	106.1
02 M SS	0.185	1.244		1.185	105.0
03 M SS	0.084	1.288		1.084	118.8
05 M SS	0.718	1.866		1.718	108.6
07 M SS	0.282	1.561		1.282	121.7
08 M SS	0.409	1.624		1.409	115.2
01 F SS	0.763	1.757	1.0	1.763	99.7
02 F SS	0.244	1.351		1.244	108.6
04 F SS	2.366	3.036		3.366	90.2
05 F SS	0.274	1.353		1.274	106.3
06 F SS	0.197	1.419		1.197	118.6
07 F SS	0.637	1.625		1.637	99.3

Table 26. TNFα selectivity results using Singulex technology.
Acceptance criteria: ACC% between 70.0. and 130.0

The method resulted not to be interferred by the complex matrix since the ACC% of each samples spiked with 1 pg/mL of biomarkers was between 90.2 to 121.7.

Based on the preliminary results, above reported, the Singulex assay was confirmed to be suitable for the quantification of TNFα in serum with a sensitivity around 1 pg/mL. The main limitation of the assay was the long duration of the assay and the critical influence of the operator precision on the assay. Due to this fact, it was decided to evaluate othe technologies for the TNFα quantification.

7.3. TNFα quantification using Mesoscale technology

The calculations to establish calibration curves and determine concentrations were carried out using the MSD DISCOVERY WORKBENCH® analysis software.

7.3.1. Calibration standard curve

Standard curve was prepared as described in *section 6.3.2.1.*. Three replicates for each calibrator standard were analyzed. The results are reported in the tables below:

Calibrator Standard	Exp Conc (pg/mL)	Raw Values	Obs Conc (pg/mL)	80.0 ≤ ACC% ≥ 120.0
STD 1	369.00	369871	364.44	98.8
		373607	368.43	99.8
STD 2	92.25	103382	95.63	103.7
		101861	94.18	102.1
STD 3	23.06	24635	22.10	95.8
		26440	23.75	103.0
STD 4	5.77	6551	5.74	99.6
		6594	5.78	100.2
STD 5	1.44	1707	1.43	99.4
		1626	1.36	94.4
STD 6	0.36	489	0.37	101.3
		471	0.35	97.0
STD 7	0.09	189	0.11	117.2
		167	0.09	96.3
STD 0	0	74	na	na
		54	na	na

Table 27. TNFα standard curve results using MSD technology. The calibration curves used to calculate analyte concentrations were established by fitting the signals from the calibrators to a 4-parameter logistic (or sigmoidal dose-response) model with a 1/Y2 weighting. Analyte concentrations were determined from the ECL signals by back-fitting to the calibration curve.

An example of STD curve generated by the DISCOVERY WORKBENCH software is shown in the figure below:
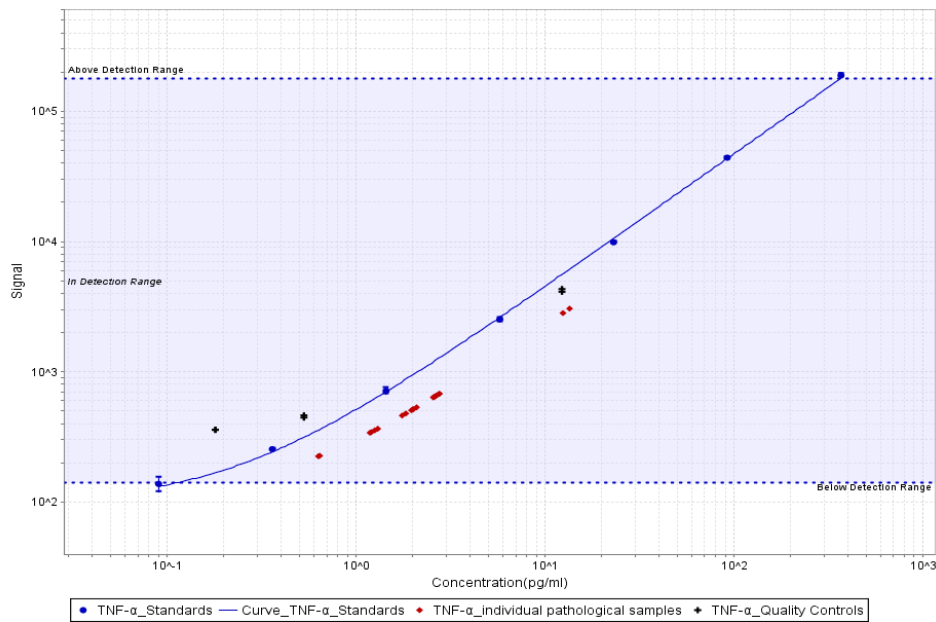
Figure 20. *example of STD curve generated by DISCOVERY WORKBENCH software.*

The standard curve was tested in each analytical run. The results obtained form the standard curves are compliant to the acceptance criteria set. Standard curves reproducibility is shown in the graph reported below:
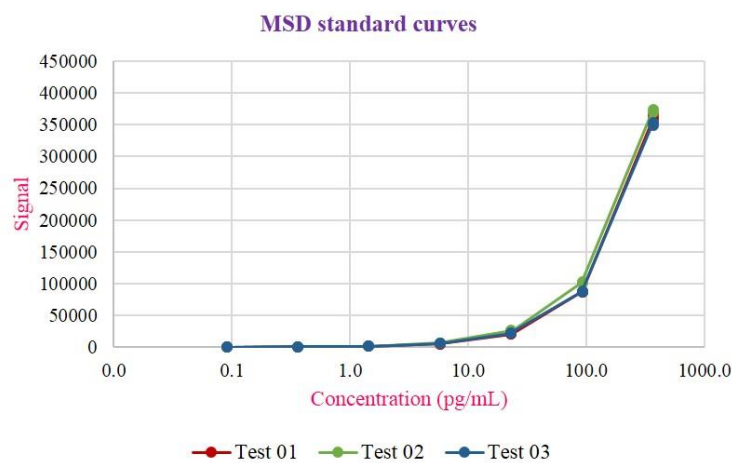
Figure 21. *Reproducibility of three STD curve analyzed using MSD technology (an example).*

7.3.2. Individual serum samples concentration

Ten human serum samples were tested to evaluate the level of the biomarker of interest in the same analytical run. The samples were tested in duplicate. The CV% between the two replicates were calculated as reported in the *paragraph 7.2.2.*. The results are reported in the table below:

Sample (Healthy volunteer)	Observed Concentration (pg/mL)	Average Conc	CV% ≤ 30.0	Sample (Healthy volunteer)	Observed Concentration (pg/mL)	Average Conc	CV% ≤ 30.0
11 M	1.24 / 1.23	1.23	0.6	08 F	0.33 / 0.50	0.42	30.0
12 M	0.86 / 0.84	0.85	2.1	12 F	0.46 / 0.39	0.43	12.4
13 M	0.36 / 0.36	0.36	1.0	15 F	0.48 / 0.34	0.41	23.0
17 M	1.31 / 1.32	1.32	0.3	17 F	0.87 / 0.79	0.83	7.0
20 M	0.69 / 0.73	0.71	4.1	19 F	0.53 / 0.49	0.51	6.6

Table 28. TNF-α Healthy volunteer endogenous levels.

The CV% resulted to be lower than 30% for all the samples except one indicating a good precision of the method. The basal levels of the biomarkers of interest were between 0.36 to 1.23 pg/mL.

7.3.3. Accuracy

Quality controls were prepared as described in *section 6.3.2.2.*.

Two independent replicates of each quality control were tested in two different analytical sessions. Accuracy (ACC %) were calculated as described in the *paragraph 7.2.3.*.

The results are reported in the tables below.

Sample ID	Obs Conc (pg/mL)	Average conc (pg/mL)	CV%	Exp conc (pg/mL)	Obs - BLK (pg/mL)	ACC%
BLK	0.52	0.51	2.8	/	/	/
	0.50					
QC Low	1.08	1.10	2.6	0.53	0.57	107.5
	1.12				0.61	115.1
QC Medium	12.24	12.25	0.1	12.30	11.73	95.4
	12.25				11.74	95.4
LLOQ	0.67	0.69	4.1	0.18	0.16	88.9
	0.71				0.2	111.1

Table 29. TNF-α spiked samples accuracy results using Mesoscale technology

Sample ID	Obs Conc (pg/mL)	Average conc (pg/mL)	CV%	Exp conc (pg/mL)	Obs - BLK (pg/mL)	ACC%
BLK	0.62	0.59	7.2	/	/	/
	0.56					
QC Low	1.18	1.180	0.0	0.53	0.59	111.3
	1.18				0.59	111.3
QC Medium	12.47	12.555	0.1	12.30	11.88	96.6
	12.64				12.05	98.0
LLOQ	0.77	0.775	0.9	0.18	0.18	100.0
	0.78				0.19	105.6

Table 30. TNF-α spiked samples accuracy results using Mesoscale technology

7.3.4. Dilution linearity

To assess linearity of the dilution a spiked sample prepared in pooled human serum was used. The sample will be tested at the MRD and after serial dilution 1:2, in Diluent 2, for five times. Two independent dilution sequences were tested. The ACC% of the observed concentration for each dilution sequence will be calculated according to the formula reported in the *paragraph 7.2.4.*.

The results are reported in the table below.

Sample ID (STD in pool)	Conc (pg/mL)	Dil fact	Conc x Dil fact	Average Conc	CV%	70.0 ≤ ACC % ≤ 130.0	total CV%
STD tq	311.69	1	311.69	354.66	9.12	84.5	
	354.66		354.66			96.1	
STD dil 1:2	206.90	2	413.80	424.66	3.62	112.1	
	217.76		435.52			118.0	
STD dil 1:4	100.35	4	401.40	405.16	1.31	108.8	
	102.23		408.92			110.8	8.4
STD dil 1:8	52.47	8	419.76	419.68	0.03	113.8	
	52.45		419.60			113.7	
STD dil 1:16	24.91	16	398.56	405.36	2.37	108.0	
	25.76		412.16			111.7	
STD dil 1:32	12.66	32	405.12	402.56	0.90	109.8	
	12.50		400.00			108.4	

Table 31. TNF-α dilution linearity using MSD technology. Conc: concentration.

The assessment was repeated twice showing a good performance in sample quantification since the ACC% of each diluted sample was between 84.5 and 113.7 and the overall CV lower than 20%.

7.3.5. Selectivity

Ten individual healthy human serum samples (5 females and 5 males, HybriDomus) were used to verify any possible interferences with the matrix. Each individual sample was run unspiked and spiked with a concentration equal to 0.53 pg/mL. Selectivity samples were prepared as described in *section 6.3.2.3.*.

The ACC% for each Ind-SS were calculated according to the formula reported in *paragraph 7.2.3.*.

The results are reported in the table below:

Sample (healthy volunteer)	Endogenous Concentration (pg/mL)	Expected Concentration (pg/mL)	Observed (spiked) Concentration (pg/mL)	70.0 ≤ ACC % ≤ 130.0
11 M SS	0.29		0.89	113.2
12 M SS	0.24		0.85	115.1
13 M SS	0.37		0.96	111.3
17 M SS	0.54		1.03	92.5
20 M SS	0.38	0.53	1.05	126.4
08 F SS	0.86		1.49	118.9
12 F SS	0.50		1.00	94.3
15 F SS	0.27		0.85	109.4
17 F SS	1.00		1.65	122.6
19 F SS	0.62		1.23	115.1

Table 32. TNF-α selectivity at QC Low concentration results using Mesoscale technology (0.53 pg/mL).

The MSD method resulted to be not interferred by the matrix since the ACC% of each samples spiked with 0,53 pg/mL of biomarkers was between 92.5 to 126.4.

Based on the preliminary results above reported, the MSD assay was confirmed to be suitable for the quantification of TNFα in serum with a sensitivity around 0,18 pg/mL lower than those obtained with the Erenna technology.

Considering the high performance of the MSD if compared to the Singulex assay, it was decided to evaluate also inter-run accuracy and precision.

7.3.6. Inter-run accuracy and precision

Four independent replicates of QC medium and QC low and six independent replicates of LLOQ were analyzed in two independent analytical runs. The following parameters were calculated:

- the mean BIAS% of the observed concentration for each level in each run (intra-run)

- the CV% of the ACC% for each level in each run (intra-run)

- the mean BIAS% and the mean CV% of the ACC% for each run (intra-run)

- the overall mean BIAS% of the observed concentration and the overall CV% of the ACC% for each level (inter-run)

The results are reported in the tables below.

Quality Control	QC Nominal Conc. (pg/mL)	QC back-calc conc. Raw (pg/mL)	BIAS %	ACC %	Mean BIAS% ≤ 30.0	RSD% ACC% ≤ 30.0	Mean BIAS% ≤ 30.0	RSD% ACC% ≤ 30.0
LLOQ	0.18	0.22	22.2	122.2	4.6	11.3	-3.0	11.9
		0.18	0.0	100.0				
		0.21	16.7	116.7				
		0.17	-5.6	94.4				
		0.17	-5.6	94.4				
		0.18	0.0	100.0				
QC Medium	0.53	0.53	0.0	100.0	-5.2	9.1		
		0.55	3.8	103.8				
		0.48	-9.4	90.6				
		0.45	-15.1	84.9				
QC Low	12.30	11.76	-4.4	95.6	-12.1	6.3		
		10.72	-12.8	87.2				
		10.63	-13.6	86.4				
		10.13	-17.6	82.4				

Table 33. TNF-α Intra-run accuracy and precision results using Mesoscale technology (data from test 06)

Quality Control	QC Nominal Conc. (pg/mL)	QC back-calc conc. Raw (pg/mL)	BIAS %	ACC %	Mean BIAS% ≤ 30.0	RSD% ACC% ≤ 30.0	Mean BIAS% ≤ 30.0	RSD% ACC% ≤ 30.0
LLOQ	0.18	0.19	5.6	105.6	-1.9	9.9	3.1	8.7
		0.16	-11.1	88.9				
		0.19	5.6	105.6				
		0.15	-16.7	83.3				
		0.18	0.0	100.0				
		0.19	5.6	105.6				
QC Medium	0.53	0.62	17.0	117.0	9.9	7.5		
		0.62	17.0	117.0				
		0.54	1.9	101.9				
		0.55	3.8	103.8				
QC Low	12.30	12.87	4.6	104.6	3.6	3.3		
		13.07	6.3	106.3				
		12.13	-1.4	98.6				
		12.90	4.9	104.9				

Table 34. TNF-α Intra-run accuracy and precision results using Mesoscale technology (data from test 07)

overall mean BIAS% ≤ 20.0	0.1
Overall RSD% ACC% ≤ 30.0	11.6

Table 35. TNF-α Inter-run accuracy and precision using Mesoscale technology

The method was confirmed to be accurate and precise. Therefore, it was selected to be further optimized and validate to quantify TNFα in human serum samples.

7.4. TNFα quantification using Luminex technology

7.4.1. R&D systems kit evaluation

Data obtained during validation exercise are shown. Intra-run and inter-run accuracy and precision were evaluated.

7.4.1.1. Calibration standard curve

Standard curve was freshly prepared as described in section *6.4.2.1.*. Three replicates for each calibrator standard were analyzed. The results are reported in the tables below:

Calibrator Standard	Exp Conc (pg/mL)	Raw Values	Obs Conc (pg/mL)	80.0 ≤ ACC% ≥ 120.0
STD 1	3550.00	20617.00	4181.59*	117.8
		19201.00	3133.36	88.3
STD 2	887.50	9798.00	888.83	100.1
		9517.00	856.74	96.5
STD 3	221.88	2994.50	253.59	114.3
		25350	216.54	97.6
STD 4	55.47	547.50	52.49	94.6
		528.50	50.80	91.6
STD 5	13.87	138.00	13.97	100.7
		138.00	13.97	100.7
STD 6	3.47	47.00	3.98	114.7
		46.00	3.86	111.2
STD 7	0.87	22.00	0.74	85.1
		22.00	0.74	85.1
STD 0	0	13.50	na	na
		13.00	na	na

*Table 36. TNFα standard curve results using Lumimnex technology (R&D systems kit). The Standard concentrations on the Standard Value Card was used to calculate 4-fold dilutions for the remaining standard levels.The duplicate readings for each standard and sample were averaged and the average blank Mean Fluorescence Intensity (MFI) was subtracted. A standard curve was created by reducing the data using Bio-Plex Manager software in a five parameter logistic (5-PL) curve-fit. Since samples have been diluted, the concentration read from the standard curve was multiplied by the dilution factor. *Value extrapolated beyond standard range*

The standard curve was tested in each analytical run during validation and the results were in compliant to the acceptance criteria defined in the validation protocol. STD curves reproducibility is shown in the graph reported below:

Figure 21. *Reproducibility of STD curve analyzed using Luminex technology (R&D systems kit).The graph show an example of three STD curves generated with R&D kit.*

7.4.1.2. Intra-run and inter-run accuracy and precision

Three independent replicates of each VS (VS1, VS2 and VS3) spanning the curve range, were analyzed on each of three different days according to the method.

The following parameters were calculated to asses intra and inter-run accuracy and precision:

- the mean BIAS% of the observed concentration for each level in each run (intra-run)
- the CV% of the ACC% for each level in each run (intra-run)
- the mean BIAS% and the mean CV% of the ACC% for each run (intra-run)
- the overall mean BIAS% of the observed concentration and the overall CV% of the ACC% for each level (inter-run)

The ACC% was calculated according to the formula reported in the *paragraph 7.2.2*.

The BIAS% was calculated according to the following formula:

$$BIAS\% = \frac{ObsConc - ExpConc}{ExpConc} \times 100$$

Intra and inter-run accuracy and precision were repeated in six analytical runs due to a failure of VS2 and VS3 Mean BIAS% at Day 3. In the table below results from the first three runs are shown. Despite the fact that the intra-run for Run 03, 04, 05 and 06 did not satisfy the acceptance criteria, the inter-run accuracy and precision were satisfied as reported in *Table 38*.

Day 1 - Data from RUN 01								
Sample ID	VS NomConc (pg/mL)	VS back-calculated concentration (pg/mL)	BIAS%	ACC%	Mean BIAS% (*) Acc. Criteria: ≤ 30.0	RSD% ACC% Acc. Criteria: ≤ 30.0	Mean BIAS% (*) Acc. Criteria: ≤ 25.0	RSD% ACC% Acc. Criteria: ≤ 30.0
VS 1	177.5	151.7	-14.5	85.5	13.2	5.9	14.7	8.9
		164.2	-7.5	92.5				
		146.4	-17.5	82.5				
VS 2	29.6	22.3	-24.6	75.4	21.7	5.1		
		22.7	-23.3	76.7				
		24.5	-17.2	82.8				
VS 3	5.9	4.8	-18.9	81.1	9.3	9.2		
		5.6	-5.4	94.6				
		5.7	-3.7	96.3				
Day 2 - Data from RUN 03								
Sample ID	VS NomConc (pg/mL)	VS back-calculated concentration (pg/mL)	BIAS%	ACC%	Mean BIAS% (*) Acc. Criteria: ≤ 30.0	RSD% ACC% Acc. Criteria: ≤ 30.0	Mean BIAS% (*) Acc. Criteria: ≤ 25.0	RSD% ACC% Acc. Criteria: ≤ 30.0
VS 1	177.5	160.2	-9.7	90.3	8.8	2.0	1.0	11.2
		159.9	-9.9	90.1				
		165.7	-6.6	93.4				
VS 2	29.6	30.1	1.8	101.8	0.9	3.8		
		30.8	4.1	104.1				
		28.6	-3.3	96.7				
VS 3	5.9	6.6	11.5	111.5	10.9	13.0		
		5.7	-3.7	96.3				
		7.4	25.0	125.0				

Day 3 - Data from RUN 08								
Sample ID	VS NomConc (pg/mL)	VS back-calculated concentration (pg/mL)	BIAS%	ACC%	Mean BIAS% (*) Acc. Criteria: ≤ 30.0	RSD% ACC% Acc. Criteria: ≤ 30.0	Mean BIAS% (*) Acc. Criteria: ≤ 25.0	RSD% ACC% Acc. Criteria: ≤ 30.0
VS 1	177.5	144.0	-18.9	81.1	19.5	1.9		
		139.9	-21.2	78.8				
		144.9	-18.4	81.6				
VS 2	29.6	19.2	-35.1	64.9	33.7 (**)	1.8	36.1 (**)	25.0
		19.8	-33.1	66.9				
		19.8	-33.1	66.9				
VS 3	5.9	2.3	-61.1	38.9	55.0 (**)	17.7		
		2.5	-57.8	42.2				
		3.2	-45.9	54.1				

Table 37. Intra-Run Accuracy and Precision.
(*) Absolute value. (**) Out of acceptance criteria.

TNF-alpha	Value	Result
Overall mean BIAS% - Acc. Criteria: ≤ 20.0 (*)	16.6	Passed
Overall RSD% ACC% - Acc. Criteria: ≤ 30.0	23.2	Passed

Table 38. Inter-Run Accuracy and Precision.
(*) Absolute value.

A multiple failure was considered not acceptable therefore an investigation was opened in order to understand the source of variability.

After some assessment using multiple batches of kit, the VSs variability was supposed to be linked to an instability of the R&D Systems recombinant standards to mid-storage freezing. Therefore suitability of TNF-alpha quantification assay was evaluated by comparing fresh and frozen VS in three additional analytical runs. Acceptance criteria for TNF-alpha were not satisfied in fresh VS as well as in frozen VS as shown in the tables below.

Day 1 - Data from RUN 14								
Sample ID	VS NomConc (pg/mL)	VS back-calculated concentration (pg/mL)	BIAS%	ACC%	Mean BIAS% (*) Acc. Criteria: ≤ 30.0	RSD% ACC% Acc. Criteria: ≤ 30.0	Mean BIAS% (*) Acc. Criteria: ≤ 25.0	RSD% ACC% Acc. Criteria: ≤ 30.0
VS 1	177.5	99.3	-44.1	55.9	42.0 (**)	3.1		
		105.4	-40.6	59.4				
		103.9	-41.5	58.5				
VS 2	29.6	20.6	-30.4	69.6	30.1 (**)	1.0	29.6 (**)	15.9
		20.9	-29.3	70.7				
		20.5	-30.7	69.3				
VS 3	5.9	4.8	-18.9	81.1	16.7	4.7		
		4.8	-18.9	81.1				
		5.2	-12.2	87.8				
Day 2 - Data from RUN 15								
Sample ID	VS NomConc (pg/mL)	VS back-calculated concentration (pg/mL)	BIAS%	ACC%	Mean BIAS% (*) Acc. Criteria: ≤ 30.0	RSD% ACC% Acc. Criteria: ≤ 30.0	Mean BIAS% (*) Acc. Criteria: ≤ 25.0	RSD% ACC% Acc. Criteria: ≤ 30.0
VS 1	177.5	103.0	-42.0	58.0	40.9 (**)	1.7		
		106.6	-39.9	60.1				
		105.1	-40.8	59.2				
VS 2	29.6	17.4	-41.2	58.8	40.2 (**)	1.5	39.7 (**)	2.5
		17.8	-39.8	60.2				
		17.9	-39.5	60.5				
VS 3	5.9	3.7	-37.5	62.5	38.1 (**)	1.6		
		3.7	-37.5	62.5				
		3.6	-39.2	60.8				

Day 3 - Data from RUN 16								
Sample ID	VS NomConc (pg/mL)	VS back-calculated concentration (pg/mL)	BIAS%	ACC%	Mean BIAS% (*) Acc. Criteria: ≤ 30.0	RSD% ACC% Acc. Criteria: ≤ 30.0	Mean BIAS% (*) Acc. Criteria: ≤ 25.0	RSD% ACC% Acc. Criteria: ≤ 30.0
VS 1	177.5	112.8	-36.5	63.5	35.9 (**)	4.6	29.9 (**)	11.5
		119.4	-32.7	67.3				
		109.1	-38.5	61.5				
VS 2	29.6	20.0	-32.4	67.6	31.0 (**)	3.8		
		21.3	-28.0	72.0				
		19.9	-32.7	67.3				
VS 3	5.9	4.2	-29.1	70.9	22.9	13.9		
		4.2	-29.1	70.9				
		5.3	-10.5	89.5				

Table 39. TNF-alpha Intra-Run Accuracy and Precision using fresh VS (Investigation 1). (*) Absolute value. (**) Out of Acceptance Criteria

						Day 1 - Data from RUN 14			
Sample ID	VS NomConc (pg/mL)	VS back-calculated concentration (pg/mL)	BIAS %	ACC %	Mean BIAS% (*) Acc. Criteria: ≤ 30.0	RSD% ACC% Acc. Criteria: ≤ 30.0	Mean BIAS% (*) Acc. Criteria: ≤ 25.0	RSD% ACC% Acc. Criteria: ≤ 30.0
VS 1	177.5	102.6	-42.2	57.8	42.5 (**)	0.6	41.6 (**)	8.2
		102.3	-42.4	57.6				
		101.5	-42.8	57.2				
VS 2	29.6	17.5	-40.8	59.2	36.4 (**)	6.0		
		19.3	-34.8	65.2				
		19.6	-33.7	66.3				
VS 3	5.9	3.0	-49.3	50.7	45.9 (**)	5.4		
		3.3	-44.3	55.7				
		3.3	-44.3	55.7				
						Day 2 - Data from RUN 15			
Sample ID	VS NomConc (pg/mL)	VS back-calculated concentration (pg/mL)	BIAS %	ACC %	Mean BIAS% (*) Acc. Criteria: ≤ 30.0	RSD% ACC% Acc. Criteria: ≤ 30.0	Mean BIAS% (*) Acc. Criteria: ≤ 25.0	RSD% ACC% Acc. Criteria: ≤ 30.0
VS 1	177.5	100.9	-43.2	56.8	44.1 (**)	2.4	45.8 (**)	9.9
		100.2	-43.5	56.5				
		96.5	-45.6	54.4				
VS 2	29.6	16.4	-44.6	55.4	45.0 (**)	3.8		
		15.6	-47.3	52.7				
		16.8	-43.2	56.8				
VS 3	5.9	2.4	-59.5	40.5	48.2 (**)	18.8		
		3.4	-42.6	57.4				
		3.4	-42.6	57.4				

Day 3 - Data from RUN 16								
Sample ID	VS NomConc (pg/mL)	VS back-calculated concentration (pg/mL)	BIAS %	ACC %	Mean BIAS% (*) Acc. Criteria: ≤ 30.0	RSD% ACC% Acc. Criteria: ≤ 30.0	Mean BIAS% (*) Acc. Criteria: ≤ 25.0	RSD% ACC% Acc. Criteria: ≤ 30.0
VS 1	177.5	96.4	-45.7	54.3	42.9 (**)	4.4	44.0 (**)	6.0
		102.9	-42.0	58.0				
		104.9	-40.9	59.1				
VS 2	29.6	16.9	-42.9	57.1	42.2 (**)	3.1		
		17.7	-40.2	59.8				
		16.7	-43.5	56.5				
VS 3	5.9	3.0	-49.3	50.7	47.1 (**)	7.4		
		3.0	-49.3	50.7				
		3.4	-42.6	57.4				

Table 40. TNF-alpha Intra-Run Accuracy and Precision using frozen VS (Investigation 1). (*) Absolute value. (**) Out of Acceptance Criteria

Fresh VS	Value	Result
Overall mean BIAS% - Acc. Criteria: ≤ 20.0 (*)	33.1 (**)	Failed
Overall RSD% ACC% - Acc. Criteria: ≤ 30.0	13.6	Passed
Frozen VS	Value	Result
Overall mean BIAS% - Acc. Criteria: ≤ 20.0 (*)	43.8 (**)	Failed
Overall RSD% ACC% - Acc. Criteria: ≤ 30.0	8.4	Passed

Table 41. TNF-alpha Inter-Run Accuracy and Precision using fresh and frozen VS (Investigation 1). (*) Absolute value. (**) Out of Acceptance Criteria.

Due to the non acceptable rusults, the method validation of R&D system kit was not completed. Therefore, it was decided to evaluate the performance of the bio-rad kit.

7.4.2. Bio-rad kit method validation

After an optimization phase, the technology was selected as the most suitable to quantify the analyte of interest (TNF α) in human serum. The method was finally successfully validated. In the next paragraphs validation results are reported as comparison to the results obtained with the new technologies evaluated.

The following parameters were evaluated during the validation:
- Intra-run and inter-run accuracy and precision
- Selectivity
- Ruggedness
- Short-term stability
- Freeze-thaw cycle stability

All the back calculations were performed using the Bio-Plex Manager 5.0. All the other calculations and statistics were performed using Microsoft Excel 2013 software.

Calibration standards and VS were calculated after subtracting the average Blank (B) FI value. Unknown samples concentration were calculated after subtracting the average sample diluent HB FI value.

7.4.2.1. Calibration standard curve

Standard curve was freshly prepared for each analytical run as described in *section 6.5.2.1.*.

The ACC% of each replicate for each standard point was calculated according to the formula reported in the *paragraph 7.2.2.*.

The acceptance criteria for the analytical run effectiveness are reported below:
- The ACC% (for each replicate) of the lower limit of quantification (LLOQ) must be within 70.0 –130.0 % of its nominal concentration to be valid.
- The ACC% (for each replicate) of all the other calibration standards must be within 80.0- 120.0% of their nominal concentration to be valid.
- The curve must contain at least 75% of valid points to be valid (12/16).

- Invalid points must not be used to estimate calibration curve parameters.

- Two consecutive calibration standard concentration levels cannot be rejected.

- LLOQ or ULOQ concentration levels cannot be rejected (if the Standard Curve level corresponding to the stipulated low limit of quantitation (LLOQ) or the upper limit of quantitation (ULOQ) is eliminated from evaluation, the concentration of unknown samples must lie within the updated method range. Otherwise the run must be rejected and repeated).

The acceptance criteria were satisfied. Results are shown in the table below.

Calibrator Standard	Exp Conc (pg/mL)	Raw Values	Obs Conc (pg/mL)	80.0 ≤ ACC% ≥ 120.0
STD 1	1191.0	27353.3	1086.5	91.2
		28296.3	*1341.6	112.6
STD 2	397.0	16935.8	385.3	97.1
		17564.8	405.2	102.1
STD 3	132.3	6896.3	137.5	103.9
		6694.3	133.2	100.7
STD 4	44.1	2278.8	43.3	98.2
		2249.3	42.7	96.8
STD 5	14.7	761.3	13.9	94.6
		809.3	14.9	101.4
STD 6	4.9	291.3	5.2	106.1
		281.8	5	102.0
STD 7	1.6	90.8	1.6	100.0
		96.8	1.7	106.3
STD 8	0.5	32.8	0.6	120.0
		28.3	*0.5	100.0
STD 0	0.0	34.5	na	na
		33.0	na	na

*Table 42. TNFα standard curve results using Lumimnex technology (Bio-rad kit). The standard curve was created by reducing the data using Bio-Plex Manager software in a five parameter logistic (5-PL) curve-fit. Since samples have been diluted, the concentration read from the standard curve was multiplied by the dilution factor. *Value extrapolated beyond standard range*

The standard curve was tested in each analytical run during validation and the results were in compliant to the acceptance criteria defined in the validation protocol. STD curves reproducibility is shown in the graph reported below:

Luminex standard curves
(BIO-RAD kit)
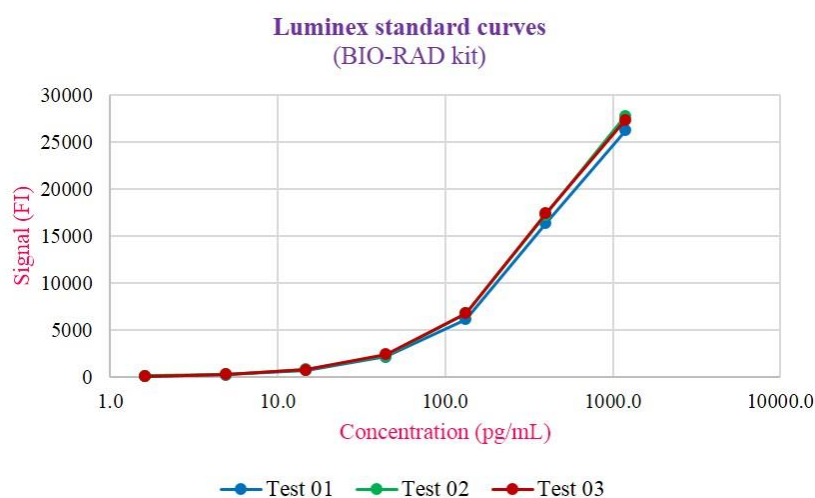
Figure 22. Reproducibility of STD curve analyzed using Luminex technology (R&D systems kit).The graph show an example of three STD curves generated with R&D kit.

7.4.2.2. Intra-run and inter-run accuracy and precision

Three independent replicates of VS1, VS2 and VS3 were analyzed in four different days, while three independent replicates of LLOQ were analysed in five different days according to the method.

The following parameters were calculated to asses intra and inter-run accuracy and precision:

- the mean BIAS% of the observed concentration for each level in each run (intra-run)
- the CV% of the ACC% for each level in each run (intra-run)
- the mean BIAS% and the mean CV% of the ACC% for each run (intra-run)
- the overall mean BIAS% of the observed concentration and the overall CV% of the ACC% for each level (inter-run)

The BIAS% and ACC% were calculated according to the formulas reported in *paragraphs 7.4.1.2.* and *7.2.2.*.

Acceptance criteria applied for intra and inter-run accuracy and precision are reported below:

- The mean BIAS% of the observed concentration in each run must be within ± 20% for VS1, VS2 and VS3 and ± 25% for the LLOQ

- The CV% of the ACC% for each level (VS1, VS2, VS3 and LLOQ) in each run must be within ± 20%;

- The mean BIAS% for each run must be within ± 20% for VS1, VS2, VS3 and ± 25% for the LLOQ

- The mean CV% of the ACC% for each run must be within ± 20% (for VS1, VS2, VS3 and LLOQ)

- The overall mean BIAS% of the observed concentration must be within ± 20% for VS1, VS2 and VS3 and ± 25% for the LLOQ

- The overall CV% of the ACC % for each level (VS1, VS2, VS3 and LLOQ) must be within ± 20%

The method resulted to be precise and accurate since all the below acceptance criteria were satisfied. Results are shown in the tables below.

Sample ID	NomConc (pg/mL)	ObsConc (pg/mL)	ACC %	BIAS %	CV ACC% (Acc. Criteria ≤ 20.0)	mean BIAS %	mean BIAS% Acc. Criteria:	Overall mean BIAS% (Acc. Criteria within ± 25.0)	Overall ACC% (Acc. Criteria ≤ 20.0)
LLOQ	2.0	1.9	95.0	-5.0	15.1	-16.7	within ± 30.0	N.A.	N.A.
		1.4	70.0	-30.0					
		1.7	85.0	-15.0					
VS3	8.0	7.5	93.8	-6.3	9.6	-13.3	within ± 25.0	-7.2	8.0
		7.1	88.8	-11.3					
		6.2	77.5	-22.5					
VS2	101.4	95.6	94.3	-5.7	2.1	-7.7	within ± 25.0		
		93.6	92.3	-7.7					
		91.7	90.4	-9.6					
VS1	3572.9	3349.3	93.7	-6.3	5.1	-0.5	within ± 25.0		
		3671.6	102.8	2.8					
		3645.0	102.0	2.0					

Table 43. *Intra-Run Accuracy and Precision (data from run 01).*

Sample ID	NomConc (pg/mL)	ObsConc (pg/mL)	ACC %	BIAS %	CV ACC% (Acc. Criteria ≤ 20.0)	mean BIAS %	mean BIAS% Acc. Criteria:	Overall mean BIAS% (Acc. Criteria within ± 25.0)	Overall ACC% (Acc. Criteria ≤ 20.0)
LLOQ	2.0	1.4	70.0	-30.0	12.7	-18.3	within ± 30.0	N.A.	N.A.
		1.8	90.0	-10.0					
		1.7	85.0	-15.0					
VS3	8.0	6.2	77.5	-22.5	4.4	-20.0	within ± 25.0	-5.5	14.9
		5.7	71.3(*)	-28.8(*)					
		6.6	82.5	-17.5					
VS2	101.4	84.7	83.5	-16.5	4.7	-11.7	within ± 25.0		
		92.6	91.3	-8.7					
		91.2	89.9	-10.1					
VS1	3572.9	3870.7	108.3	8.3	4.5	10.5	within ± 25.0		
		4147.0	116.1	16.1					
		3821.2	106.9	6.9					

Table 44. *Intra-Run Accuracy and Precision (data from run 02) (*) Out of acceptance criteria, excluded from the elaboration.*

Sample ID	NomConc (pg/mL)	ObsConc (pg/mL)	ACC %	BIAS %	CV ACC% (Acc. Criteria ≤ 20.0)	mean BIAS %	mean BIAS% Acc. Criteria:	Overall mean BIAS% (Acc. Criteria within ± 25.0)	Overall ACC% (Acc. Criteria ≤ 20.0)
LLOQ	2.0	2.7	135(*)	35(*)	4.9	-27.5	within ± 30.0	N.A.	N.A.
		1.4	70.0	-30.0					
		1.5	75.0	-25.0					
VS3	8.0	7.1	88.8	-11.3	2.1	-9.2	within ± 25.0	-4.2	11.1
		7.3	91.3	-8.8					
		7.4	92.5	-7.5					
VS2	101.4	89.7	88.5	-11.5	3.7	-11.3	within ± 25.0		
		93.4	92.1	-7.9					
		86.7	85.5	-14.5					
VS1	3572.9	4275.6	119.7	19.7	9.5	7.8	within ± 25.0		
		3657.0	102.4	2.4					
		3625.5	101.5	1.5					

Table 45. Intra-Run Accuracy and Precision (data from run 03) (*) Out of acceptance criteria, excluded from the elaboration.

Sample ID	NomConc (pg/mL)	ObsConc (pg/mL)	ACC %	BIAS %	CV ACC% (Acc. Criteria ≤ 20.0)	mean BIAS %	mean BIAS% Acc. Criteria:	Overall mean BIAS% (Acc. Criteria within ± 25.0)	Overall ACC% (Acc. Criteria ≤ 20.0)
LLOQ	2.0	1.8	90.0	-10.0	8.6	-11.7	within ± 30.0	N.A.	N.A.
		1.9	95.0	-5.0					
		1.6	80.0	-20.0					
VS3	8.0	7.0	87.5	-12.5	5.2	-9.2	within ± 25.0	-5.0	8.3
		7.7	96.3	-3.8					
		7.1	88.8	-11.3					
VS2	101.4	92.8	91.5	-8.5	2.2	-6.4	within ± 25.0		
		96.9	95.6	-4.4					
		95.0	93.7	-6.3					
VS1	3572.9	4035.7	113.0	13.0	13.1	3.4	within ± 25.0		
		1937.3(*)	54.2(*)	-45.8					
		3355.1	93.9	-6.1					

Table 46. Intra-Run Accuracy and Precision (data from run 04). (*) Out of acceptance criteria, excluded from the elaboration.

Sample ID	NomConc (pg/mL)	ObsConc (pg/mL)	ACC %	BIAS %	CV ACC% (Acc. Criteria ≤ 20.0)	mean BIAS %	mean BIAS% Acc. Criteria:	Overall mean BIAS% (Acc. Criteria within ± 25.0)	Overall ACC% (Acc. Criteria ≤ 20.0)
LLOQ	2.0	2.1	105.0	5.0	14.9	-3.3	within ± 30.0	N.A.	N.A.
		2.1	105.0	5.0					
		1.6	80.0	-20.0					

Table 47. Intra-Run Accuracy and Precision (data from run 05).

Sample ID	Overall mean BIAS%	Overall mean BIAS% Acc. Criteria:	Overall ACC% CV% (Acc. Criteria ≤ 20.0)	Run used
LLOQ	-13.7	within ±30%	14.1	Run 01,02,03,04,05
VS3	-12.3	within ±25%	7.1	Run 01,02,03,04
VS2	-9.3	within ±25%	3.9	Run 01,02,03,04
VS1	1.2	within ±25%	7.9	Run 01,02,03,04

Table 48. Inter-Run Accuracy and Precision.

7.4.2.3. Selectivity

Twelve individual healthy human serum samples (6 females and 6 males, HybriDomus) and twelve individual cancer patient samples (from different indications, Seralab) were used to verify any possible interferences of the sample.

The un-spiked and spiked samples were tested as a single point.

The ACC% for each Ind-SS were calculated according to the formula reported in *paragraph 6.5.2.3.*.

The method was demonstrated to be selective for the analyte of interest since more than 2/3 (at least 8 out of 12) of healthy human individual samples and more than 2/3 (at least 8 out of 12) of the cancer patient samples showed an ACC% between 80.0 and 120.0.
 The results are shown in the table below.

Matrix type	Sample ID	Results as un-spiked	Spiked NomConc (pg/mL)	Spiked ObsConc (pg/mL)	ACC% (Acc.criteria 8/12 with ACC% between 80-120% each population)	Result
Healthy human Sera	01M-G1017088	BLLOQ	101.4	88.9	87.7	11/12 PASSED
	02M-G1017089	BLLOQ	101.4	107.0	105.5	
	03M-G1017090	BLLOQ	101.4	117.5	115.9	
	04M-G1017091	BLLOQ	101.4	125.7	124.0 (*)	
	05M-G1017092	BLLOQ	101.4	114.1	112.5	
	06M-G1017093	BLLOQ	101.4	118.7	117.1	
	02F-G1017109	BLLOQ	101.4	107.5	106.0	
	04F-G1017111	BLLOQ	101.4	104.7	103.3	
	06F-G1017113	BLLOQ	101.4	84.3	83.1	
	07F-G1017114	BLLOQ	101.4	96.0	94.7	
	08F-G1017115	BLLOQ	101.4	87.7	86.5	
	09F-G1017116	BLLOQ	101.4	119.1	117.5	
Cancer Patient Sera	01-BRH1119439	BLLOQ	101.4	106.4	104.9	09/12 PASSED
	02-BRH1119438	BLLOQ	101.4	75.3	74.3 (*)	
	03-BRH1119437	BLLOQ	101.4	114.6	113.0	
	04-BRH1119433	BLLOQ	101.4	112.0	110.5	
	05-BRH1119434	BLLOQ	101.4	79.7	78.6 (*)	
	06-BRH1119435	BLLOQ	101.4	121.9	120.2 (*)	
	08-BRH1119426	BLLOQ	101.4	119.3	117.7	
	13-BRH1119431	BLLOQ	101.4	105.6	104.1	
	14-BRH1119432	BLLOQ	101.4	91.7	90.4	
	17-BRH1119425	BLLOQ	101.4	92.2	90.9	
	19-BRH1119420	BLLOQ	101.4	91.7	90.4	
	20-BRH1119421	BLLOQ	101.4	92.2	90.9	

Table 49. Selectivity (data from run 05). (*) Out of acceptance criteria, excluded from the elaboration.

7.4.2.4. Ruggedness

Three independent replicates of each VS (VS1, VS2 and VS3) used to assess intra and inter-assay accuracy and precision were analyzed on a different day by a second operator according to the method.

For each VS, the following parameters were calculated:

- Mean ACC% for the VS sample processed by a second operator (Op_2)
- Mean ACC% for the VS processed by first operator (Op_1) in one of the three runs produced to assess intra and inter-assay accuracy and precision.

$$RUG\% = \frac{Mean\ ACC\%\ VS\ Op_2}{Average\ (Mean\ ACC\%\ VS\ Op_1\ ;\ Mean\ ACC\%\ VS\ Op_2)} \times 100$$

The assay was considered unaffected by different operators since the RUG% was between 80.0 and 120.0. The result is shown in the table below.

Sample ID	NomConc (pg/mL)	ObsConc (pg/mL)	ACC%	Mean ACC%	Mean ACC% Op₁ (data from RUN 03)	Ruggedness Acc. Criteria: 80.0 ≤RUG%≤ 120.0
VS3	8.0	7.9	98.8	94.2	90.9	101.8
		7.5	93.8			
		7.2	90.0			
VS2	101.4	93.8	92.5	92.7	88.7	102.2
		94.5	93.2			
		93.6	92.3			
VS1	3572.9	3510.9	98.3	99.6	107.9	96.0
		3717.9	104.1			
		3448.0	96.5			

Table 50. Ruggedness (data from run 09).

7.4.2.5. Short term stability

One aliquot of each VS (VS1, VS2 and VS3) prepared for intra and inter-run accuracy and precision was kept at room temperature for 2 and 4 hours. Three independent replicates were tested according to the method. Results were averaged for each concentration at each time point.

The stability of each concentration at each cycle were calculated as follows:

$$STAB\% = \frac{Averaged\ ObsConc}{ExpConc} \times 100$$

Short-term stability of each VS was confirmed up to 4 hours at room temperature since the STAB% was between 80.0 and 120.0. Results are shown in the table below.

2 hour RT				
Sample ID	NomConc (pg/mL)	ObsConc (pg/mL)	meanObsConc (pg/mL)	mean STAB% (Acc. Criteria: $75 \le STAB\% \le 125$)
VS3	8.0	7.2 / 7.9 / 7.9	7.7	96.3
VS2	101.4	95.8 / 96.8 / 94.5	95.7	94.4
VS1	3572.9	4429.9 / 3760.0 / 3999.3	4063.1	113.7
4 hour RT				
Sample ID	NomConc (pg/mL)	ObsConc (pg/mL)	meanObsConc (pg/mL)	mean STAB% (Acc. Criteria: $75 \le STAB\% \le 125$)
VS3	8.0	6.9 / 6.8 / 7.2	7.0	87.5
VS2	101.4	88.2 / 91.8 / 90.9	90.3	89.1
VS1	3572.9	4414.3 / 4147.4 / 4244.6	4268.8	119.5

Table 51. Short term stability (data from run 10).

7.4.2.6. Freeze-Thaw cycle stability

VS (VS1, VS2 and VS3) used for intra and inter-run accuracy and precision assessment were frozen at -80°C and thawed. The freeze-thaw cycle was repeated five times. Three independent replicates of each VS sample were tested after each freeze-thaw cycle and

analyzed in the same analytical run according to the method. Results were averaged for each concentration at each cycle.

The stability of each concentration at each cycle for VS1, VS2 and VS3 were calculated as reported in *paragraph 7.4.2.5.* for short term stability.

The freeze-thaw stability of each VS was confirmed up to the four cycles since the CYCL% (STAB%) was between 80.0 and 120.0. The results are shown in the table below.

1st cycle				
Sample ID	**NomConc (pg/mL)**	**ObsConc (pg/mL)**	**meanObsConc (pg/mL)**	**mean CYCL% (Acc. Criteria: $75 \leq CYCL\% \leq 125$)**
VS3	8.0	7.5 7.3 6.9	7.2	90.0
VS2	101.4	82.5 85.2 87.6	85.1	83.9
VS1	3572.9	4374.7 3714.4 4195.2	4094.8	114.6
2nd cycle				
Sample ID	**NomConc (pg/mL)**	**ObsConc (pg/mL)**	**meanObsConc (pg/mL)**	**mean CYCL% (Acc. Criteria: $75 \leq CYCL\% \leq 125$)**
VS3	8.0	5.2 6.4 6.5	6.0	75.0
VS2	101.4	76.8 83.1 76.4	78.8	77.7
VS1	3572.9	3954.1 4232.2 4401.3	4195.9	117.4

3rd cycle				
Sample ID	NomConc (pg/mL)	ObsConc (pg/mL)	meanObsConc (pg/mL)	mean CYCL% (Acc. Criteria: 75 ≤ CYCL% ≤ 125)
VS3	8.0	6.3 5.8 6.4	6.2	77.5
VS2	101.4	91.6 82.4 86.2	86.7	85.5
VS1	3572.9	4209.6 4347.9 4281.1	4279.5	119.8
4th cycle				
Sample ID	NomConc (pg/mL)	ObsConc (pg/mL)	meanObsConc (pg/mL)	mean CYCL% (Acc. Criteria: 75 ≤ CYCL% ≤ 125)
VS3	8.0	5.6 6.5 6.8	6.3	78.8
VS2	101.4	89.8 96.7 90.1	92.2	90.9
VS1	3572.9	4177.6 4108.0 3875.8	4053.8	113.5
5th cycle				
Sample ID	NomConc (pg/mL)	ObsConc (pg/mL)	meanObsConc (pg/mL)	mean CYCL% (Acc. Criteria: 75 ≤ CYCL% ≤ 125)
VS3	8.0	5.5 5.8 6.4	5.9	73.8 (*)
VS2	101.4	77.3 81.4 77.4	78.7	77.6
VS1	3572.9	4255.3 4077.1 3909.2	4080.5	114.2

Table 52. Freeze and Thaw stability (data from run 08). (*) Out of acceptance criteria, considered not stable.

8. Conclusions

Development and validation of analytical method for biomarker quantification involves the selection of the most suitable technology to quantify the biomarkers of interest with theappropriate level of precision, accuracy and sensitivity. The sensitivity required is an information usually obtained by project rappresentative of the Function in the scientific project team and which is based on the literature expected levels of biomarker, after drug administration.

In particular, for TNFα quantification in clinical human serum samples, the main requirement was to select the most sensitive technology to quantify with accuracy and precision the biomarker.

The technologies available in the laboratory were evaluated for this purpose: AlphaLISA, Singulex and Luminex. In addition Mesoscale Discovery technology was evaluated during a demo session. Immuoassay kits from different suppliers were also tested.

The performance of the different kits were analyzed in according to the internal procedure for analytical method set-up, optimization and validation at Clinical Biomarkers department and in accordance to the international guidelines.

As the first technology evaluated, AlphaLISA, did not shown good results for the standard curve calculation and considering the preliminary results not promising it was decided to not proceed to futher optimization.

On the contrary, the Singulex technology was demonstrated to be promising in terms of standard curves performance allowing a selectivity quantification of the biomarker with good accuracy and precision and a sensitivity of around 0.05 pg/mL (preliminary data, LLOQ not validated). Unfortunately, this technology is not easy to use and reagents are very expensive. Some of the very extensive procedural steps are critical and even little changes in handling could impact the assay performance. For these reasons, this technology was also excluded. Luminex technology was finally evaluated using two kits: as a first choice a R&D systems kit was selected. After a positive optimization phase concluded with a method release, the validation of the critical parameter selected

(intra-run accuracy and precion, LLOQ, short-term stability, freeze and thaw cycle stability, selectivity and ruggedness) was started. Intra-run accuracy and precision did not fulfill the acceptance criteria and, an internal investigation to understand the root cause was performed. The outcome of the investigation confirmed the variability of the results due to a non stable reagent provided with the kit, therefore the kit was not further developed and the the validation was stopped.

A second Luminex kit from BIO-RAD company was optimize and validated with good accuracy and precision and a sensitivity of 2.0 pg/mL confirming the goodness of the Luminex technology for biomarker analyses.

In order to have a more complete picture of the availble technology for Biomarkers method development, a demo session of the Mesoscale Discovery was planned within the Clinical Biomarkers lab. Preliminary results generated with this technology, suggested a future possible use of MSD for Biomarkers quantification. A good accuracy and precision and a very high sensitivity (around 0.18 pg/mL) allowed us to decided to buy the equipment and validate MSD TNF-α kit as future assay in substitution of the previously validated from Bio-Rad.

The activity was not completed since the company decided to reorganize Clinical biomarkers activities by moving the entire activities to another Merck site.

PART II

Viral Clearance: viral purification method development & viral titration method validation.

9. Introduction

9.1. Biotechnology drugs and viral safety

Biotechnology drugs are products derived from characterised cell lines of human or animal sources such as blood products, recombinant products, vaccine, animal or human tissue or fluids derived products.

The regulations for licensing biotechnology products stipulate that cell banks, biologically derived raw materials, and bulk harvest must be controlled and tested for viral safety in addition to the downstream process (24). According to the Q5A(R1) Guideline "*Viral safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin*" defined by ICH International Conference on Harmonization (USA - Japan - Europe), the viral safety of biotechnology products derived from human or animal origin cell lines should be demonstrated before the registration and demand for marketing of these products (25).

The risk of viral contamination is a feature common to all biotechnology products derived from cell lines. Such contamination could have serious clinical consequences and can arise from the contamination of the original source of the cell lines themselves (cell substrates) or from adventitious introduction of virus during production (25).

Regarding Viruses That Could Occur in the Master Cell Bank (MCB), cells may have latent or persistent virus infection (e.g. herpesvirus) or endogenous retrovirus which may be transmitted vertically from one cell generation to the next, since the viral genome persists within the cell. Such viruses may be constitutively expressed or may unexpectedly become expressed as an infectious virus (25).

Viruses can be introduced into the MCB by several routes such as derivation of cell lines from infected animals; use of virus to establish the cell line; use of contaminated biological reagents such as animal serum components and contamination during cell handling (25).

Moreover, adventitious viruses can be introduced into the final product during production by several routes including, but not limited to, the following: the use of contaminated biological reagents such as animal serum components; the use of a virus for the induction of expression of specific genes encoding a desired protein; the use of a contaminated reagent, such as a monoclonal antibody affinity column; the use of a contaminated excipient during formulation; contamination during cell and medium handling. Monitoring of cell culture parameters can be helpful in the early detection of potential adventitious viral contamination (25).

To date, however, biotechnology products derived from cell lines have not been implicated in the transmission of viruses. Nevertheless, it is expected that the safety of these biotechnological products with regard to viral contamination can be assured by the application of a virus testing program and assessment of virus removal and inactivation achieved by the manufacturing process.

Three principal, complementary approaches have evolved to control the potential viral contamination of biotechnology products. These include: selecting and testing cell lines and other raw materials, including media components, for the absence of undesirable viruses which may be infectious and/or pathogenic for humans; assessing the capacity of the production processes to clear infectious viruses; and testing the product at appropriate steps of production for absence of contaminating infectious viruses (25).

9.1.1. Cell line qualification: testing for viruses

Cell lines used in the production process must be qualified. An important part of qualifying a cell line for use in the production of a biotechnology product is the appropriate testing for the presence of virus. Many tests for retroviruses, non-endogenous or adventitious viruses are suggested to be done, using different assays (e.g. antibody production, in vivo and in vitro virus screen, Transmission Electronic Microscope (TEM), Polymerase chain reaction (PCR), retrovirus infectivity), on MCB,

Working Cell Bank (WCB) and cells at the limit of in vitro cell age used for production. The limit of in vitro cell age used for production should be based on data derived from production cells expanded under pilot-plant scale or commercial-scale conditions to the proposed in vitro cell age or beyond. Generally, the production cells are obtained by expansion of the WCB; the MCB could also be used to prepare the production cells. Cells at the limit of in vitro cell age should be evaluated once for those endogenous viruses that may have been undetected in the MCB and WCB. Testing for non-endogenous viruses in MCB should include *in vitro* and *in vivo* inoculation tests and other specific tests such as species-specific tests (e.g. the mouse antibody production (MAP) test) to detect possible contaminating viruses (24, 25).

After the production from qualified cell lines, bulk harvest containing biotechnology drugs undergo a purification process (downstream process) to obtain the final drugs to be formulated for the market. This purification process involves different steps such as affinity chromatography or other chromatography types, pH inactivation and nanofiltration. It is important to evaluate and to characterize the virus removal or inactivation during this process in order to assure the viral safety of biotechnology products (25). This is the aim of viral clearance studies.

9.2. Viral Clearance

A Viral Clearance study should evaluate the capability of selected steps of the production purification process and of the overall purification process (total viral clearance) to remove and/or inactivate a broad spectrum of virus type, including viruses that are known to contaminate or have the potential to contaminate the raw materials, and those that can be introduced during manufacturing, as mentioned above. Viral clearance studies should demonstrate the reduction, in a logarithmic scale, of the concentration (titer) of viruses after the purification step evaluated.

In order to conduct a viral clearance study, it is necessary and required by regulatory authorities, to proportionally scale-down to a laboratory scale (downscale process) some steps of the industrial process for drug purification. The downscale process of the industrial purification process is required to avoid the introduction of viruses in the industrial plant and to limit the amount of viruses used for each study. The validation studies of the comparability between full-scale and downscale processes are performed by the manufacturing site. After this validation, downscale viral clearance studies could be performed in a separate laboratory from the production site.

In viral clearance studies is not required to perform all the purification steps in linear order as in the industrial process, every step should be individually evaluated. Some steps selected for the down-scale viral clearance study are highligted in the figure below.
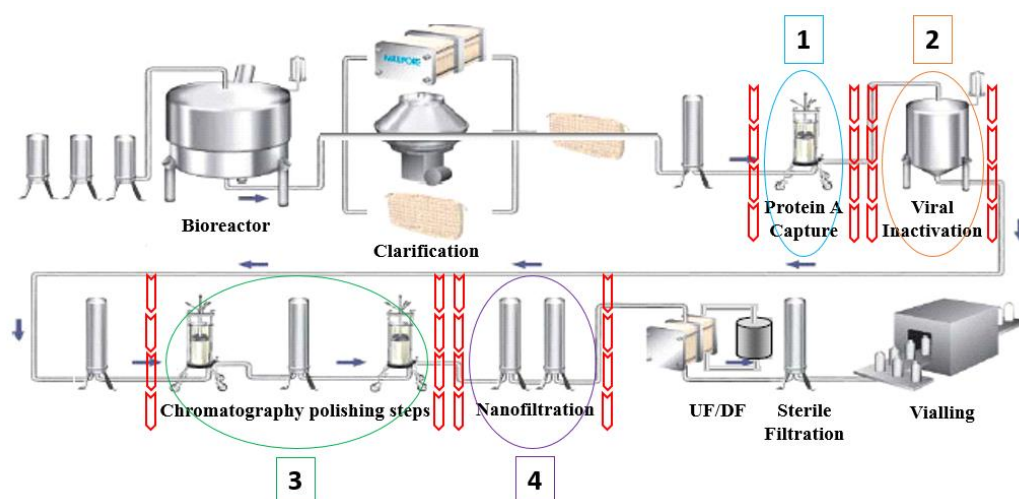
Figure 23. Industrial process for the production of biotechnology products general scheme. Steps evaluated during viral clearance studies are highlighted (1: protein A chromatography, 2: viral inactivation step, 3: AEX and CEX xhromatography, 4: nanofiltration step).

Viral clearance consists in the deliberate addition (spiking) of significant amount of a highly purified virus to the "crude" material and/or to different fractions obtained during the various process steps (starting materials, process intermediates) and demonstrating its removal or inactivation during the subsequent steps. The reduction of virus infectivity

may be achieved by removal (e.g. physical removal) of virus particles or by inactivation of viral infectivity (e.g. pH inactivation) (25).

The amount of virus added to the starting material or to the process intermediate for the downscale purification step to be studied should be as high as possible, in order to determine the capacity of the production step to inactivate/remove viruses adequately. However, the virus spike should be added such that the composition of the production material is not altered (the maximum volume of the virus allowed for the spiking is 10%) (24).

Viruses for viral clearance evaluation studies should be chosen to resemble viruses which may contaminate the product and to represent a wide range of physico-chemical properties of viruses, in order to test the ability of the system to eliminate viruses in general. Viruses used during viral clearance studies fall into three categories (25):

- ❖ *Relevant virus*: virus, or virus of the same species as an identified virus, that are known or likely to contaminate the cell substrate or any other raw materials used in the production process;

- ❖ *Specific model virus:* virus closely related (same genus or family) to a known or suspected virus that may contaminate the cell substrate or raw materials used in the production process, having similar physical and chemical properties (e.g. SV40, parainfluenza virus, herpes virus);

- ❖ *Non-specific model virus*: virus used to characterize the capability of the purification process to remove and/or inactivate viruses in general, i.e. to characterize the robustness of the process at eliminating or inactivating viruses.

Several viruses with different physical and chemical properties could be used. It is not necessary to test all type of viruses. Preference should be given to viruses that show a significant resistance to physical and/or chemical treatments. The number and type of viruses to be included in the studies depends on the type of cell line selected for drug production, the origin of the raw materials used in the production process and the

development status of the product (i.e. early clinical development or late clinical development).

If, in a biotechnology product, expressed in a well-characterized mammalian cell line, no virus has been detected, or only rodent retrovirus, or non-pathogenic retrovirus-like particles have been detected, and the production process does not involve raw materials of animal origin, the viral clearance studies should demonstrate:

- In clinical development Phase I to II: the removal and/or inactivation of at least two relevant or specific model viruses.

- In clinical development Phase III: the removal and/or inactivation of five relevant or specific model viruses. However, more than two viruses may be required, depending on the manufacturing process and the clinical study design (dose, duration, number of patients);

- When drug applying for marketing authorization: a combination of relevant specific and non-specific model viruses should be used to demonstrate the robustness of virus removal and/or inactivation. The selection should cover a wide range of biophysical and biochemical properties (enveloped vs. non-enveloped, DNA vs. RNA, sensitivity to chemical inactivation, etc.). At least four different viruses should be included in the panel.

In all cases, the selection of type and number of viruses to be included in a Viral Clearance Validation Study should be justified. This justification may be supported by the risk analysis associated with the evaluation of the process safety.

Example of Viruses used in Viral Clearance Studies for biotechnology products in:

- Phase I / Phase II are Murine Leukemia Virus (MuLV), Minute Virus of Mice (MVM);

- Phase III are Parainfluenza Virus (hPIVs), Reovirus 3 and Pseudorabies virus / Suid herpesvirus 1 (PRV / SuHV1) in addition to the previous ones.

In this thesis is described the development of a method purification process and the titration assay validation for Mo.A.MuLV, a virus used for Phase I, II and III Viral Clearance studies.

The Viral Clearance Study should be repeated for each virus to be tested and each study is specific for the caracteristics of the virus consiedered. As mentioned above, two or more viruses for each study are required, depending on the clinical phase in which the biotechnology product is under evaluation. Only one kind of virus for each analytical run is tested.

Every process purification step (AEX, CEX, protein A Chromatografy or nanofiltration etc..) is evaluated with two replicates (two analytical runs, for statistic reasons) for each type of virus involved in the Study.

Viral clearance studies are conducted according to ICH (Q5A R1) described above and according to other guidelines such as *EU Guideline for Good Manufacturing Practice (GMP) for Medicinal Products for Human and Veterinary Use*, volume 4 of Eudralex and the collection of rules and regulation governing medicinal products in the European Union. Therefore, the European Medicines Agency (EMA) plays an important role in harmonization and coordination of GMP (24, 25, 26, 27, 28, 29, 30).

GMP, which have the force of law, require that manufacturers, processors, and packagers of drugs, medical devices, some food, and blood take proactive steps to ensure that their products are safe, pure, and effective.

GMP regulations require a quality approach to manufacturing, enabling companies to minimize or eliminate instances of contamination, mix-ups, and errors. This, in turn, protects the consumer from purchasing a product that is not effective or even dangerous. GMP regulations address issues including recordkeeping, personnel qualifications, sanitation, cleanliness, equipment verification, process validation and complaint handling.

GMP requires that the analytical methods used during product- and process-development activities or in characterization studies should be scientifically sound (e.g., specific, sensitive and accurate) and provide results that are reliable. New analytical technology and modifications to existing technology are continually being developed and can be used for these purposes. The use of these methods is particularly appropriate when they reduce risk by providing greater understanding or control of the product quality. In addition, there should be assurance of proper equipment function for laboratory experiments and the procedures for analytical method, equipment maintenance, documentation and calibration practices should be documented or described.

10. Aim of the study

The PhD study purpose was to develop a method for obtaining purified virus stock characterized by high titer and low concentration of impurities (e.g. cell protein). Different techniques should be applied for viral purification, in line with characteristic of different viruses (e.g. MVM, MuLV, Reovirus 3, Parainfluenza 3, and PRV). In particular, for this PhD, the project was focused on Ma.A.MuLV (Moloney Amphotropic Murine Leukemia Virus) purification.

Furthermore, validation of Mo.A.MuLV titration according to GMP (Good Manufacturing Practice) principles and international guidelines, e.g. ICH Q2 (R1), was performed.

11. Preparation (set-up) of high titer and highly purified Mo.A.MuLV viral stocks.

The quality of viral stocks used in viral clearance studies is an essential parameter in order to determine and evaluate the effectiveness of the viral clearance step under evaluation.

Viral stocks used in viral clearance studies should have clearly defined characteristics (24, 28). These characteristics are highly dependent on the viral propagation and purification method. In particular, highly purified virus stocks, defined as Virus Production Lot (VPL), should have the characteristics reported below:

- ❖ **High titer**: this allow not to excessively dilute the starting material. In addition, starting a viral clearance step with an high titer of virus allow to demonstrate an higher Logarithmic Reduction Factor (LRF) at the end of the step;

- ❖ **High purity grade**: in particular, this characteristic is very important during some critical steps, such as nanofiltration, in which the presence of aggregates, protein or DNA coming from the viral propagation process could have a strong impact on the good success of the step.

As described above, the PhD was focused on the development of a method for Mo.A.MuLV purification and titration.

Moloney Amphotropic Murine Leukemia Virus (Mo.A.MuLV) is a medium dimension (80-100 nm) virus belonging to the Retroviridae family of RNA lipidic enveloped viruses. The virus characteristics are summarized in the table below.

Charactetistics	Mo/A-MuLV
Name	*Murine Leukemia Virus*
Family	*Retroviridae*
Host	Murine / Mammalian
Genome	RNA, single strand
Lipidic envelope	yes
Diameter size	80-110 nm
Physico-chemical treatment resistance	Low
Category	Specific virus
Cell line used for propagation	NIH/3T3 Mus Dunni
Cell line used for titration	PG-4 (S+L-)

Table 53. Mo.A.MuLV characteristics.

In order to obtain Moloney Amphotropic Murine Leukemia virus (Mo.A.MuLV) highly purified viral stocks with high titers, precise propagation and purification steps sequences are required. In particular, purification steps are performed to reduce impurity content coming from propagation steps (e.g. cells impurities).

In the paragraphs below are reported the propagation and purification steps set-up and the final operating method defined for the preparation of MuLV highly purified viral stocks.

Different propagation and purification conditions have been tested in order to evaluate and to determine the conditions that allow to obtain a high titer of virus with low impurities concentration.

The conditions related to the virus propagation, tested in the set-up phase, are reported below:

❖ Different cells split ratio before virus infection and split numbers during the purification;

❖ Different incubation temperature during virus propagation;

❖ Different virus concentration (pfu/mL) for initial cells infection;

Regarding virus purification the following condition was evaluated:

❖ Different ultracentrifugation speed and different duration time;

11.1. Propagation method development

The propagation of one aliquot of Mo.A.MuLV (ATCC®, cat n° VR-1450) was started with the infection of NIH/3T3 (mouse embryonal fibroblasts, ATCC® CRL-1658™) cells. NIH/3T3 are the cells suggested to be used for MuLV propagation by ATCC. Four cells flasks were infected with one vial of Moloney Amphotropic Murine Leukemia Virus, 4-fold diluted in the culture media (DMEM 10% FBS). The flasks were incubated for one hour at $37.0 \pm 1.0°C$ and 5.0 ± 1.0 CO_2 in the incubator. After one hour, 20 mL of fresh medium was added to each flask.

After seven days of incubation (as recommended by the virus supplier), the virus propagation was stopped according to the following procedure. The viral suspension was centrifuged in order to eliminate cells impurities, the supernatant was collected and homogenized. Aliquots were prepared and stored at $-80 \pm 10°C$. One aliquot was titrate to evaluate the virus titer. Briefly, the titration consists in a 3-fold serial dilution of the sample (from 3^{-1} to 3^{-33}). Three 96-well plates containing PG4 cells at a concentration of $1.0 * 10^5$ cell/mL (100 μl/well), were infected with the dilution obtained. The cells were incubated for six days and, after this time, the viral infectivity was evaluated by the observation of the presence of foci in each well using an optical microscope. The detail description of the titration method is reported in *paragraphs 4.3.2. and 4.3.3..*

The results of the titration assay is expressed as $Log_{10}TCID_{50}/mL$, Tissue culture infective dose. It is the amount of virus that will produce pathological changes in 50% of cell cultures inoculated.

The viral titer obtained from the propagation described above was 4.52 $Log10TCID50/mL$ ($3.14 * 10^4$ pfu/mL).

As the titer obtained was low, after the first purification step on NIH/3T3 cells, virus propagation was continued with Mus Dunni cells (ATCC® CRL-2017™), mouse skin fibroblasts. Mus Dunni are able to support MuLV replication, as reported in literature *(31)* and in the cells CoA. The virus propagation set-up using Mus Dunni cells, started with the evaluation of influences of different cells split ratio on the virus propagation process.

11.1.1. Different cells split ratio before viral infection and cells splits number during propagation

The day before the viral infection, different Mus Dunni cells flasks were prepared with different split ratio: 1:5, 1:20 and 1:50. One Mus Dunni flask for each different cells split was infected with the virus (one virus aliquot obtained from the previous purification on NIH/3T3 cells was used) at a concentration of 10 ffu/mL: the cells medium was removed and replaced with 2 mL of virus suspension diluted in 10 mL of culture media (McCoy's 5A medium 10% FBS). In addition, 300 μl of Hexadimethrine Bromide (Polybrene, 8 μg/mL) was added to the flasks. Polybrene is used to increase the efficiency of viral infection in the cells. Hexadimethrine bromide acts by neutralizing the charge repulsion between virions and sialic acid on the cell surface (32). The flasks were finally incubated at $37.0 \pm 1.0°C$ and 5.0 ± 1.0 CO_2 in the incubator.

Cells splits are required during MuLV propagation on Mus Dunni, as the cells exhibit cytopathic effects when infected with the virus. Therefore, after two or three incubation days, a 5 or 10-fold cells split was performed. The split was repeated five times (for a total of six splits except for the 1:5 cell split in which five total splits were performed) every two incubation days after each split. The cells medium was collected at every split (except for the cell with 1:5 split before infection) and centrifuged (1500 rpm for 10 minutes) to discard the cells impurities. The supernatant was homogenized, aliquoted and stored at $-80 \pm 10°C$. Each condition were tested by sample titration on PG4 (S+L-) cells to obtain the viral titer. The results are reported in the table below:

Split ratio before infection	Split ratio after infection	Split number after infection	LogTCID50/ml
1:5	1:5	5	7.02 ± 0.28
1:20	1:10	1	5.89 ± 0.34
		2	7.14 ± 0.20
		3	7.79 ± 0.29
		4	7.20 ± 0.21
		5	6.85 ± 0.28
		6	6.90 ± 0.28
1:50	1:10	1	6.01 ± 0.26
		2	8.09 ± 0.32
		3	8.16 ± 0.29
		4	8.09 ± 0.30
		5	8.16 ± 0.21
		6	7.98 ± 0.27

Table 54. Titration results using different split during propagation process.

The tests were repeated several time obtaining the same results. Therefore, at the end of the set up phase, according to the results obtained, the split with ratio 1:50 and 5 splits after infection were selected as the best conditions for MuLV propagation process (titre obtained 8.16 ± 0.21).

11.1.2. Different incubation temperature

After the infection with 10 ffu/mL of virus (in 2 mL), Mus Dunni flasks were incubated at $37.0 \pm 1.0°C$ or $32.0 \pm 1.0°C$ in the incubator with 5.0 ± 1.0 CO_2 % for one hour. After one hour, 30 mL of culture media were added to every cells flask and the cells were incubated at $37.0 \pm 1.0°C$ or $32.0 \pm 1.0°C$ in the incubator with 5.0 ± 1.0 CO_2 % for 12 days. Within this period, a cell split was performed every two or three days.

The results are shown in the table below:

Incubation temperature	Cells split ratio before infection	Titration result (LogTCID50/ml)
37°C	1:20	6.85 ± 0.28
	1:50	8.16 ± 0.21
32°C	1:20	7.98 ± 0.29
	1:50	7.92 ± 0.30

Table 55. Titration results using different incubation temperature during propagation process.

The test was repeated several times confirming the data obtained. Therefore, at the end of the set up phase, according to the results obtained, the incubation of the cell flasks at 37°C was selected as the best condition for MuLV propagation process.

11.1.3. Different virus concentration used for the initial infection

Mus Dunni cells were infected with different concentration of viral suspension: 10, 100 and 1000 ffu/mL (contained in 2 mL of culture medium). After the infection, 8µg/mL Polybrene were added to the cells. The cells were incubated at 37.0 ± 1.0°C in the incubator with 5.0 ± 1.0 CO_2 % for one hour. After one hour, 30 mL of culture media were added to every cells flask and the cells were incubated at 37.0 ± 1.0°C in the incubator with 5.0 ± 1.0 CO_2 % for 12 days. Every two days a 5-fold cells split were performed. The cells culture media of each infection were collected and centrifuged to eliminate cells impurities. The supernatant was homogenized and aliquoted. The aliquots were stored at −80 ± 10°C. Each condition was tested by titration on PG4 (S+L-) cells. The results are reported below:

Viral concentration used for infection (ffu/mL)	Virus titration before purification (crude)	Titration result (LogTCID50/ml)
10		8.16 ± 0.31
100	Crude	7.98 ± 0.14
1000		7.86 ± 0.31

Table 56. Titration results using different virus concentration for cells infection. ffu: focus forming unit.

The test was repeated several times confirming the data obtained. Therefore, at the end of the set up phase, according to the results obtained, the cells infection with 10 ffu/mL of virus was selected for MuLV propagation process, as the difference between the conditions tested was not significant (less than 0.5 Log: difference considered only related to assay variability). Therefore, the concentration of virus used for cells infection was not a critical parameter for the propagation process.

11.2. Purification process

11.2.1. Different ultracentrifugation speed

Ultracentrifugation is the most critical step of the purification process, since ultracentrifuge speed determines virus pellet formation and, at the same time, could have an impact (decrease) on virus infectivity. For these reason, different ultracentrifuge speed were evaluated during purification method development.

The purification process starts with the supernatant collection from Mus Dunni cells after the viral propagation. The suspension containing the cells media and the virus was centrifuged (1800 rpm for 10 minutes) and filtrated on 0.45 μm filter to discard cells impurities. The viral suspension was then ultracentrifuged for 2 hours at 4°C using different settings: 20000 rpm and 25000 rpm (Ultracentrifuge Optima XPN-80 Backman Coulter, rotor SW 32 Ti). The results are reported in the table below:

Ultracentrifuge speed (rpm)	Virus titration after purification (pure)	Titration result ($Log_{10}TCID50/ml$)
20000	Pure	7.62
25000		7.14

Table 57. Titration results of MuLV purification process using different ultracentrifuge speeds.

The test was repeated several times confirming the data obtained. Therefore, at the end of the set up phase, according to the obtained results, the lower ultracentrifuge speed (20000 rpm) was selected for the Mo.A.MuLV purification process.

In addition, at the end of the purification steps, it was decided to filter the viral suspension obtained, in order to reduce aggregates in the final virus bank. Considering the dimension of Mo.A-MuLV virus, a 0.22 µm filter was used to obtain a complete removal of virus aggregates and allowing , at the same time, the single virus particles to pass through the filter.

11.3. Final Methods

11.3.1. Mo.A.MuLV propagation method

Moloney Amphotropic Murine Leukemia Virus (Mo.A-MuLV) should be initially propagated on NIH-3T3 cells to prepare the MVB (Master Virus Bank) as described below.

One vial of NIH-3T3 cells were thawed and cultured in sterile 75 cm^2 flasks until a maximum of twelve passages using DMEM 10% FBS culture media. The day before the infection, a 4-fold cells split were performed. Four cells flasks are required for viral infection. Cells monolayer should be at 60-70% of confluence at the infection day.

One original Moloney Amphotropic Murine Leukemia Virus (Mo.A-MuLV) was thawed at $37.0°C \pm 1.0°C$ and was added to 7 mL of DMEM 10%FBS.

The culture medium was removed from the flasks, cells monolayer was washed with PBS (3 mL) and infected with 2 mL of viral suspension. Cell flasks were incubated at $37.0°C \pm 1.0°C$ with $5.0 \pm 1.0\%$ CO_2 for one hour. After one hour, 18 mL of culture media (DMEM 10% FBS) were added to each flask. The flasks were finally incubated at $37.0°C \pm 1.0°C$ with $5.0 \pm 1.0\%$ CO_2 for seven days.

After seven days, cell supernatant was collected and centrifuged at 1000 rpm for 10 minutes in order to eliminare cells impurities. The solution obtained was homogeneized and aliquotes (about 1 mL each) were prepared in cryovials and stored at $-80°C \pm 10°C$ to obtain a Mo.A MuLV (VR-1450) Master Virus Bank (MVB). The MVB obtained

should be qualified according to the internal procedure and to the international guidelines.

The MVB should be further propagated on Mus Dunni cells in order to obtain highly purified viral stocks. Mo.A.MuLV propagation method on Mus Dunni and following purification method are reported in the paragraph below.

One vial of Mus Dunni cells were thawed and cultured in sterile 150 cm^2 flasks until a maximum of twelve passages using McCoy's 5A Medium 10%FBS culture media. Two days before the infection, a 50-fold cells split were performed. Three cells flasks are usually required for viral infection. The cells were incubated at 37.0°C ± 1.0°C with 5.0 ± 1.0% CO$_2$ for 24 hours. The day before the infection, 8 µg/mL Polybrene was added to each flask. The cells were incubated at 37.0°C ± 1.0°C with 5.0 ± 1.0% CO$_2$ for one day. After this time, cells were infected with 300 pfu/ml of Mo.A.MuLV MVB (30 mL each flask). The cells were incubated at 37.0°C ± 1.0°C with 5.0 ± 1.0% CO$_2$ for two-three days until the cells total confluence. The cells were split every two days for a total of five splits to allow the virus propagation and amplification.

11.3.2. Mo.A.MuLV purification method

After the viral propagation on Mus Dunni, supernatants from cells flasks were collected and centrifuged at 1000 rpm for 10 minuti at 4°C. Supernatant from each flask were pooled, homogeneized and filtered with 0.45 µm filter to remove any cell impurities still present in the viral suspension. Supernatant aliquots were then prepared in ultracentrifugation tubes using a siringe and avoid foaming during tube filling.

Tubes need to be paired and weight balanced each other (max weight difference of 0.07 g) for the ultracentrifuge weight sensitivity. The viral suspension was ultracentrifuged at 20000 rpm for 2 hours at 4°C. This conditions is required to allow the formation of virus pellet and separation from the supernatant. At the end of the centrifugation, the tubes were opened and the supernatant was removed. The pellet obtained was resuspended with TNE buffer. The final resuspension volume should be ⅓ of the

supernatant volume collected form the culture cell flasks. Using a lower resuspension volume allows to recover any loss due to the purification process.

Initially a very low TNE volume should be used to resuspend the pellet in order to disrupt the aggregates as much as possible. The pooled suspension obtained from the ultracentrifugation was homogeneized and stored at 5.0 ± 3.0 °C overnight or for at least four hours to further disrupt virus aggregates.

After 24 hours, the remaining TNE volume was added to the suspension to achieve the total volume needed.

The suspension was then sonicated in a ultrasound water bath for 60 seconds.

Finally, the suspension was filtered with a 0.22 µm PES Membrane filter to remove any viral aggregates. The obtained suspension was homogeneized and aliquots (of about 1 mL) were prepared in cryovials and stored at -80°C ± 10°C.

All the purification steps are summarized in the scheme reported below:

Centrifugation
(10 min, 1'000 rpm, 4°C)
+ 0,45 µm filtration

Ultracentrifugation
(2 h, 20'000 rpm, 4°C)

Resuspension with TNE
+ overnight at 5 ± 3°C
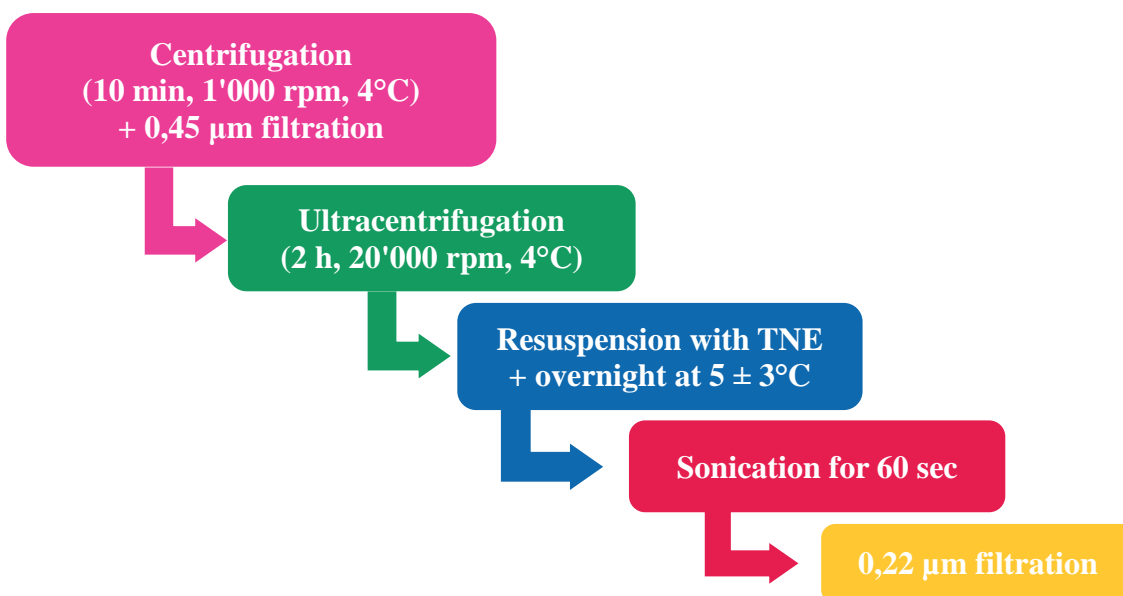
Sonication for 60 sec

0,22 µm filtration

Figure 24. Mo.A.MuLV purification steps scheme.

The collected vials make up a Virus Production Lot (VPL). The VPL should be qualified according to the internal procedure and to the international guidelines, before their use in Viral Clearance studies.

12. Validation of a titration method for Mo.A.MuLV

Different paramenters such as cells concentration used in the titration plates, different titration plates incubation days were tested during a set-up phase. After the method development, the method was finally validated. In the next paragraphs are reported the results coming from the method validation.

According to ICH Q2(R1) guideline and internal procedures, the "Mo.A-MLV (Moloney Amphotropic Murine Leukemia Virus) Titration Assay" method is considered a quantal assay. Therefore, it is possible to determinate the concentration of the virus in a sample by serially diluting it and infect detector cells with the different dilutions prepared. After an incubation time of six days, the effect of the virus on the detector cells was observed using an optical microscope. Following a statistical formula (Spermann-Kaber) it is possible to calculate the initial virus titer, expressed in $Log_{10}TCID_{50}/mL$. The detailed method is reported in the next paragraphs.

Validated parameters are listed below:

- ❖ Accuracy;
- ❖ Precision:
  - o Repeatability;
  - o Intermediate precision;
- ❖ Linearity;
- ❖ Robustness:
  - o at different incubation time;
  - o with different culture media;
  - o at different pH.

Specificity of the method was assessed in each titration plate (96-well plate) by comparing the "positive" wells (wells in which the presence of the virus is revealed with morphological changes on cells monolayer) with the negative control (cells not infected with the virus, the well contains only the cells and their culture media) in each titration

plate. The comparison was performed with the observation of each well using an optical microscope.

The tests on the described above parameters were conducted under a Test Protocol to check the reliability of the analytical method as used in the "BQC-Viral Clearance" laboratory.

During validation the reliability of the method was demonstrated with respect to set efficiency characteristics chosen depending on the type of method and on its specific application. The availability of qualification, calibration and traceability documentation for, respectively, equipment/systems, laboratory equipment and standards used for carrying out method validation testing was also verified (according to the guidelines).

12.1. Description of the method

The "Mo.A-MuLV virus (Moloney Amphotropic Murine Leukemia Virus) Titration Assay" method allows determination of the viral titer of Mo.A-MuLV suspensions that induce morphological changing (focus forming plaque) on a monolayer of detector cells PG4(S+L-) (cat astrocytes). The titer is expressed as $TCID_{50}$/ml (Tissue Culture Infective Dose). It is the amount of virus that will produce pathological change in 50% of cell cultures inoculated.

12.1.1. Principles of the method

The Mo.A-MuLV virus (Moloney Amphotropic Murine Leukemia Virus) Titration Assay is a quantal method that allows determination of the concentration of virus required to infect 50% of the cultured cells, i.e. the $TCID_{50}$ of the viral suspension.

The titer of Mo.A-MuLV can be determined by assessing the effect induced on PG4(S+L-) cells (cat astrocytes: connective tissue cells, see *table below*), an embryonal cat cell line stable infected with the Moloney, isolated from Murine Sarcoma Virus. This

virus is activated after a simultaneous infection with retrovirus as Murine Leukemia Virus.

Cell line	Species	Type	Tissue
PG4 (S+L-)	*Felis catus*, cat	Astrocytes	Brain

Table 58. PG4 (S+L-) cell line origin.

Replication of Murine Sarcoma Virus induces morphological changes on cellular monolayer (focus forming plaque) as shown in the *figures below*.
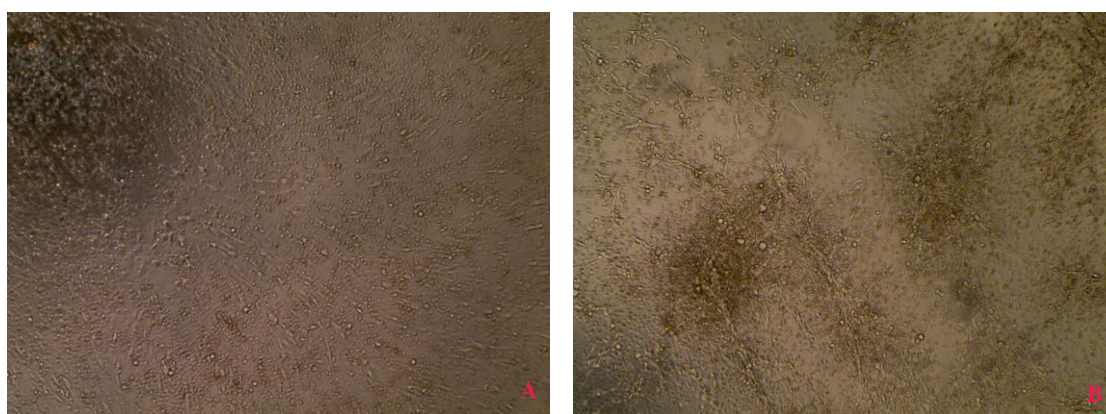
Figure 25. The picture show the intact cell monolayer (A, left) and the morphological changes of the cells infected with Mo.A.MuLV (B, right)

The method is based on assessing the effect induced on the cell monolayer by various viral suspension scalar dilutions.

12.2. Materials and methods

The following analytical apparatures/equipment/reference standards/solutions were used during method validation:

❖ **Test substance**

Validation was tested using viral suspensions of Mo.A-MuLV obtained by propagating an original vial of Mo.A-MuLV (VR-1450 ATCC) on MUS DUNNI cells (CRL-2017™) and titrated it on PG4 (S+L-) cells (CRL-2032).

❖ **Material**

- Polypropylene high capacity plates
- Flat-bottom 96-well low evaporation plates
- Trough
- Single and multi-channel pipettes (15 – 1200 μL dosing range)
- Tips for 50, 300 and 1200 μl pipettes
- Sterile tubes

❖ **Reagents**

- McCoy's 5A Medium culture medium 2% FBS
- McCoy's 5A Medium culture medium 2% FBS with 1% Penicillin-Streptomycin and 1% Amphotericin
- McCoy's 5A Medium culture medium 10% FBS
- Phosphate Buffer Saline (PBS)
- Polybrene 0.8 mg/ml
- HCl 1M
- NaOH 1M
-

❖ **Equipment**

- CO_2 incubator
- Optical microscope
- Refrigerator
- -20°C freezer
- -80°C freezer

- Biological laminar Hood

12.3. Analytical procedure

All reagents and solution preparation were reported in a specific laboratory record book, according to GMP requirements.

The reagents preparation are reported below.

12.3.1. Solution preparations

❖ **McCoy's 5A Medium 10% FBS** preparation (for cell culturing): this medium was used for culturing and plating PG4(S+L-) cells. The culture medium was prepared according to an internal procedure starting from commercially available Lyophilyzed medium (Sigma, cat n°M4892). McCoy's 5A Medium 10% FBS was prepared by dissolving an aliquot of lyophilized medium, formulated to contain 11.9 grams of powder per liter of medium, in 1000 ml of ultrapure water. Solution obtained is supplemented with 2.2 g/L sodium bicarbonate and then it is filtered on 0.22 µm filter to obtain a sterile medium. 100 ml of filtered medium are substituted with 100 ml of FBS, in order to obtain a medium added of 10% serum. The sterile medium expired after one month after preparation.

❖ **McCoy's 5A Medium 2% FBS** preparation (for viral growth)
This culture medium was used for titrating Mo.A-MLV on PG4(S+L-) cells. The culture medium was prepared according to an internal procedure starting from commercially available Lyophilyzed medium (Sigma, cat n°M4892). McCoy's 5A Medium 2% FBS was prepared as described for the McCoy's 5A medium 10% FBS (see above) except for the volume of serum. 20 ml of filtered medium are substituted with 20 ml of FBS instead of 100 mL, in order to obtain a medium

added of 2% serum. The sterile medium expired after one month after preparation.

❖ **McCoy's 5A Medium 2% FBS with antibiotic and antifungal agent preparation (for viral growth)**

This culture medium was used for titrating Mo.A-MLV on PG4 (S+L-) cells. The medium contains antibiotic and antifungal agent (1% Penicillin-Streptomycin and 1% Amphotericin B).

The culture medium was prepared according to an internal procedure starting from commercially available Lyophilyzed medium (Sigma, cat n°M4892). McCoy's 5A Medium 2% FBS with antibiotic and antifungal was prepared as described for the McCoy's 5A Medium 2% FBS. In addition, before filtering the medium, 1% Penicillin-Streptomycin and 1% Amphotericin B were added to the medium (10 mL of each solution for 1 Lt of medium). The sterile medium expired after one month after preparation.

❖ **Hexadimethrine bromide (Polybrene) 0.8 mg/mL solution preparation**

The Hexadimethrine bromide solution was prepared by weigh 8 mg of Hexadimethrine bromide and dissolve it in 10 mL of ultrapure water. The solution is then filtered on 0.22 µm filter to obtain a sterile solution. The sterile solution expired after one month after preparation.

12.3.2. Moloney Amphotropic Murine Leukemia virus (Mo.A.MuLV) dilutions preparation

The viral suspension (Mo.A-MuLV) obtained following propagation on MUS DUNNI cells was diluted with 1:3 scalar dilutions.

Each titration consists of three plates (flat bottom low evaporation 96-well plates) of PG4(S+L-) cells (plated at a concentration of $1.0*10^5$ cell/mL, 100µl/well):

Plate 1 → infection with dilutions from 3^{-1} to 3^{-11}, last column negative control;

Plate 2 → infection with dilutions from 3^{-12} to 3^{-22}, last column negative control;

Plate 3 → infection with dilutions from 3^{-23} to 3^{-33}, last column negative control

For all titrations (Plates 1, 2 and 3) the dilutions were prepared in a high capacity polypropylene plate as described in the procedure below (see the plate format in the *Table 59*).

1) The first 6 rows of the high capacity plate were filled with 600 µl of McCoy's 5A culture medium 2% FBS

→ rows A and B were used to infect Plate 1 (dilutions from 3^{-1} to 3^{-11} + negative control)

→ rows C and D were used to infect Plate 2 (dilutions from 3^{-12} to 3^{-22} + negative control)

→ rows E and F were used to infect Plate 3 (dilutions from 3^{-23} to 3^{-33} + negative control)

2) 300 µl of Mo.A-MuLV viral suspension (Stock) were added to row A column 1, and 300 µl of Mo.A-MuLV viral suspension (Stock) to row B column 1, obtaining a suspension diluted 1:3. Pipette at least 8 times to obtain a homogenous suspension.

3) 300 µl were taken from rows A and B column 1 (well A1 and well B1) and added to rows A and B column 2 (well A2 and well B2). The suspension was homogenized by pipetting at least 8 times. The transfer was repeated until reaching column 11 (column 12 was skipped → negative control) obtaining the dilutions from 3^{-1} to 3^{-11}.

4) 300 µl were taken from rows A and B column 11 (well A11 and well B11) and transferred to the 2 wells in rows C and D column 1 (C1 and D1). The suspension was homogenized by pipetting at least 8 times. The transfer was repeated until

reaching column 11 (column 12 was skipped → negative control) obtaining the dilutions from 3^{-12} to 3^{-22}.

5) 300 µl were taken from rows C and D column 11(well C11 and well D11) and transferred to the 2 wells in rows E and F column 1 (E1 and F1). The suspension was homogenized by pipetting at least 8 times. The transfer was repeated until reaching column 11 (column 12 was skipped → negative control) obtaining the dilutions from 3^{-23} to 3^{-33}.

6) After scalar dilution preparation, 6 µL of Polybrene 0.8 mg/ml were added in each well to obtain a final concentration of 8 µg/ml.

The plate layout reported below was followed for the dilution plate (high capacity plate) prepared as described in the paragraph above:

	1	2	3	4	5	6	7	8	9	10	11	12	
A	600 µL McCOY'S 2%FBS + 300 µL STOCK MuLV(3^{-1})	600 µL McCOY'S 2%FBS + 300 µL WELL A1 (3^{-2})	600 µL McCOY'S 2%FBS + 300 µL WELL A2 (3^{-3})	600 µL McCOY'S 2%FBS + 300 µL WELL A3 (3^{-4})	600 µL McCOY'S 2%FBS + 300 µL WELL A4 (3^{-5})	600 µL McCOY'S 2%FBS + 300 µL WELL A5 (3^{-6})	600 µL McCOY'S 2%FBS + 300 µL WELL A6 (3^{-7})	600 µL McCOY'S 2%FBS + 300 µL WELL A7 (3^{-8})	600 µL McCOY'S 2%FBS + 300 µL WELL A8 (3^{-9})	600 µL McCOY'S 2%FBS + 300 µL WELL A9 (3^{-10})	600 µL McCOY'S 2%FBS + 300 µL WELL A10 (3^{-11})	600 µL McCOY'S 2%FBS C-	1° PLATE
B	600 µL McCOY'S 2%FBS + 300 µL STOCK MuLV(3^{-1})	600 µL McCOY'S 2%FBS + 300 µL WELL B1 (3^{-2})	600 µL McCOY'S 2%FBS + 300 µL WELL B2 (3^{-3})	600 µL McCOY'S 2%FBS + 300 µL WELL B3 (3^{-4})	600 µL McCOY'S 2%FBS + 300 µL WELL B4 (3^{-5})	600 µL McCOY'S 2%FBS + 300 µL WELL B5 (3^{-6})	600 µL McCOY'S 2%FBS + 300 µL WELL B6 (3^{-7})	600 µL McCOY'S 2%FBS + 300 µL WELL B7 (3^{-8})	600 µL McCOY'S 2%FBS + 300 µL WELL B8 (3^{-9})	600 µL McCOY'S 2%FBS + 300 µL WELL B9 (3^{-10})	600 µL McCOY'S 2%FBS + 300 µL WELL B10 (3^{-11})	600 µL McCOY'S 2%FBS C-	
C	600 µL McCOY'S 2%FBS + 300 µL WELL A11 (3^{-12})	600 µL McCOY'S 2%FBS + 300 µL WELL C1 (3^{-13})	600 µL McCOY'S 2%FBS + 300 µL WELL C2 (3^{-14})	600 µL McCOY'S 2%FBS + 300 µL WELL C3 (3^{-15})	600 µL McCOY'S 2%FBS + 300 µL WELL C4 (3^{-16})	600 µL McCOY'S 2%FBS + 300 µL WELL C5 (3^{-17})	600 µL McCOY'S 2%FBS + 300 µL WELL C6 (3^{-18})	600 µL McCOY'S 2%FBS + 300 µL WELL C7 (3^{-19})	600 µL McCOY'S 2%FBS + 300 µL WELL C8 (3^{-20})	600 µL McCOY'S 2%FBS + 300 µL WELL C9 (3^{-21})	600 µL McCOY'S 2%FBS + 300 µL WELL C10 (3^{-22})	600 µL McCOY'S 2%FBS C-	2° PLATE
D	600 µL McCOY'S 2%FBS + 300 µL WELL B11 (3^{-12})	600 µL McCOY'S 2%FBS + 300 µL WELL D1 (3^{-13})	600 µL McCOY'S 2%FBS + 300 µL WELL D2 (3^{-14})	600 µL McCOY'S 2%FBS + 300 µL WELL D3 (3^{-15})	600 µL McCOY'S 2%FBS + 300 µL WELL D4 (3^{-16})	600 µL McCOY'S 2%FBS + 300 µL WELL D5 (3^{-17})	600 µL McCOY'S 2%FBS + 300 µL WELL D6 (3^{-18})	600 µL McCOY'S 2%FBS + 300 µL WELL D7 (3^{-19})	600 µL McCOY'S 2%FBS + 300 µL WELL D8 (3^{-20})	600 µL McCOY'S 2%FBS + 300 µL WELL D9 (3^{-21})	600 µL McCOY'S 2%FBS + 300 µL WELL D10 (3^{-22})	600 µL McCOY'S 2%FBS C-	
E	600 µL McCOY'S 2%FBS + 300 µL WELL C11 (3^{-23})	600 µL McCOY'S 2%FBS + 300 µL WELL E1 (3^{-24})	600 µL McCOY'S 2%FBS + 300 µL WELL E2 (3^{-25})	600 µL McCOY'S 2%FBS + 300 µL WELL E3 (3^{-26})	600 µL McCOY'S 2%FBS + 300 µL WELL E4 (3^{-27})	600 µL McCOY'S 2%FBS + 300 µL WELL E5 (3^{-28})	600 µL McCOY'S 2%FBS + 300 µL WELL E6 (3^{-29})	600 µL McCOY'S 2%FBS + 300 µL WELL E7 (3^{-30})	600 µL McCOY'S 2%FBS + 300 µL WELL E8 (3^{-31})	600 µL McCOY'S 2%FBS + 300 µL WELL E9 (3^{-32})	600 µL McCOY'S 2%FBS + 300 µL WELL E10 (3^{-33})	600 µL McCOY'S 2%FBS C-	3° PLATE
F	600 µL McCOY'S 2%FBS + 300 µL WELL D11 (3^{-23})	600 µL McCOY'S 2%FBS + 300 µL WELL F1 (3^{-24})	600 µL McCOY'S 2%FBS + 300 µL WELL F2 (3^{-25})	600 µL McCOY'S 2%FBS + 300 µL WELL F3 (3^{-26})	600 µL McCOY'S 2%FBS + 300 µL WELL F4 (3^{-27})	600 µL McCOY'S 2%FBS + 300 µL WELL F5 (3^{-28})	600 µL McCOY'S 2%FBS + 300 µL WELL F6 (3^{-29})	600 µL McCOY'S 2%FBS + 300 µL WELL F7 (3^{-30})	600 µL McCOY'S 2%FBS + 300 µL WELL F8 (3^{-31})	600 µL McCOY'S 2%FBS + 300 µL WELL F9 (3^{-32})	600 µL McCOY'S 2%FBS + 300 µL WELL F10 (3^{-33})		
G													
H													

Table 59. High capacity plate format for the titration of three 96-well plates. Rows A and B (highlighted in pink) are used for plate 1 titration, rows C and D (highlighted in green) for plate 2 titration and rows E and F (highlighted in blue) for plate 3 titration (see paragraph below).

12.3.3. Titration procedure

On the day prior to titration, the PG4 (S+L-) cells plates were prepared as described below.

The PG4(S+L-) cells were cultured with McCoy's 5A culture medium 10 % FBS in 75 cm^2 flasks. the cells were detached with trypsin (Sigma, cat. N°T3924), centrifuged at 1000 g for 10 minutes, resuspended in a known volume (3 or 5 mL depending on the size of the pellet) and then counted with a nucleocounter. 3 flat bottom 96-well low evaporation plates were prepared (Plates 1, 2 and 3) by dispensing 100 µl of the resulting cell suspension (concentration $1.0*10^5$ cells/ml) in each well of each plate as shown in the table below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS **C-**
B	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS **C-**
C	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS **C-**
D	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS **C-**
E	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS **C-**
F	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS **C-**
G	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS **C-**
H	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS	100 µL PG4(S+L-) McCOY'S 10%FBS **C-**

Table 60. *Titration plate scheme. The cells are plated the day before the infection at a concentration of $1 * 10^5$ cells/mL. Each well contains 100 µl of cell suspension. The cells are cultured with McCoy's 5A medium supplied with 10%FBS.*

The plates were incubated at $37.0\pm1.0°C$ with $5.0\pm1.0\%CO_2$ for 24 ± 2 hours.

After about 24h, the plates were removed from the incubator and the content was emptied by turning the plate upside down into a trough containing sodium hypochlorite, for inactivation. The cell monolayer was washed with 50 µl of PBS (each well) to get rid of any residues of serum and dead cells. The plates was again emptied by turning upside down into a trough containing sodium hypochlorite and, finally, the plates 1, 2 and 3 were infected as described below.

Plate 1 infection :

1) 100 µl of the content of the high capacity plate row A were aspirated using a multichannel pipette and dispensed in Plate 1 row A, so that the content of the tip used for aspirating from column 1 was dispensed in Plate 1 column 1.

2) The transfer was repeated a further 3 times dispensing respectively in row B, C and D of Plate 1 (for a total of four plate 1 rows filled from one high capacity plate row).

3) 100 µl of the content of the high capacity plate row B were aspirated using a multichannel pipette and dispensed in Plate 1 row E, so that the content of the tip used for aspirating from column 1 is dispensed in column 1 of Plate 1.

4) The transfer was repeated a further 3 times dispensing respectively in row F, G and H of Plate 1 (for a total of four plate 1 rows filled from one high capacity plate row).

The high capacity plates rows used to prepare plate 2 are highlighted in pink in the *Table 59*.

Plate 1 content are reported in the table below.

	1 MULV 3^{-1}	2 MULV 3^{-2}	3 MULV 3^{-3}	4 MULV 3^{-4}	5 MULV 3^{-5}	6 MULV 3^{-6}	7 MULV 3^{-7}	8 MULV 3^{-8}	9 MULV 3^{-9}	10 MULV 3^{-10}	11 MULV 3^{-11}	12 C-
A	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-1}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-2}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-3}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-4}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-5}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-6}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-7}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-8}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-9}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-10}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-21}	PG4(S+L-) in 100 µL McCOY'S 2%FBS
B	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-1}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-2}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-3}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-4}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-5}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-6}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-7}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-8}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-9}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-10}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-21}	PG4(S+L-) in 100 µL McCOY'S 2%FBS
C	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-1}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-2}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-3}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-4}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-5}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-6}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-7}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-8}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-9}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-10}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-21}	PG4(S+L-) in 100 µL McCOY'S 2%FBS
D	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-1}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-2}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-3}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-4}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-5}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-6}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-7}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-8}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-9}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-10}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-21}	PG4(S+L-) in 100 µL McCOY'S 2%FBS
E	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-1}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-2}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-3}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-4}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-5}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-6}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-7}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-8}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-9}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-10}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-21}	PG4(S+L-) in 100 µL McCOY'S 2%FBS
F	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-1}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-2}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-3}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-4}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-5}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-6}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-7}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-8}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-9}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-10}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-21}	PG4(S+L-) in 100 µL McCOY'S 2%FBS
G	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-1}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-2}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-3}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-4}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-5}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-6}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-7}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-8}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-9}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-10}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-21}	PG4(S+L-) in 100 µL McCOY'S 2%FBS
H	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-1}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-2}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-3}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-4}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-5}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-6}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-7}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-8}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-9}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-10}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-21}	PG4(S+L-) in 100 µL McCOY'S 2%FBS

*Table 61. Plate 1 content scheme. Each well contains PG4 (S+L-) cells at a concentration of 1.0 *10^5 cells/mL in 100µl of McCoy's 5A medium supplied with 2%FBS, antibiotical and antifungal agent(penicillin-streptomycin and amphotericin B). In addition to the medium, 8 µg/mL Polybrene was added to the plate. The deep volume plate row A was used to fill the rows A, B, C and D of the titer plate 1 (highlighted in dark pink); the row B was used to fill the rows E, F, G and H of the titer plate 1 (highlighted in soft pink). The column 12 is the negative control: only the cells and the culture media are present in it.*

Plate 2 infection:

1) 100 µl of the content of the high capacity plate row C were aspirated using a multichannel pipette and dispensed in Plate 2 row A, so that the content of the tip used for aspirating from column 1 was dispensed in Plate 2 column 1.

2) The transfer was repeated a further 3 times always taking from row C and dispensing respectively in row B, C and D of Plate 2 (for a total of four plate 2 rows filled from one high capacity plate row).

3) 100 µl of the content of the high capacity plate row D were aspirated using a multichannel pipette and dispensed in Plate 2 row E, so that the content of the tip used for aspirating from column 1 is dispensed in column 1 of Plate 2.

4) The transfer was repeated a further 3 times always taking from row D and dispensing respectively in row F, G and H of Plate 2 (for a total of four plate 2 rows filled from one high capacity plate row).

The high capacity plates rows used to prepare plate 2 are highlighted in the *Table 59*.

Plate 2 content are reported in the table below.

	1 MULV 3^{-12}	2 MULV 3^{-13}	3 MULV 3^{-14}	4 MULV 3^{-15}	5 MULV 3^{-16}	6 MULV 3^{-17}	7 MULV 3^{-18}	8 MULV 3^{-19}	9 MULV 3^{-20}	10 MULV 3^{-21}	11 MULV 3^{-22}	12 C-
A	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-12}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-13}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-14}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-15}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-16}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-17}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-18}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-19}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-20}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-21}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-22}	PG4(S+L-) + McCOY'S 2%FBS
B	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-12}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-13}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-14}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-15}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-16}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-17}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-18}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-19}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-20}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-21}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-22}	PG4(S+L-) + McCOY'S 2%FBS
C	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-12}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-13}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-14}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-15}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-16}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-17}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-18}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-19}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-20}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-21}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-22}	PG4(S+L-) + McCOY'S 2%FBS
D	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-12}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-13}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-14}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-15}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-16}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-17}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-18}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-19}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-20}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-21}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-22}	PG4(S+L-) + McCOY'S 2%FBS
E	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-12}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-13}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-14}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-15}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-16}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-17}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-18}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-19}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-20}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-21}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-22}	PG4(S+L-) + McCOY'S 2%FBS
F	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-12}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-13}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-14}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-15}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-16}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-17}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-18}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-19}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-20}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-21}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-22}	PG4(S+L-) + McCOY'S 2%FBS
G	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-12}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-13}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-14}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-15}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-16}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-17}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-18}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-19}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-20}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-21}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-22}	PG4(S+L-) + McCOY'S 2%FBS
H	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-12}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-13}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-14}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-15}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-16}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-17}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-18}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-19}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-20}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-21}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-22}	PG4(S+L-) + McCOY'S 2%FBS

Table 62. Plate 2 content scheme. Each well contains PG4 (S+L-) cells at a concentration of 1.0 *10^5 cells/mL in 100μl of McCoy's 5A medium supplied with 2%FBS, antibiotical and antifungal agent(penicillin-streptomycin and amphotericin B). In addition to the medium, 8 μg/mL Polybrene was added to the plate. The deep volume plate row A was used to fill the rows A, B, C and D of the titer plate 1 (highlighted in dark pink); the row B was used to fill the rows E, F, G and H of the titer plate 1 (highlighted in soft pink). The column 12 is the negative control: only the cells and the culture media are present in it.

Plate 3 infection:

1) 100 µl of the content of the high capacity plate row E were aspirated using a multichannel pipette and dispensed in Plate 3 row A, so that the content of the tip used for aspirating from column 1 was dispensed in Plate 3 column 1.

2) The transfer was repeated a further 3 times dispensing respectively in row B, C and D of Plate 3 (for a total of four plate 1 rows filled from one high capacity plate row).

3) 100 µl of the content of the high capacity plate row F were aspirated using a multichannel pipette and dispensed in Plate 3 row E, so that the content of the tip used for aspirating from column 1 is dispensed in column 1 of Plate 1.

4) The transfer was repeated a further 3 times dispensing respectively in row F, G and H of Plate 3 (for a total of four plate 1 rows filled from one high capacity plate row).

The high capacity plates rows used to prepare plate 3 are highlighted in the *Table 59*.

Plate 3 content are reported in the table below.

	1	2	3	4	5	6	7	8	9	10	11	12
	MULV 3^{-23}	MULV 3^{-24}	MULV 3^{-25}	MULV 3^{-26}	MULV 3^{-27}	MULV 3^{-28}	MULV 3^{-29}	MULV 3^{-30}	MULV 3^{-31}	MULV 3^{-32}	MULV 3^{-33}	C-
A	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-12}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-24}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-25}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-26}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-27}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-28}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-29}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-30}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-31}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-32}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-33}	PG4(S+L-) + McCOY'S 2%FBS
B	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-12}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-24}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-25}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-26}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-27}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-28}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-29}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-30}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-31}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-32}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-33}	PG4(S+L-) + McCOY'S 2%FBS
C	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-12}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-24}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-25}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-26}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-27}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-28}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-29}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-30}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-31}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-32}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-33}	PG4(S+L-) + McCOY'S 2%FBS
D	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-12}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-24}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-25}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-26}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-27}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-28}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-29}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-30}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-31}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-32}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-33}	PG4(S+L-) + McCOY'S 2%FBS
E	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-12}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-24}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-25}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-26}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-27}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-28}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-29}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-30}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-31}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-32}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-33}	PG4(S+L-) + McCOY'S 2%FBS
F	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-12}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-24}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-25}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-26}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-27}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-28}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-29}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-30}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-31}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-32}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-33}	PG4(S+L-) + McCOY'S 2%FBS
G	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-12}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-24}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-25}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-26}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-27}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-28}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-29}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-30}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-31}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-32}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-33}	PG4(S+L-) + McCOY'S 2%FBS
H	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-12}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-24}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-25}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-26}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-27}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-28}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-29}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-30}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-31}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-32}	PG4(S+L-) + McCOY'S 2%FBS + MuLV 3^{-33}	PG4(S+L-) + McCOY'S 2%FBS

Table 63. Plate 3 content scheme. Each well contains PG4 (S+L-) cells at a concentration of $1.0 *10^5$ cells/mL in 100µl of McCoy's 5A medium supplied with 2%FBS, antibiotical and antifungal agent(penicillin-streptomycin and amphotericin B). In addition to the medium, 8 µg/mL Polybrene was added to the plate. The deep volume plate row A was used to fill the rows A, B, C and D of the titer plate 1 (highlighted in dark pink); the row B was used to fill the rows E, F, G and H of the titer plate 1 (highlighted in soft pink). The column 12 is the negative control: only the cells and the culture media are present in it.

Once the 3 plates have been infected, were placed in an incubator at $37.0\pm1.0°C$ in $5.0\pm1.0\%$ CO_2 for 6 days.

After 6 days, the evaluation of the plates were done using an optical microscope to assess whether or not there were morphological changes (focus forming plaque) on cell monolayer. As the titration is a quantal assay, the plates evaluation is not quantitative: a well is considered "positive" if there are morphological changes on cells monolayer, independently from the number of the foci observed in the well.

12.4. Results

12.4.1. Calculating results

Results were calculated by visual examination using an optical microscope to assess the cell monolayer integrity. By using the optical microscope the operator has defined in which wells there were a cytopathic effect on the cell monolayer and has completed the validated Excel file "*Viral Titer by Spearman Karber Formula*" to achieve the $Log_{10}TCID_{50}$ (A **mean**).

The $Log_{10}TCID50$ (A mean), where A is the Log_{10} of the titer for a certain volume analyzed, was calculated using the formula below:

$$\mathbf{Log_{10}TCID_{50}\ (A\ mean)} = X_o - (d/2) + d\textstyle\sum pi$$

The excel file calculates also the Standard Deviation which can be used to obtain the Maximum (**A max**) and Minimum (**A min**) Titer.

The calculation to obtain the Standard Deviation is:

$$\mathbf{Sd} = d2 \textstyle\sum (\ pi\ (1\text{-}pi))\ /\ (ni\text{-}1)$$

The calculations to obtain **A max** and **A min** are:

$$\textbf{Amax} = \text{Log}_{10}\text{TCID50 } (\textbf{A mean}) - (2* \text{ Sd})$$
$$\textbf{Amin} = \text{Log}_{10}\text{TCID50 } (\textbf{A mean}) + (2* \text{ Sd})$$

$X_0 = \text{Log}_{10}$ of the reciprocal of the highest dilution that produces 100% of infection

$d = \text{Log}_{10}$ of the dilution factor

pi = proportion of wells with a positive result at each single dilution.

Σ pi = sum of pi starting from the highest dilution with 100% of infection.

ni= number of replicates

12.4.2. Validity of results

The test is considered valid if:

- ❖ The difference between A max and Log_{10}TCID50 (A mean) is ≤ 0.5 Log and if
- ❖ The difference between Log_{10}TCID50 (A mean) and A min is ≤ 0.5 Log

The test is therefore considered valid when the Log_{10}TCID50/ml is ± 0.5 Log with respect to A min and A max, as required also in the "Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin Q5A(R1)-Appendix 3". Otherwise the whole assay has to be repeated. If also the results of the second assay do not meet the acceptance criteria, a deviation should be opened and managed in the validation protocol.

Acceptance criteria for all parameters were established based on the requirement of ICH Q5A (R1) "*Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin*" *(25)*.

The results obtained from the validation tests are reported in the paragraphs below.

12.4.3. Precision: Repeatability

To check repeatability, 5 samples prepared from the starting pool were tested in duplicate:

- ❖ Pure (undiluted)
- ❖ Diluted 1:10
- ❖ Diluted 1:100
- ❖ Diluted 1:1000
- ❖ Diluted 1:10000

The test was run in parallel (two different titers for each sample) by two operators in two independent analytical sessions. The difference between the titers of each analysis on the same sample expressed as $Log_{10}TCID_{50}/ml$ measured in 2 titrations done by the same operator in parallel should fall within a ±0.5 Log_{10} range. This criterion was established based on the requirement of ICH Q5A (R1) Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin (25). The results are shown in the table below:

Operator	Dilution	Titration	Titer measured ($Log_{10}TCID_{50}/ml$)	Difference between titers measured ($Log_{10}TCID_{50}/ml$)
Operator 1	INITIAL TITER (A)	1	7.86	0.36
		2	8.22	
	1:10 (B)	1	7.20	0.06
		2	7.26	
	1:100 (C)	1	6.13	0.24
		2	5.89	
	1:1000 (D)	1	5.12	0.30
		2	4.82	
	1:10000 (E)	1	3.74	0.12
		2	3.86	

Operator	Dilution	Titration	Titer measured ($Log_{10}TCID_{50}/ml$)	Difference between titers measured ($Log_{10}TCID_{50}/ml$)
Operator 2	*INITIAL TITER* (A)	1	7.86	0.06
		2	7.80	
	1:10 (B)	1	6.90	0.05
		2	6.85	
	1:100 (C)	1	5.65	0.06
		2	5.71	
	1:1000 (D)	1	4.70	0.06
		2	4.64	
	1:10000 (E)	1	3.39	0.06
		2	3.45	

Table 64. Precision results (repeatability).

Repeatability was confirmed since all the results were compliant with the acceptance criteria.

12.4.4. Intermediate precision

Five samples, prepared from the starting pool, were tested in duplicate to check intermediate precision:

- ❖ Pure (undiluted)
- ❖ Diluted 1:10
- ❖ Diluted 1:100
- ❖ Diluted 1:1000
- ❖ Diluted 1:10000

The test was run in parallel (two different titrations for each sample) by two operators in two independent analytical sessions.

The difference between the Mean Values of each analysis (expressed as $Log_{10}TCID_{50}/ml$) carried out throughout the validation phase (two different operators carried out two independent analytical sessions each day) should be less than or equal to $0.5 Log_{10}$.

The results are shown in the table below:

Dilution	Titration	Titer measured ($Log_{10}TCID_{50}$/ml)	Difference between titers measured ($Log_{10}TCID_{50}$/ml)
Initial titer (A)	*Op.1/T.1*	7.86	0.00
	Op.2/T.1	7.86	
	Op.1/T.1	7.86	0.06
	Op.2/T.2	7.80	
	Op.1/T.2	8.22	0.36
	Op.2/T.1	7.86	
	Op.1/T.2	8.22	0.42
	Op.2/T.2	7.80	
1:10 (B)	*Op.1/T.1*	7.20	0.30
	Op.2/T.1	6.90	
	Op.1/T.1	7.20	0.35
	Op.2/T.2	6.85	
	Op.1/T.2	7.26	0.36
	Op.2/T.1	6.90	
	Op.1/T.2	7.26	0.41
	Op.2/T.2	6.85	
1:100 (C)	*Op.1/T.1*	6.13	0.48
	Op.2/T.1	5.65	
	Op.1/T.1	6.13	0.42
	Op.2/T.2	5.71	
	Op.1/T.2	5.89	0.24
	Op.2/T.1	5.65	
	Op.1/T.2	5.89	0.18
	Op.2/T.2	5.71	
1:1000 (D)	*Op.1/T.1*	5.12	0.42
	Op.2/T.1	4.70	
	Op.1/T.1	5.12	0.48
	Op.2/T.2	4.64	
	Op.1/T.2	4.82	0.12
	Op.2/T.1	4.70	
	Op.1/T.2	4.82	0.18
	Op.2/T.2	4.64	

Dilution	Titration	Titer measured ($Log_{10}TCID_{50}$/ml)	Difference between titers measured ($Log_{10}TCID_{50}$/ml)
1:10000 (E)	Op.1/T.1	3.74	0.35
	Op.2/T.1	3.39	
	Op.1/T.1	3.74	0.29
	Op.2/T.2	3.45	
	Op.1/T.2	3.86	0.47
	Op.2/T.1	3.39	
	Op.1/T.2	3.86	0.41
	Op.2/T.2	3.45	

Table 65. Intermediate precision results.

Intermediate precision was confirmed since all the results were compliant with the acceptance criteria.

12.4.5. Accuracy

Five samples prepared from the starting pool were tested in duplicate to check accuracy:

- ❖ Pure
- ❖ Diluted 1:10
- ❖ Diluted 1:100
- ❖ Diluted 1:1000
- ❖ Diluted 1:10000

The test was run in parallel (two different titrations for each sample) by two operators in two independent analytical sessions.

The difference between the titer expected for the sample and the titer (mean $Log_{10}TCID_{50}$/ml) obtained using this method should fall within a ± 0.5 $Log_{10}TCID_{50}$ range. This criterion was established based on the requirement of ICH Q5A (R1) Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin.

The results are shown in the table below:

Operator	Dilution	Titer expected ($Log_{10}TCID_{50}/ml$)	Titration	Titer measured ($Log_{10}TCID_{50}/ml$)	Difference between the titer measured ($Log_{10}TCID_{50}/ml$) and that expected ($Log_{10}TCID_{50}/ml$)
Operator 1	INITIAL TITER (A)	N.A.	1	7.86	N.A. to be used as reference value
			2	8.22	N.A. to be used as reference value
	1:10 (B)	7.16	1	7.20	0.16
			2	7.26	0.22
	1:100 (C)	6.16	1	6.13	0.09
			2	5.89	0.15
	1:1000 (D)	5.16	1	5.12	0.08
			2	4.82	0.22
	1:10000 (E)	4.16	1	3.74	0.30
			2	3.86	0.18
Operator 2	INITIAL TITER (A)	N.A.	1	7.86	N.A. to be used as reference value
			2	7.80	N.A. to be used as reference value
	1:10 (B)	7.10	1	6.90	0.07
			2	6.85	0.02
	1:100 (C)	6.10	1	5.65	0.18
			2	5.71	0.12
	1:1000 (D)	5.10	1	4.70	0.13
			2	4.64	0.19
	1:10000 (E)	4.10	1	3.39	0.44
			2	3.45	0.38

Table 66. Accuracy results.

Accuracy was confirmed since all the results were compliant with the acceptance criteria

12.4.6. Robustness at different incubation times

For each analytical session the method was run as described in *paragraph 4.3.2.* until the plates incubation at $37.0\pm1.0°C$ in $5.0\pm1.0\%$ CO_2 using the undiluted sample (A). The titrations were stopped at three different times: 5, 6 and 7 days after the beginning of incubation. The correct time between the start and end of titration is 6 days from the start of incubation; therefore the titer achieved in this test will be used as a reference.

The difference in titer ($LogTCID_{50}/mL$) of the same plate read after 5 and 6 days should fall within a ±0.5 Log_{10} range and the difference in titer (Mean Value) of the same plate read after 6 and 7 days should fall within a ±0.5 Log_{10} range.

The results are shown in the table below:

Operator	Titration	Reference titer ($Log_{10}TCID_{50}/ml$) Day 6	Titer measured ($Log_{10}TCID_{50}/ml$)		Difference between the titer measured ($Log_{10}TCID_{50}/ml$) and that expected (Day 6) ($Log_{10}TCID_{50}/ml$)
Operator 1	1	7.86	Day 5	7.80	0.06
			Day 7	8.16	0.30
	2	8.22	Day 5	8.22	0.00
			Day 7	8.57	0.35
Operator 2	1	7.86	Day 5	7.74	0.12
			Day 7	8.16	0.30
	2	7.80	Day 5	7.62	0.18
			Day 7	7.98	0.18

Table 67. Robustness results (with different incubation times).

Robustness with different incubation times was confirmed since all the results were compliant with the acceptance criteria.

12.4.7. Robustness with different culture media

The "Mo.A-MuLV virus (Moloney Amphotropic Murine Leukemia Virus) Titration Assay " method was run as described in in *paragraph 4.3.2* except for the culture medium used to prepare the dilution plates; in fact, in this case the intention was to assess any variation in determining the titer using McCoy's 5A culture medium 2% FBS with and without antibiotic and undiluted sample (A).

The culture medium used to validate this method was McCoy's 5A 2% FBS without antibiotic; therefore the titer obtained with this test is used as a reference.

The difference in titer ($LogTCID_{50}/ml$) of the same sample titrated with McCoy's 5A culture medium 2%FBS without antibiotic and McCoy's 5A culture medium 2% FBS with 1% Penicillin-Streptomycin and 1% Amphotericin should fall with a ±0.5 Log_{10} range.

The results are shown in the table below:

Operator	Titration	Reference titer ($Log_{10}TCID_{50}/ml$) McCoy's 5A culture medium 2% without antibiotic	Titer measured ($Log_{10}TCID_{50}/ml$) McCoy's 5A culture medium 2% with antibiotic	Difference between titer measured (McCoy's 5A culture medium with antibiotic) ($Log_{10}TCID_{50}/ml$) and titer expected (McCoy's 5A culture medium without antibiotic) ($Log_{10}TCID_{50}/ml$) (Difference within ± 0.5Log_{10} range)
Operator 1	1	7.86	8.16	0.30
	2	8.22	8.28	0.06
Operator 2	1	7.86	8.04	0.18
	2	7.80	7.92	0.12

Table 68. Robustness results (with different culture media).

Robustness with different culture media was confirmed since all the results were compliant with the acceptance criteria.

12.4.8. Robustness at different pH

The "Mo.A-MuLV virus (Moloney Amphotropic Murine Leukemia Virus) Titration Assay" method was run as described in *paragraph 4.3.2* except for the culture medium used to prepare the dilution plates; in fact, in this case the intention was to assess the stability of a virus diluted 1:1000 (sample) with culture media at pH 6.0±0.2 and of a virus diluted 1:1000 (sample) with culture media at pH 8.0±0.2.
The virus sample used to validate this method was a virus diluted 1:1000 (sample) in McCoy's 5A 2% FBS at pH 7.2-7.4; therefore the titer obtained with this test is used as a reference.

The difference in titer (LogTCID$_{50}$/ml) of a sample prepared with McCoy's 5A 2% FBS at pH 7.2-7.4 and of the sample prepared with McCoy's 5A 2% FBS at pH 6.0±0.2 should fall with a ±1 Log$_{10}$ range. In addition, the difference in titer (LogTCID$_{50}$/ml) of a sample prepared with McCoy's 5A 2% FBS at pH 7.2-7.4 and of the sample prepared with McCoy's 5A 2% FBS at pH 8.0±0.2 should fall with a ± 1.0 Log$_{10}$ range.

The results are shown in the table below:

Operator	Titration	Reference titer ($Log_{10}TCID_{50}/ml$) McCoy's 5A medium 2% pH 7.2-7.4	Culture media and pH used	Titer measured ($Log_{10}TCID_{50}/ml$)	Difference between measured titer ($Log_{10}TCID_{50}/ml$) and reference titer
Operator 1	1	5.12	McCoy's 5A Medium 2% pH 6.0±0.2	4.34	0.78
			McCoy's 5A Medium 2% pH 8.0±0.2	4.40	0.72
	2	4.82	McCoy's 5A Medium 2% pH 6.0±0.2	4.34	0.48
			McCoy's 5A Medium 2% pH 8.0±0.2	4.34	0.48
Operator 2	1	4.70	McCoy's 5A Medium 2% pH 6.0±0.2	4.10	0.60
			McCoy's 5A Medium 2% pH 8.0±0.2	4.22	0.48
	2	4.64	McCoy's 5A Medium 2% pH 6.0±0.2	4.16	0.48
			McCoy's 5A Medium 2% pH 8.0±0.2	4.22	0.42

Table 69. Robustness results (at different pH).

Robustness at different pH was confirmed since all the results comply with the acceptance criteria.

12.4.9. Linearity

Five samples obtained from the starting pool were tested in duplicate to check linearity:

- ❖ Pure
- ❖ Diluted 1:10
- ❖ Diluted 1:100
- ❖ Diluted 1:1000

❖ Diluted 1:10000

The test was run in parallel (two different titrations for each sample) by two operators in two independent analytical sessions.

The coefficient of determination (R^2) of the straight line (described by a linear equation $y=ax+b$) obtained in a graph where the x axis shows the expected sample titers (Initial Titer (A), 1:10 dilution (B), 1:100 dilution (C), 1:1000 dilution (D) and 1:10000 dilution (E)) and the y axis the titers achieved using this method, should be greater than or equal to 0.98. This criterion was established based on the results produced during the setup phase.

The results are shown in the table below:

Operator	Titration	R^2
Operator 1	Titration 1	0.99
	Titration 2	1.00
Operator 2	Titration 1	1.00
	Titration 2	1.00

Table 70. Linearity results (repeatability).
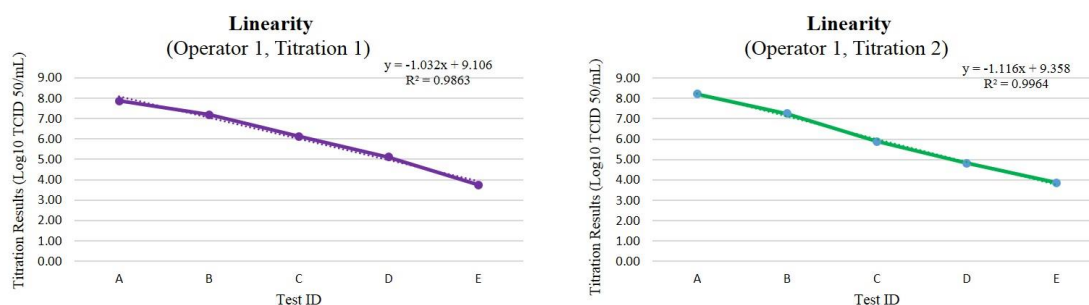
The graphs produced are shown below:

Figure 26. The graphs show precision results obtained by operator 1 in two different titration.
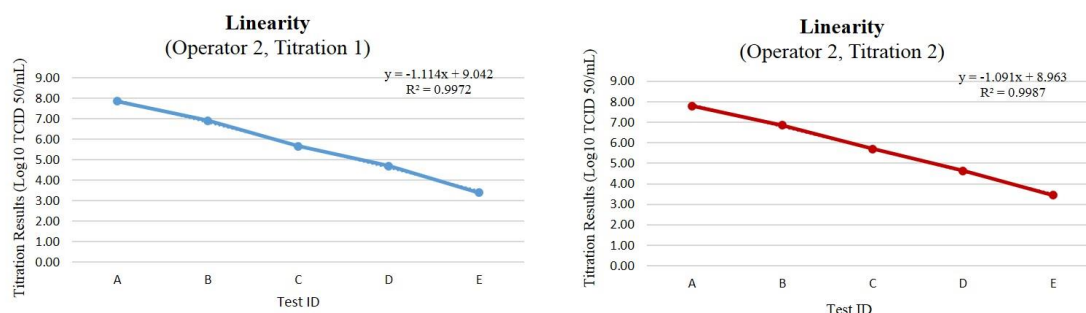
Figure 27. The graphs show precision results obtained by operator 2 in two different titration.

Linearity was confirmed since all the results were compliant with the acceptance criteria.

12.5. Titration method validation conclusions

Following the activities carried out during the validation process, the "Mo.A-MuLV virus (Moloney Amphotropic Murine Leukemia Virus) Titration Assay" method is considered validated with respect to the parameters examined and their acceptance criteria.

Precision, accuracy, robustness and linearity were checked during the method validation process and the obtained results were in line with the acceptance criteria established in the Test Protocol.

The "Mo.A-MuLV virus (Moloney Amphotropic Murine Leukemia Virus) Titration Assay" method will be used to determine the Murine Leukemia Virus (MuLV) titer of viral stocks and samples generated in Viral Clearance Validation Studies.

13. Conclusions

Given the intrinsic limits of sensitivity of methods for determining viral contaminants, special tests done on the Drug Substance cannot guarantee the absolute safety of the product. In accordance with Regulatory Authority Guidelines (e.g. ICH Q5A (R1)), a pharmaceutical company that produces biotechnological products is required to apply a three-barrier approach aimed at demonstrating that the product is safe with respect to viral contaminations. This approach includes:

1) selecting cell lines and raw materials (in particular those derived from animals) tested for the absence of viruses that may be infective or pathogenic for humans;

2) analyzing raw intermediates from the fermentation process to confirm the absence of contamination by adventitious viruses;

3) demonstrating that the purification process (Down Stream Process – DSP) has the intrinsic ability to remove or inactivate viruses efficiently from the product.

Viral Clearance studies are performed to demonstrate the efficacy of the third barrier. This activity consists in reducing to laboratory scale (scaled-down model) some steps of the purification process known for their ability to chemically inactivate or physically remove viral particles, spiking purification intermediates of the single steps with model virus having a known titer (concentration) and determining the downstream residual viral titer.

A critical point for viral clearance validation studies is the selection of model viruses (relevant viruses, specific model viruses and non-specific model viruses). The number and type of viruses to include in a Viral Clearance study depend on the type of cell line, on the origin of the raw materials used in the production process and on the product's stage of development (initial or final clinical development). The quality of viral stocks to be used during Viral Clearance Validation studies depends on the method used to

isolate them. In any case, this quality is crucial for the studies to be performed correctly. Some key aspects should be taken into consideration:

- viral stocks should have as high a titer as possible in order to avoid excessive dilution of the purification intermediate involved, which could change its properties and compromise the applicability of the validation study;

- viral spikes must not contain aggregated particles. These aggregates could compromise the results of Viral Clearance Validation studies. For example, the retention of viruses by nanofiltration could be affected; in addition, it has been shown that virus aggregates are more resistant to chemical inactivation. Before being used in the study, viral spikes must therefore be appropriately pre-filtered on filters with a porosity depending on the size of the viral particles (e.g. MuLV suspensions consisting of particles having a diameter between 80 and 100 nm must be pre-filtered with a 0.2 µm filter);

- the purity of viral stocks should be as high as possible, in particular for some steps such as nanofiltration that are extremely sensitive to impurities such as proteins and DNA, etc.. Since viral stocks are prepared starting from cell cultures, the purification method used to isolate the viral stocks must be as selective as possible to avoid any impact on the performance of the process step to be assessed during the Viral Clearance Validation study.

Efficient and reliable infectivity tests should be available for all the viruses included in the Viral Clearance Validation study.

Each quantification (titration) test should be carried out with a sufficient number of replicates to assure adequate statistical validity of the results. The result is expressed as Log10 of the TCID50, which represents the concentration of virus able to infect 50% of the corresponding replicates in the plate. The titer reduction factor, or Log Reduction Factor (LRF), is the difference between the titer upstream and downstream from the step.

For all these reasons, the PhD project has lead to a method development for the purification of one of the viruses used in Viral Clearance studies (Mo.A.MuLV) for Phase I/II and to a method validation for its titration. Both the purification method and the titration method were described in dedicated internal procedures (as required from guidelines and internal procedures). These procedures are used for preparation and titration of Mo.A.MuLV stocks used in the BQC-Viral Clearance laboratory during phase I/II studies.

The next steps will be the development of methods for purification and titration of the remaining viruses required for phase III Viral clearance studies.

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