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Method development for biotechnological drugs

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General Introduction

A biopharmaceutical product, also known as biological, is considered any pharmaceutical drug produced, extracted or synthetized from biological sources. They are virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, proteins (including antibodies), nucleic acids (DNA, RNA or antisense oligonucleotides), applicable to the prevention, treatment, or cure of a disease or condition of human beings (FDA (Food and Drug Administration) 2006).

Analytical method development and validation play important roles during all the lifecycle of biopharmaceuticals (Berkowitz, et al. 2012). The biopharmaceutical manufacturing process (*Figure 1*) encompasses the cell line production and raw materials going into the process, cell culture/fermentation and the purification process, the bulk active product and the formulation, filling and packaging of the final drug (Hesse and Wagner 2000).

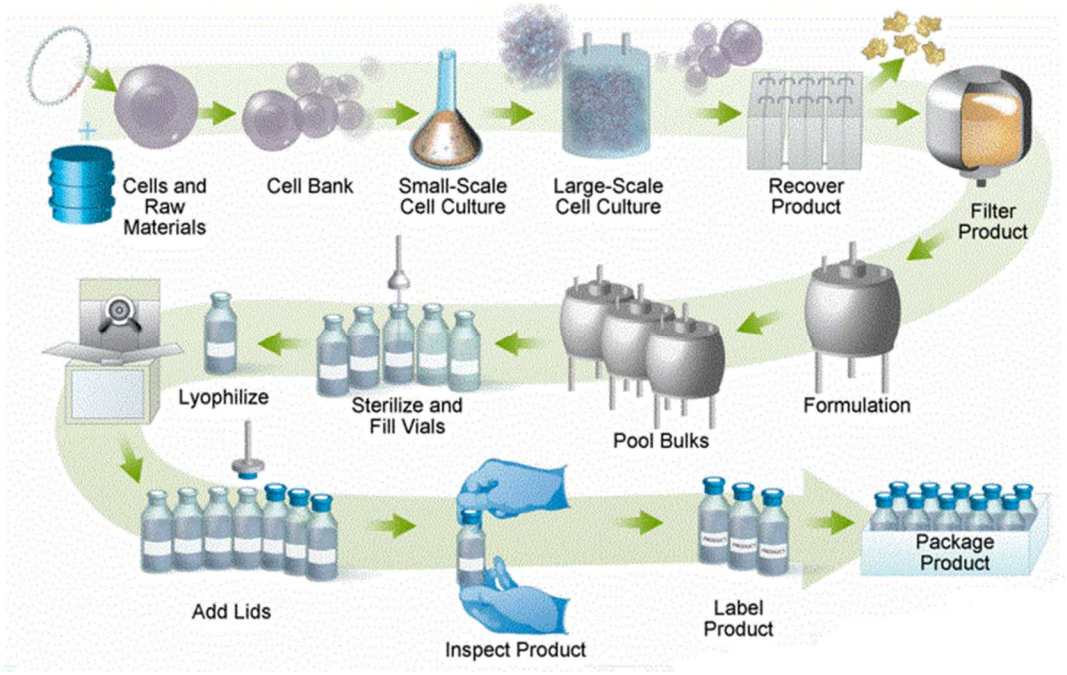

Figure 1 - Biopharmaceutical Manufacturing Process: adapted from (Genzyme Corporation s.d.)

Each of these stages requires samples to be taken and data generated to determine their acceptable quality. Moreover, the bulk and final product must be analyzed for identity, to distinguish and characterize the specific cell line from the non-cellular components, purity and safety, to confirm that the product does not contain residual process reagents or contamination, and potency, to measure the biological function relevant to treating the intended clinical indication (FDA (Food and Drug Administration) 2008) (Carmen, et al. 2012). To develop a comprehensive profile of the product, multiple aspects require analysis (Shintani 2013).

For this reason, the health authorities, as Food and Drug Administration (FDA) in the United States and European Medicine Agency (EMA) in Europe, have edited guidelines to have a detailed understanding of production processes and the development of control mechanisms that will ensure product quality. Regarding the quality of products, regulators explain how a method needs to be developed and validated, and which analytical procedures need to be in place.

The FDA defines analytical procedures as analysis "developed to test a defined characteristic of the drug substance or drug product against established acceptance criteria for that characteristic" (FDA (Food and Drug Administration) 2015). According to the International Conference on Harmonization (ICH), the most common types of analytic procedures are: (1) identification tests, (2) quantitative tests of the active moiety in samples of active pharmaceutical ingredient (API) or drug product or other selected component(s) in the drug product, (3) quantitative tests for impurity content, (4) limit tests for the control of impurities (ICH (International Conference on Harmonization) 1995).

Identification tests are intended to ensure the identity of analyte in a sample. This is normally achieved by comparison of a certain property of the sample to that of a reference standard (FDA (Food and Drug Administration) 2015) (ICH (International Conference on Harmonization) 1999) (ICH (International Conference on Harmonization) 2000).

Testing for impurities can be either a quantitative test or a limit test for the impurity in a sample. Either tests are intended to accurately reflect the purity characteristics of the sample (FDA (Food and Drug Administration) 2015).

Quantitative tests of the active moiety in samples are intended to measure the analyte present in a given sample and its activity. An analytical procedure measures biological activity of a test substance based on a specific, functional, biological response of a test system. These assays include *in-vitro* methods such as cell culture assays and *in-vivo* assays involving animal models (FDA (Food and Drug Administration) 2015).

To ensure compliance with quality and safety standards, many countries have published compendia, or *pharmacopeias*, that describe official test methods for numerous marketed drug products (Breaux, Jones and Boulas 2003). Many historical biological products, such as plasma fractionation products and vaccine products do have monograph listings that must be followed for product release (EDQM (European Directorate for the Quality of Medicines & Healthcare) 2017) (EMA (European Medicine Agency) 2011).

However, most modern biotechnology products are new molecular entities and therefore do not have monograph listings. In the absence of compendial methods, manufacturers of these products must develop and validate their own (non-compendial) analytical methods and product specifications (FDA (Food and Drug Administration) 2015) (Nicoară, et al. 2014).

Moreover, the compendial methods follow sometimes obsolete procedures, no longer abreast with new technologies. Therefore, a continuous method development with cutting-edge tools using state of the art knowledge ensures a constant improvement in product quality and safety.

Compendial method for virus identification

Introduction

An example of a compendial analytical method for impurities is the investigation of viral contamination in raw materials, used in the biopharmaceutical manufacturing process (*Figure 1*) (Garnick 1998) (Mackay and Kriz 2010).

The cell cultures can be contaminated by viruses, which were present in the animal derived materials used in manipulation or for the growth of the cells (Merten 2002) (Eloit 1999). These types of materials include serum or trypsin. Fetal, new born or adult bovine sera as medium additive is the most widespread animal derived material used today (Eloit 1999). Trypsin, mainly from pig pancreas, is a very important detachment agent for all adherent cells. Several zoonotic viruses are known and can be transmitted from animal sources. For this reason, detecting the presence of viral extraneous agent can avoid the contamination of the whole biopharmaceutical process.

In the 9 Code of Federal Regulations Part 113 (9 CFR 113.47/53), the FDA reports the panel of viruses needed to be tested and the different methods that should be used. In the list, there are: Bovine virus diarrhea virus, Reovirus, Rabies virus, Bluetongue virus, Bovine adenovirus, Bovine parvovirus, Bovine respiratory syncytial virus, Porcine adenovirus, Porcine parvovirus and Transmissible gastroenteritis virus (US Government Printing Office 2000).

Inoculation of the lysate coming from the cell bank in detector cell lines able to be infected, will highlight the presence of a contamination with those viruses. By a cytopathic assay (CPE) and a hemagglutination assay (HA) it is possible to highlight the presence of these adventitious agents; using immunofluorescence assay with FITC-conjugate antibodies these viruses can be identified and the nature of the contamination clarified.

All samples need to be compared with a positive control for assuring the reliability of results. To have these viruses as positive controls in the test, they have been propagated and titrated using both cytopathic effect and immunofluorescence assay. Moreover, using an automatic image multi-plate reader the immunofluorescence assay protocol has been simplified. A method able to count in an automatic way the infected cells, assuring reliability of results, has been set-up. This allow to improve the method with new technology and to reduce errors during the immunofluorescence observation.

Materials and Methods

Cell culture

The following cell lines were obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK): BT (ECACC 89031603, bovine turbinate), SCP (ECACC 89101302, ovine brain choroid plexus), ST (ECACC 92040221, porcine testis, fetal), EBTr (ECACC 87090202, bovine fetal trachea) and Vero C1008 [Vero 76, clone E6, Vero E6] (ECACC 85020206, monkey african green kidney). The MDBK (ATCC CCL-22™, bovine kidney) were obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA).

The BT were cultured in Minimum Essential Medium (MEM) (MERCK KGaA, Darmstadt, Germany) supplemented with 10% horse serum (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The ST, SCP and EBTr were cultured in MEM supplemented with 10% fetal bovine serum (GE Healthcare, Little Chalfont, UK). The Vero C1008 and MDBK were cultured with Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum. All the cells were cultured without antibiotics at 37°±1°C in a humidified atmosphere with 5%±1% CO_2.

For all cell lines were created a Master Cell Bank (MCB) and a Working Cell Bank (WCB).

Viruses

The following viruses were obtained from ATCC: Bovine herpesvirus 1 (IBR/BHV-1, ATCC® VR-188™), Bovine parainfluenza virus (PI-3, ATCC® VR-281™), Bovine adenovirus 1 (BAV-1, ATCC® VR-313™), Bovine virus diarrhea virus 1 (BVDV (CE), ATCC® VR-534™), Bovine adenovirus 3 (BAV-3, ATCC® VR-639™), Porcine parvovirus NADL-2 (PPV, ATCC® VR-742™), Bovine parvovirus (BPV, ATCC® VR-767™), Reovirus 3 (REO-3, ATCC® VR-824™), Bovine respiratory syncytial virus (BRSV, ATCC® VR-1339™), Bovine virus diarrhea virus 1 (BVDV (nCE), ATCC® VR-1561™), Transmissible gastroenteritis virus, porcine (TGEV, ATCC® VR-1740™). The Porcine adenovirus (PAdV, RVB-0071) and the Border disease virus (BDV, RVB-1077) were obtained from Friedric-Loeffler-Institut (FLI, Greifswald, Germany).

Viruses propagation

The propagation for viruses with cytopathic effect was done as described in the following: 162cm^2 Corning® TC Flasks (Corning, New York, USA) with confluent cell lines were inoculated with 1 mL of

virus suspension and incubated for 1 hour at 37°±1°C in a humidified atmosphere with 5%±1% CO_2. After 1 hour flasks were filled at 30 mL with virus growth medium and incubated at 37°±1°C in a humidified atmosphere with 5%±1% CO_2. When the cytopathic effect was evident, supernatant with progeny virus were centrifuged at 600 rcf for 10 minutes to eliminate all cell debris. Then supernatants were aliquoted and stored at -80°C until the use.

The virus VR-188 was grown in EBTr cells for 3 days using MEM supplemented with 2% fetal bovine serum. The virus VR-281 was grown in BT cells for 9 days using MEM supplemented with 10% horse serum. The virus VR-313 was grown in MBDK cells for 8 days using MEM supplemented with 10% horse serum. The virus VR-534 was grown in BT cells for 9 days using DMEM supplemented with 10% horse serum. The virus VR-639 was grown in BT cells for 4 days using MEM supplemented with 10% horse serum. The virus VR-742 was grown in ST cells for 5 days using MEM supplemented with 10% fetal bovine serum. The virus VR-767 was grown in BT cells for 11 days using MEM supplemented with 10% horse serum. The virus VR-824 was grown in Vero C1008 cells for 6 days using MEM supplemented with 2% fetal bovine serum. The virus VR-1339 was grown in BT and MDBK cells using DMEM supplemented with 2% horse serum; in BT cells for 6 days and in MDBK cells for 17 days. The virus RVB-0071 was grown in ST cells for 4 days using MEM supplemented with 2% fetal bovine serum. The virus RVB-1077 was grown in SCP cells for 6 days using MEM supplemented with 2% fetal bovine serum. The virus VR-1740 was grown in ST cells for 3 days using MEM supplemented with 1 µg/mL TPCK-treated trypsin (Merck KGaA). The VR-1740 aliquots collection was done as the normal procedure and after 3 cycles of freezing and thawing.

The propagation for viruses with no cytopathic effect was done as the propagation of cytopathic virus, and stopped as indicated from the supplier. The virus VR-1561 was grown in BT cells for 7 days using MEM supplemented with 10% horse serum.

Virus titration for cytopathic effect

The viruses were titrated on the same cell lines and for the same time period used for the propagations. The cell lines were seeded in two 96 well tissue culture flat bottom plates at the concentration of $1.5x10^4$ cells/well in 100 µL virus growth medium and incubated overnight at 37°±1°C in a humidified atmosphere with 5%±1% CO_2. For the BT cell line, different concentrations were tested and the best found was $2x10^4$ cells/well in 100 µL virus growth medium (data not shown). After 24 hours, the virus suspensions were prepared and diluted 10-fold from 1:10 to $1:10^{10}$ in virus growth medium and 25 µL of each dilution was transferred to the confluent cell lines. After

1 hour, 100 µL of virus growth media were added in each well and plates incubated at 37°±1°C in a humidified atmosphere with 5%±1% CO_2. At the end of titration, supernatants were removed, and the infected cells were fixed with 50 µL/well of Methanol (Merck KGaA) for 10 minutes. After fixation, the monolayers were stained with Crystal Violet Solution (Merck KGaA) for at least 5 minutes. The plates were then washed with running water and dried. The stained plates were observed under microscope to evaluate the presence of lysis plaque on the monolayer. For every titration, there were wells for the cell line growth monitoring. The $TCID_{50}$ calculation were performed with *Spearman-Karber* method, using the formula:

$$\log_{10} \mathrm{TCID50} = X_0 - \left(d/2\right) + d \sum pi$$

Starting from the $TCID_{50}$, the viral title in pfu/mL was calculated using the Poisson distribution.

Antibodies and control slides

The following antibodies FITC-conjugate were obtained from Veterinary Medical Research & Development (VMRD, Pullman, Washington, USA): Bovine viral diarrhea virus (BVDV) Direct FA Conjugate, Infectious Bovine Rhinotracheitis virus (IBR) Direct FA Conjugate, Bovine Parainfluenza Type 3 (PI-3) Direct FA Conjugate, Bovine Respiratory Syncytial virus (BRSV) Direct FA Conjugate, Bovine Parvovirus (BPV) Direct FA Conjugate, Porcine Parvovirus (PPV) Direct FA Conjugate, Reovirus Direct FA Conjugate, Transmissible Gastroenteritis virus (TGEV) Direct FA Conjugate, Bluetongue virus (BTV) Direct FA Conjugate, Bovine Adenovirus Type 3 (BAV-3) Direct FA Conjugate and Porcine Adenovirus (PAV) Direct FA Conjugate. The Monoclonal Anti-Rabies, FITC was obtain from Sifin Diagnostic GmbH (Berlin, Germany). The WS363 monoclonal antibody to pestiviruses (BDV specific) was obtain from Animal and Plant Health Agency (APHA Scientific, Addlestone, UK).

The positive and negative control slides were obtained from VMRD: Bovine viral diarrhea virus (BVDV) FA Control Slide, Infectious Bovine Rhinotracheitis virus/Bovine herpesvirus Type 1 (IBR/BHV-1) FA Control Slide, Bovine Parainfluenza Virus Type 3 (PI-3) FA Control Slide, Bovine Respiratory Syncytial Virus (BRSV) FA Control Slide, Bovine Parvovirus (BPV) FA Control Slide, Porcine Parvovirus (PPV) FA Control Slide, Reovirus (REO) FA Control Slide, Transmissible Gastroenteritis Virus (TGEV) FA Control Slide, Bluetongue virus (BTV) IFA Substrate Slide, Rabies Recombinant Nucleoprotein (rNP) FA Control Slide and Bovine Adenovirus Type 3 (BAV-3) FA Control Slide.

The fixation was done using Methanol/Acetone solution 1:1 (Merck KGaA) or with PFA 4% solution (Merck KGaA). The washing steps in the immunofluorescence protocols were performed with

phosphate buffered saline 1x (PBS 1x) (Merck KGaA) or with PBS 1x supplemented with 0.05% TWEEN® 20 (Merck KGaA). The permeabilization step was executed with PBS 1x supplemented with 0.1% TWEEN® 20. The saturation of unspecific binding sites was done with PBS 1x supplemented with 10% fetal bovine serum. For the nuclei staining DAPI, FluoroPure™ grade (Thermo Fisher Scientific) was used and the slide mounted with ProLong® Gold Antifade Mountant (Thermo Fisher Scientific).

Virus titration with immunofluorescence

The virus titration with immunofluorescence was done using two different protocols. The first one was performed using chamber slides, the second one using 96 well tissue culture black, white bottom plates. As in cytopathic effect titration, cells were seeded the day before and infected with 10-fold or 5-fold dilutions of viruses from 1:10 to $1:10^{10}$. At the end of titration, the supernatants were removed from slide or from the plates and the protocol for immunofluorescence performed. The chamber slides were washed with PBS 1x and fixed with Methanol/Acetone solution 1:1 at -20°C for 20 minutes. After the fixation, slides were washed and stained with conjugate antibodies and DAPI for 30 minutes. The mounting was performed using the ProLong® Gold Antifade Mountant and slides left to dry until the observation under a fluorescence microscope. For plates, different protocols of immunofluorescence were tested and the most efficient is the following one. Plates were washed with PBS 1x and fixed with PFA 4% solution at room temperature for 10 minutes. After fixation, the cells were permeabilized with PBS 1x supplemented with 0.1% TWEEN® 20 for 10 minutes and then washed with PBS 1x supplemented with 0.05% TWEEN® 20. The saturation of unspecific binding sites was performed using PBS 1x supplemented with 10% fetal bovine serum for 30 minutes. After that, the cells were stained with conjugate antibodies and DAPI for 30 minutes. The wells were washed and left in PBS 1x at 2°-8°C until the observation using the multi-plate reader Cytation3 Microplate Imager (BioTek Instrument, Winooski, Vermont, USA).
For the immune-titration, $FAID_{50}$ calculation was performed with *Spearman-Karber* method, using the formula:

$$\log_{10} \mathrm{FAID50} = X_0 - \left(d/2\right) + d \sum pi$$

Results

Virus antibodies tested on control slides

Each virus was propagated and the efficacy of the virus conjugated antibodies with control slide (positive and negative wells) was tested before the titration. The FITC-conjugate antibodies were specific and able to recognize cytoplasmic (*Figure 2A and 2E*), nuclear (*Figure 2B and 2D*) and syncytial (*Figure 2C*) viruses. In *Figure 2* some examples of immunofluorescence on control slides are shown.

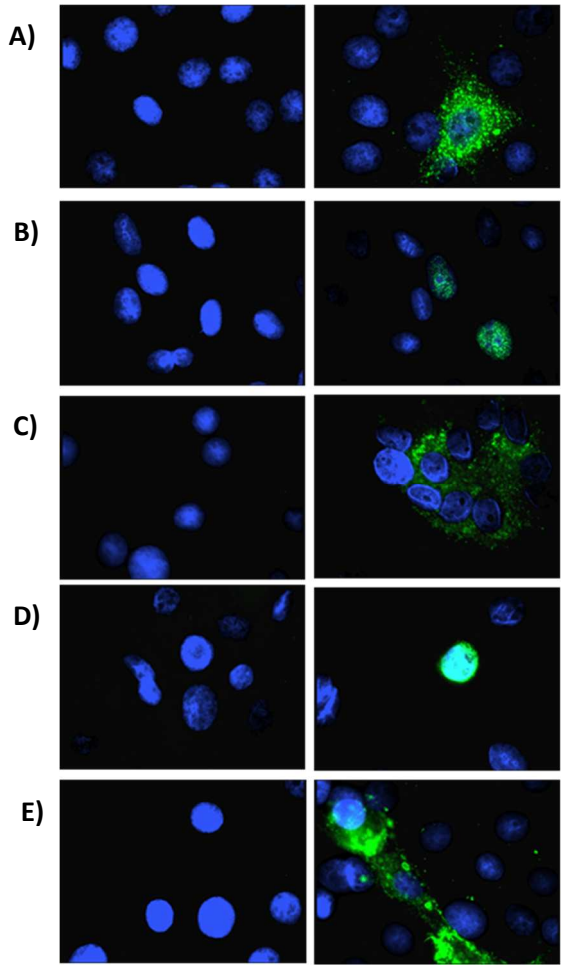

Figure 2- Immunofluorescence of viruses: A) PI-3 cytoplasmic virus negative and positive control B) PPV nuclear virus negative and positive control C) TGEV syncytial virus negative and positive control D) BPV nuclear virus negative and positive control E) BRSV cytoplasmic virus negative and positive control

Comparison of virus titration using cytopathic effect vs. immunofluorescence

The viruses were propagated as described before, and titer obtained using both cytopathic effect and immunofluorescence with the chamber slides protocol. The results of *Table 1* show that with the immunofluorescence method the titers obtained were higher compared to the cytopathic effect method. For the cytopathic effect, the plate observation was always done by the same operator, because it was clear that the operator's expertise could influence the results. By contrast, the positive signal in immunofluorescence is clear and cannot be misinterpreted. Moreover, the immunofluorescence method is direct because it recognized the presence of virus in cell culture and not the effect as in the cytopathic effect.

This was true for all viruses except for VR-742 and VR-1740, where the titer obtained with the immunofluorescence was lower than the titer with cytopathic effect.

For the viruses VR-639 and RVB-1077, the propagation was not possible. Different protocols were performed but no results were obtained.

For the virus VR-767, the titer was not clear and we decided to use it without dilution.

	Original Concentration ($TCID_{50}$ units/mL)	*Cytopathic effect ($TCID_{50}$ units/mL)*	*Immunofluorescence ($FAID_{50}$ units/mL)*
VR-188 – IBR/BHV-1	$2.8x10^7$	$1x10^8$	$1.8x10^8$
VR-281 – PI-3	$8.9x10^6$	$1.3x10^6$	$1.3x10^7$
VR-313 – BAV-1	$1.6x10^5$	$5.2x10^4$	$1.3x10^6$
VR-534 – BVDV (CE)	$1.6x10^6$	$3.3x10^6$	$3.1x10^6$
VR-639 – BAV-3	$1.6x10^5$	No expansion	
VR-742 – PPV	$2.8x10^8$	$1.1x10^9$	$6.3x10^7$
VR-767 – BPV-1	$8.9x10^5$	No effect	
VR-824 – REO-3	$8.9x10^8$	$7.2x10^7$	$1.3x10^8$
VR-1339 – BRSV	$1.6x10^5$	$4.1x10^5$ (BT)	$1.3x10^6$ (BT)
	$1.6x10^5$	No expansion on MDBK	
VR-1561 – BVDV (nCE)	$2.8x10^7$	No CE	$6.3x10^6$
VR-1740 – TGEV	$7.9x10^5$	$2.7x10^4$	$3.9x10^2$
RVB-0071 – PadV	N.A.	$2.1x10^4$	$1.3x10^6$
RVB-1077 - BDV	N.A.	No expansion	

Table 1 – Virus Titration: initial concentration as well as newly propagated concentration ($TCID_{50}$ and $FAID_{50}$) are indicated.

Virus titration using Cytation3

After the titer determination of each virus, the optimization of the immunofluorescence protocol was done. All the immunofluorescence titrations were repeated in 96 well tissue culture black, white bottom plates and examined using the multi-plate reader Cytation3 Microplate Imager. The instrument is able to capture photos of each well of the plate and to analyze those providing parameters as mean value of fluorescence, area and number of cells, etc. A protocol working with all the viruses tested, cytoplasmic, syncytial or nuclear, was set-up. Moreover, it could count infected cells, distinguishing them from debris or antibodies aggregates. To do that, initially the count of DAPI positive nuclei was performed. Then a count of green cells was done setting-up a threshold obtained as mean of positive signals. After that other parameters were added to the analysis to eliminate too low (background) or too high (aggregates or debris) values. At this point all the positive signals that didn't match with cells, highlighted by DAPI, were eliminated. To complete the analysis also the parameter of the cell area was added to eliminate false positives. The values obtained by this analysis were evaluated using two different statistical methods (Standard deviation and JMP).

To provide a more precise method, also different virus dilutions were tested, for example 10-fold and 5-fold dilution. The results were very similar (*Figure 3*). However with lower dilution rate it was possible to better discriminate which was the lowest concentration at which the virus had an effect.

A)

B)

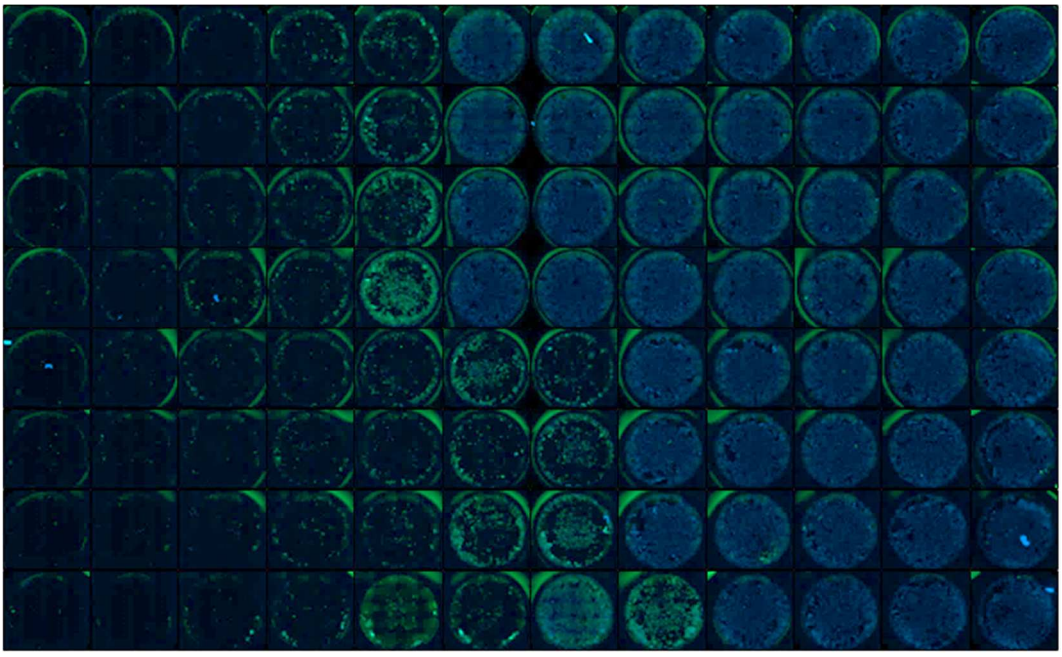

Figure 3 – Virus titration using Cytation3: A) 10-fold and B) 5-fold virus dilutions were tested

Discussion

The regulatory authorities request different test to ensure the safety of biopharmaceutical compounds. For example, the viruses that should be tested to avoid contaminations originating from raw material are listed in the 9 CFR 113. The presence of bovine and porcine viruses has to be examined by cytopathic and an immunofluorescence assay. We propagated and titrated the viruses present in the 9 CFR 113 list. The titration was done using both methods, namely cytopathic effect and immunofluorescence. However, it is clear that the procedure is laborious and time consuming. For this reason, the immunofluorescence protocol was implemented using the multi-plate reader Cytation3 Microplate Imager. Via this instrument, we were able to combine the specificity of immunofluorescence, due to the specificity of the conjugated antibody, with the fast analysis of a cytopathic effect assay, due to the use of a 96-well plate instead of slides. In addition, the new assay decreases the probability of errors by an operator during the analysis.

Obviously the 9 CFR procedure, at the moment, doesn't address the exhaustive list of viruses that are potential contaminants in animal derived raw materials and the method is long and time consuming (Sekura, et al. 2011). New technologist methods are now in place (Maroudam, et al. 2014) to overcome these problems, waiting for an update of the guideline. Our method is an example how it is possible to combine a compendial method and requests by regulators with new technologies to reach efficiently the aim of guidelines.

Non compendial methods for Immuno-oncology drugs

In recent years, Immuno-oncology has become one of the most promising and fastest growing areas of cancer research and drug development (Pandya, et al. 2016) (Tan, et al. 2016) (Latteyer, et al. 2016)

The aim of cancer immunotherapy is to boost the body's own response against cancer, and block mechanisms that prevent anti-tumor immunity (Montserrat Rangel-Sosa, Aguilar-Córdova and Rojas-Martínez 2017) (Shang, et al. 2017) (Janiczek, et al. 2017).

Under normal conditions the immune system is able to prevent and control the tumor growth by its effector cells, like natural killer cells, T and B cells. Due to the specificity of their T cell receptor the T cells are able to recognize transformed cells and control their growth (*Figure 4A*). Despite of that, the tumor cells have a variety of strategies to escape from this so called immune surveillance (*Figure 4B-D*). Two examples for tumor "escape" are the loss of target antigen expression, avoiding the recognition of tumor cells (*Figure 4B*), and the modulation of the immune functions of effector cells, through an increase of immune-inhibitors, leading to a suppression of immune responses (*Figure 4D*) (Lisiecka and Kostro 2016) (Vinay, et al. 2015) (Domschke, et al. 2016) (Beatty e Gladney 2015).

The strategies currently in place for cancer immunotherapy involve the stimulation of immune effector mechanisms and the limitation of the suppressive mechanisms described before. The approaches include the use of cancer vaccines (Guo, et al. 2013), which should immunize patients against tumor specific proteins, adoptive T cell therapy (Perica, et al. 2015), in which tumor-specific T cells are infused into cancer patients to fight against the cancer, and antibodies against immune-inhibitors (Cogdill, Andrews and Wargo 2017), able to release effector cells from the inhibition.

Often biopharmaceutical products have more than one function. An example for this are monoclonal antibodies (*Figure 5*). When multiple functional domains are part of the product's critical characteristics, it is necessary to assure that both (or all) of the domains meet physical and functional specifications for quality control. The nature of the procedures utilized for product potency testing, range from simple ligand binding or enzymatic procedures to *in-vivo* animal assays. Recently, the authorities' requests for a single bioassay covering the real and complete *mode of action* (*Table 2*) of these new drugs have increased.

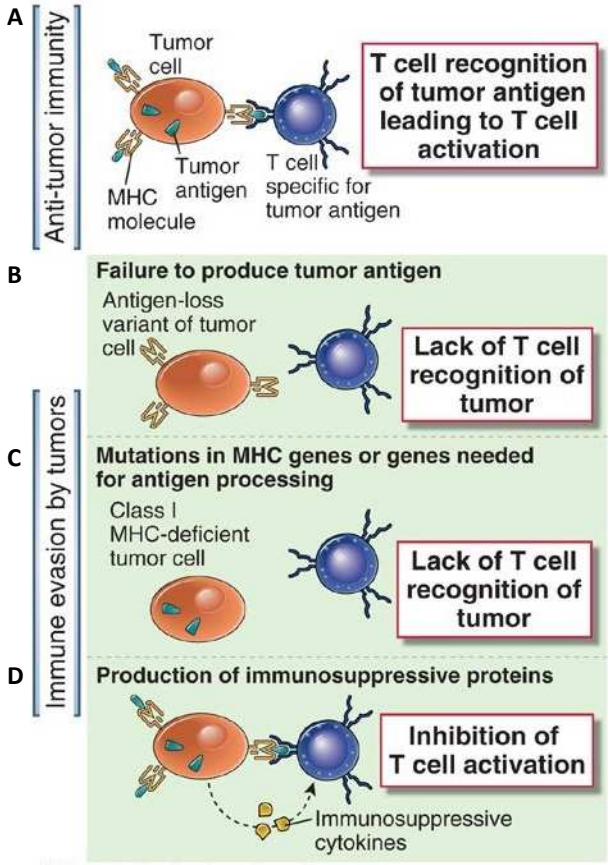

Figure 4 – Mechanisms of tumor escape. (Abbas, Lichtman e Pillai 2017). A) Anti-tumor immunity with the recognition of tumor cells by T cells. B) C) D) Different strategies of immune evasion put in place by tumors

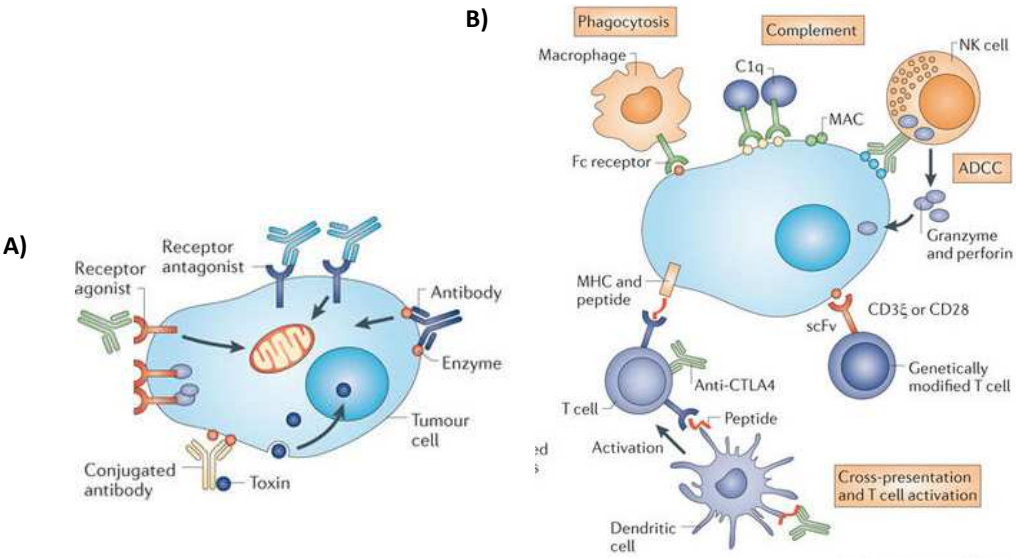

Figure 5– Different functions of an immunotherapy antibody: A) direct tumor cell killing B) Immuno-mediated tumor cell killing. The figure is adapted from Scott, Wolchok and Old 2012.

Due to the complexity and the incomplete knowledge of immunological mechanisms some of the new biologics are being tested using only simple binding assays up to now. To overcome this insufficient testing, we are developing novel cell-based assays that mimic and quantify the anti-cancer immune response reactivated by the immune-oncology drugs.

The new assays are intended to be used for biologics with a T cell related mode of action and are therefore based on the characteristics of T cells. The *selected T cells* (*Table 2*) are cells from the adaptive immune system, and are distinguished from other cells by their capability to recognize "non-self" molecules (antigens). Antigens are molecules that are capable of inducing an immune response.

T lymphocytes are able to recognize antigen through specific receptor molecules present on their surfaces. These receptors are called T cell receptors (TCRs). Each T lymphocyte has a TCR specific for a unique peptide sequence called epitope of one particular antigen, providing the extremely specificity of T cells. The interaction between the receptor and the antigen epitope is only possible if it is exposed through the MHC Major Histocompatibility Complex class I or II molecules (MHC-I, MHC-II).

All nucleated cells are expressing MHC class I molecules which present peptides derived from endogenous proteins degraded by proteasome to cytotoxic T cells (CD8+). These endogenous proteins can also derived from virus, intracellular microbial infection, or mutation of self-protein. The cells that present these non-self antigen epitopes can be recognized and killed by CD8+ to maintain the health of the body. In contrast, the MHC II is only expressed by APCs, which phagocytize exogenous peptides to present them to T helper cells (CD4+). These cells are then able to initiate the humoral immune system response or activate CD8+ T cells. A special form of presentation is the cross-presentation (Joffre, et al. 2012). In this case, particular APCs present exogenous peptides throw the MHC I for the cytotoxic T cells activation.

The binding between the antigen peptides exposed through MHC molecules and the TCR in combination with binding of *co-stimulatory factors* (*Table 2*) induces T cell activation (*Figure 6*). The recognition activates the T cells so that they start to proliferate. The T cells can then bind with their TCR to the MHC/epitope complex on the target cells and lyse them and/or produce important cytokines (Kalergis, et al. 2001) (Nel 2002).

The aim of the novel methods is to use these characteristics of T cells to mimic the T cell related mode of actions of new biologics *in-vitro*.

Definitions:
Mode of Action: also called Mechanism of Action, describes functional or anatomical changes at the cellular level, resulting from the exposure of a living organism to a substance. It is the pharmacological effect produces by the drug substance in a cell *Selected T cells*: The development of T cells takes place in the thymus. Precursors of T cells migrate from the bone marrow and mature in the thymus. Developing T cells pass through a series of stages, as the **Positive Selection**, in which only those T cells whose receptors can recognize antigens presented by self MHC molecules can mature and all other developing T cells die before reaching maturity; and **Negative selection**, in which thymocytes that recognize self are deleted from the repertoire (Janeway, Travers and Walport 2001). *Co-stimulator factors*: For T cell activation two signals are required. The first signal, which is antigen-specific, is provided through the TCR which interacts with peptide-MHC molecules on the membrane of APCs. A second signal, the co-stimulatory signal, is antigen nonspecific and is provided by the interaction between co-stimulatory molecules expressed on the membrane of APCs and the T cell. One of the best characterized co-stimulatory molecules expressed by T cells is CD28, which interacts with CD80 (B7.1) and CD86 (B7.2) on the membrane of APC. Another costimulatory receptor expressed by T cells is ICOS (Inducible Costimulator), which interacts with ICOS-L. T cell co-stimulation is necessary for T cell proliferation, differentiation and survival. Activation of T cells without co-stimulation may lead to T cell anergy, T cell deletion or the development of immune tolerance (Goronzy and Weyand 2008).

Table 2 - Definitions

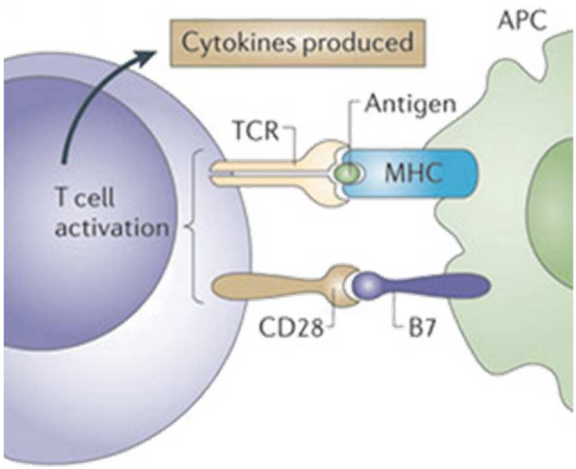

Figure 6 – Overview of T cell activation: adapted from Sharma, et al. 2011

Cancer Vaccines

The cancer vaccines can be distinguished in preventive and therapeutic vaccines. The preventive vaccines are administered to healthy people to keep cancer from developing (Berzofsky, et al. 2004). The therapeutic vaccines on the other hand are a type of immunotherapy and they boost the immune system to fight cancers. They can stop tumors from growing or spreading, and prevent cancer relapse, after cancer treatments (Berzofsky, et al. 2004).

While preventative cancer vaccines have shown significant success with cancer of viral origin such as human papillomavirus (HPV), therapeutic cancer vaccines have struggled to achieve the same success (Mellman, Coukos and Dranoff 2011). The main purpose of cancer vaccines is to supply immunogenic tumor antigens that can further stimulate effector T cells and drive anti-tumor immunity. However, it has become clear that in order to be effective, cancer vaccines should overcome the immune tolerance acquired by tumor cells by e.g. downregulating the expression of tumor antigens (Guo, et al. 2013). This requires large quantities of tumor antigens presented by antigen presenting cells, in order to activate and boost the lymphocyte response against the tumor.

Cancer vaccine formulations can include tumor lysates, purified tumor antigens, whole tumor cells, tumor cells genetically engineered to express immune-stimulatory cytokines, or DNA/RNA molecules encoding various tumor antigens (*Figure 7*) (Yaddanapudi, Mitchell and Eaton 2013).

In our company, an anti-tumor vaccine against a molecule overexpressed in cancer cells has been developed. I have worked on the development of a method using a specific T cell clone as test system to quantify and mimic the reaction of T lymphocytes activated in the human body after vaccination. In this assay, the cancer vaccine was processed by APCs, and through cross-presentation exposed via MHC-I.

The extremely sensitive T cell clone is expected to detect potential differences in vaccine batches, and this could be analyzed in a quantitative ELISA assay.

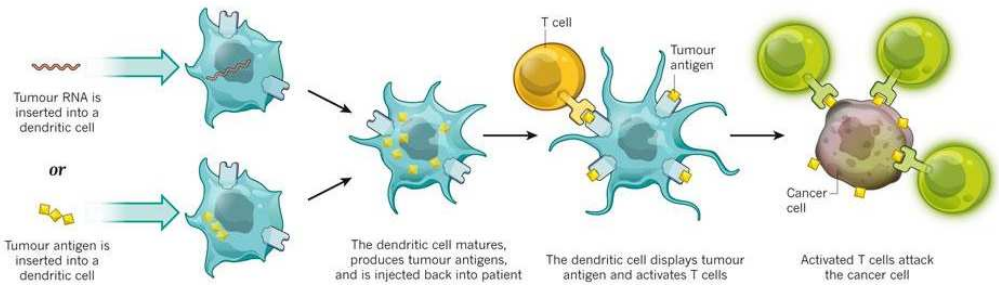

Figure 7 – How the cancer vaccines work: adapted by Gravitz 2011

Immune Checkpoint Inhibitors

There is more and more evidence that the primary mechanism by which tumors evade the immune system is by engaging immune checkpoints (Granier, et al. 2017).

These proteins limit the strength and duration of immune responses, and are normally involved in maintaining *self-tolerance* (*Table 3*) and immune homeostasis (Pardoll 2012). The tumor cells use these mechanisms to suppress the activation of T cells and escape from the killing. Therefore, one immunotherapeutic approach is to develop agents that specifically target key molecules in immune inhibitor pathways, releasing the T cells from the inhibition and promoting anti-tumor immune function (Shih, Arkenau and Infante 2014) (Kyi and Postow 2014).

In 2011 the FDA approved the first checkpoint inhibitor drug. Ipilimumab is a monoclonal anti-CTLA-4 antibody (*Figure 8*) authorized as a first line therapy for metastatic melanoma (Weber, et al. 2008) (Hodi 2010). CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4) is an inhibitory receptor that downregulates the T cell activation competing with the co-stimulatory receptor CD28 for CD80/CD86 on APCs (McCoy e Le Gros 1999). Since the approval of Ipilumimab, multiple other immune checkpoint pathway molecules have been targeted for cancer therapy and are being evaluated in preclinical tumor models or clinical trials (Farkona, Diamandis and Blasutig 2016). These include PD1 (Programmed cell death protein 1) and its ligands PD-L1/L2 (Programmed death-ligand 1 and 2), LAG3 (Lymphocyte activation gene 3) and TIM3 (T cell immunoglobulin and mucin domain-containing 3) proteins and a number of other receptors and proteins.

These new immuno-biopharmaceuticals have the same targets and similar mechanism of action that cannot be fully examined with the canonical analytical methods. Moreover, it is necessary to develop different tests for all these products because their mechanisms of action are different.

To overcome the problem, an innovative bioassay has been developed. The two main actors of this bioassay are a specific T cell line expressing the inhibitory receptor and an antigen-presenting cell expressing the respective ligand. The two cell lines have to be stable to guarantee assay that can be maintain under control and deliver reliable results. The interaction between T cells and APCs with or without the antibody produces an easily to be evaluated immune response like cytokine secretion.

Definition
Self-tolerance: the immune system is able to discriminate between self and non self antigen. At the T cell level, this is achieved by inactivation and deletion of clones recognizing peptides of self proteins presented by MHC molecules. This balance of immunological defense and self-tolerance is critical to normal physiological function and overall health (Sakaguchi, et al. 1995).

Table 3 - Definition

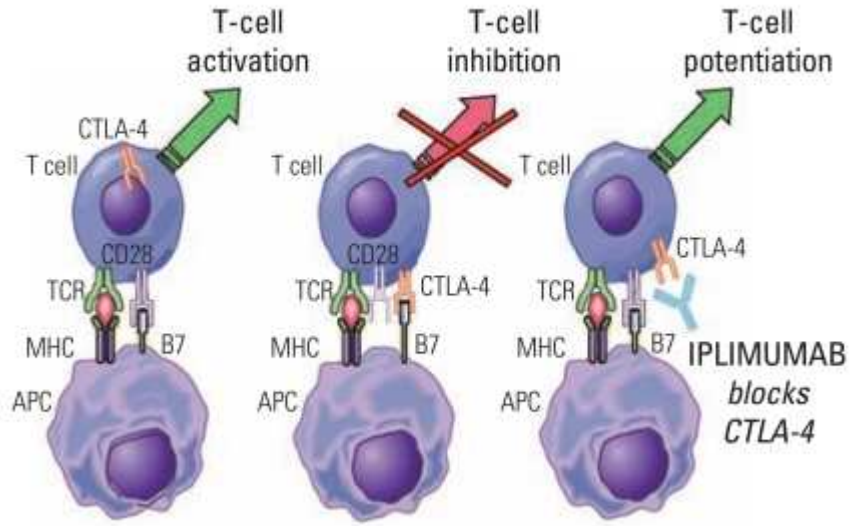

Figure 8 – Ipilimumab mode of action: after the T cell inhibition, due to the immune checkpoint inhibitor CTLA-4, the Ipilimumab antibody is able to revert the inhibition, potentiating the T cell activation. Figure adapted from Saijo, 2012.

Materials and Methods

T cell clone selection

The T cell clone generation was performed in accordance with protocols present in Conrad, et al. 2008.

For the preparation of dendritic cells the following protocol was followed. The isolation of CD14+ monocytes with CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) was performed using the supplier protocol. The CD14+ cells were isolated from HLA-A2+ peripheral blood mononucleic cells (PBMC) supplied by Stemcell Technologies (Vancouver, Canada). Briefly, PBMCs were thawed and resuspended in the MACS buffer, a solution containing PBS 1x, 0.5% bovine serum albumin (BSA) (Merck KGaA) and 2mM EDTA (Merck KGaA), and the CD14 MicroBeads added. The suspension was incubated at 2-8°C and in the meantime a magnetic column was prepared with different washing steps. After the incubation, the cells and beads suspension was added to the column. The flow-through containing all cells but the CD14+ ones was frozen in human serum (Thermo Fisher Scientific) with 10% DMSO (Merck KGaA). They were seeded in a 6 well plate at the concentration of $2x10^6$ cells/mL and differentiated with X-Vivo 10 (Lonza, Basel, Switzerland) supplemented with 10% human serum, 1600U/µL GM-CSF (R&D Systems, Minneapolis, Minnesota, USA) and 1000U/µL IL-4 (R&D Systems). The CD14+ cells were incubated at 37° ± 1°C in a humidified atmosphere with 5 ± 1% CO_2. Every two to three days 1 mL X-Vivo 10 medium supplemented with 10% human serum, 1600U/µL GM-CSF and 1000U/µL IL-4 was added to each well. After one week the cells had differentiated into immature dendritic cells as was visible from their shape under microscope. They were matured with X-Vivo 10 supplemented with 10% human serum, 1600U/µL GM-CSF, 1000U/µL IL-4 and 100U/well LPS (InvivoGen, San Diego, California, USA). The dendritic cells were incubated at 37° ± 1°C in a humidified atmosphere with 5 ± 1% CO_2 for two days.

The day of T cell stimulation, the dendritic cells were collected and loaded with a specific peptide epitope, e.g. from CMVpp65 peptide NLVPMVATV (HLA-A*0201) (IBA GmbH, Göttingen, Germany) and β-2 microglobulin (MP biomedicals, Santa Ana, California, USA) for 2 hours in AIM V® Medium (Thermo Fisher Scientific). After the loading, dendritic cells were washed and seeded at the concentration of $5x10^3$ cells/well in 96 well round bottom plates with AIM V® Medium supplemented with 5% human serum, 10ng/mL IL-12 (R&D Systems) and 1000U/mL IL-6 (R&D Systems). The T cells were isolated from the HLA-A2+ PBMCs without CD14+ cells frozen after the CD14+ isolation using Human CD8+ Isolation kit (Miltenyi Biotec). Briefly, PBMCs were thawed and treated with the CTL Anti-Aggregate Wash™ (Cellular Technology Limited C.T.L., Cleveland, Ohio,

USA) to avoid the aggregation of dead cells. After the treatment the cells were resuspended in the MACS buffer and CD8+ T Cell Biotin-Antibody Cocktail added. The suspension was incubated at 2-8°C and in the meantime, a magnetic column was prepared with different washing steps. After the incubation, the cells and beads suspension was added to the column. The CD8+ cells that passed through the column were collected and counted. The cells were re-suspended in AIM V® Medium and seed in 96 well round bottom plates at the concentration of $1x10^5$ cells/well with dendritic cells already loaded and prepared.

The 96 well round bottom plates with CD8+ and loaded dendritic cells were incubated at 37° ± 1°C in a humidified atmosphere with 5 ± 1% CO_2. After one week, the T cells were stimulated again with dendritic cells loaded with the specific peptide, following the same protocol with AIM V® Medium supplemented with 5% human serum, 2.5ng/mL IL-7 (R&D Systems), 50U/mL IL-2 (R&D Systems) and 1.25ng/mL IL-15 (R&D Systems).

After two weeks from the first stimulation an enzyme-linked immunospot (BD™ ELISPOT Human IFN-γ ELISPOT Set, BD Biosciences, Franklin Lakes, New Jersey, USA) with the HLA-A2+ T2 cell loaded with the relevant or an irrelevant peptide epitope as target cells was performed (see IFN-γ and TNF-α ELISPOT assays protocol). Wells that showed a response only to the specific peptide were chosen for cloning by limiting dilution. A pool of $4x10^4$ PBMCs and $1x10^5$ EBV-transformed B cells, already treated with Mitomycin C or irradiated (see T cells expansion protocol), were added in each well of 96 well round bottom plates. One single T cell per well was added. The cells were kept in culture with RPMI 1640 (Thermo Fisher Scientific) supplemented with 10% human serum, 30ng/mL anti-human CD3 (BioLegend, San Diego, California, USA), 100U/mL IL-2 and 2.5ng/mL IL-15 at 37° ± 1°C in a humidified atmosphere with 5 ± 1% CO_2. After two weeks from the cloning a second enzyme-linked IFN-γ immunospot with T2 cells loaded with relevant or irrelevant peptide was performed. Wells positive only for the relevant peptide were expanded.

T cells expansion

The T cell expansion was done using different protocols. The cells were expanded with a mixture of PBMCs and EBV transformed B cells treated with Mitomycin C (Merck KGaA) or γ-irradiated, otherwise with Dynabeads® Human T-Activator CD3/CD28/CD137 (Thermo Fisher Scientific).

For the expansion with Mitomycin C, the reagent was resuspended at 0.5mg/mL in PBS 1x. The PBMCs and the EBV-transformed B cells were treated for 120 minutes and 90 minutes respectively with 25µg/mL of Mitomycin C solution. After the treatment, the cells were washed for three time in RPMI 1640 medium and added to T cell clones. T cell clones were expanded with $2.5x10^7$ of PBMCs

and $5x10^6$ of EBV-transformed B cells in 25mL of RPMI 1640 medium supplemented with 10% human serum, 30ng/mL anti-human CD3, 100U/mL IL-2 and 2.5ng/mL IL-15 $1x10^5$.

For the expansion with γ-irradiated feeder cells, the PBMCs were irradiated at 30 Grey and the EBV-transformed B cells at 60 Grey. T cell clones were expanded with $2.5x10^7$ of PBMCs and $5x10^6$ of EBV-transformed B cells in 25mL of RPMI 1640 medium supplemented with 10% human serum, 30ng/mL anti-human CD3, 100U/mL IL-2 and 2.5ng/mL IL-15 $1x10^5$.

For the expansion using Dynabeads® Human T-Activator CD3/CD28/CD137 the protocol provided by the supplier was used. Briefly, the beads were washed in a washing buffer prepared with PBS 1x supplemented with 0.1% human serum and 2 mM EDTA. After the washing, the beads were separated from the washing buffer with DynaMag15 magnet. The beads were then added to the cells in a beads:T cells ratio of 1:10. The T cells were cultured in RPMI 1640 medium supplemented with 10% human serum, 30ng/mL anti-human CD3, 100U/mL IL-2 and 2.5ng/mL IL-15.

TCR transduction

The two α and β chains vectors of the specific TCR were kindly offered by the Billerica site of Merck KGaA. For the retrovirus production, the following protocol was used. A DNA mixture of DMEM, p10A1 (Clontech) and pMX-TCR or pMX-GFP (Cell Biolabs, San Diego, California, USA) was prepared and combined with the Lipofectamine® 2000 (Merck KGaA). After the time for the complex forming, the solution was added to the packaging cell line GP293 (Clontech, Mountain View, California, USA), and incubated at 37° ± 1°C in a humidified atmosphere with 5 ± 1% CO_2. After the overnight, the medium was replaced with DMEM supplemented with 10% fetal bovine serum, and the cells incubated at 37° ± 1°C in a humidified atmosphere with 5 ± 1% CO_2. After 36h the viruses formed in GP293 were collected, and used to transduce the CD8+ cells. The lymphocytes were isolated using the Human CD8+ Isolation kit, and then stimulated in a 6 well plate coated with 5 μg/ml anti-CD3 and 1 μg/ml anti-CD28 (R&D Systems) for 3 days before the infection. The transduction was performed adding the virus to CD8+ and centrifuging for 90 minutes at 2000 rpm and 32°C. After the centrifugation the cells and viruses were incubated at 37° ± 1°C in a humidified atmosphere with 5 ± 1% CO_2 overnight, and the day after the medium replaced with RPMI 1640 supplemented with 10% human serum

IFN-γ and TNF-α ELISPOT assays

The enzyme-linked immunospot (ELISPOT) assay is an *in-vitro* method used for the detection and enumeration of individual cells that secrete a protein of interest (*Figure 9*) (Helms, et al. 2000). The specificity and sensitivity of the ELISPOT assay derives from the use of high affinity capture and detection antibodies and enzyme-amplification. Although originally developed for analyzing specific antibody-secreting cells (Sedgwick e Holt 1983) (Czerkinsky, et al. 1983), the assay has been adapted for measuring the cells that produce and secrete other effector molecules, such as cytokines (Ronnblom, et al. 1988) (Nordströma e Ferrua 1992) (Fujihashi, et al. 1993). The sensitivity of the assay lends itself to measurement of even very low frequencies of analyte-producing cells (e.g., 1/300,000) (Helms, et al. 2000). The number of spots correlate with the number of cells producing cytokines and not with the amount of cytokines produced. For this reason it is a qualitative and not a quantitative assay test as the ELISA.

Antigen-specific IFN-γ and TNF-α releasing level were determined by using BD™ ELISPOT Human IFN-γ and TNF-α ELISPOT assays according to the manufacturer's instructions. Briefly, 96-well PVDF plates were coated with diluted antibodies overnight at 4°C (*Figure 9A1*). The wells were blocked with RPMI 1640 supplemented with 10% fetal bovine serum for 2 hours at 37°C. In the meantime, the APCs (T2, HEK293, dendritic cells) were stimulated with specific or unspecific peptides (10µg peptide). After the blocking, the T cells suspension was seed into dedicated wells and incubated for 30 minutes at 37° ± 1°C in a humidified atmosphere with 5 ± 1% CO_2 to allow the T cells to settle down and scatter evenly over the filter membrane. Then add the loaded APCs and incubate overnight at 37° ± 1°C in a humidified atmosphere with 5 ± 1% CO_2 (*Figure 9A2*). At the end of incubation, the plate was washed with deionized water and wash buffer, prepared with PBS 1x containing 0.05% TWEEN® 20. A solution of biotin-labeled detection antibodies was added to plates and incubated for 2 hours at room temperature (*Figure 9A3*). Subsequently plates were washed and the HRP-conjugated streptavidin working solution was added for another 1 hour at room temperature (*Figure 9A4*). AEC substrate final solution was added after a washing step to each well for 5 minutes in the dark at room temperature (*Figure 9A5*). Color development was stopped by thoroughly rinsing both sides of the PVDF membrane with demineralized water (*Figure 9A6*). The plates were dried in the dark at room temperature overnight. The spots were counted by ELISPOT Analyzer (C.T.L., ImmunoSpot® Series S 5 Versa, S5VERSA-04-9070) (*Figure 9A7*).

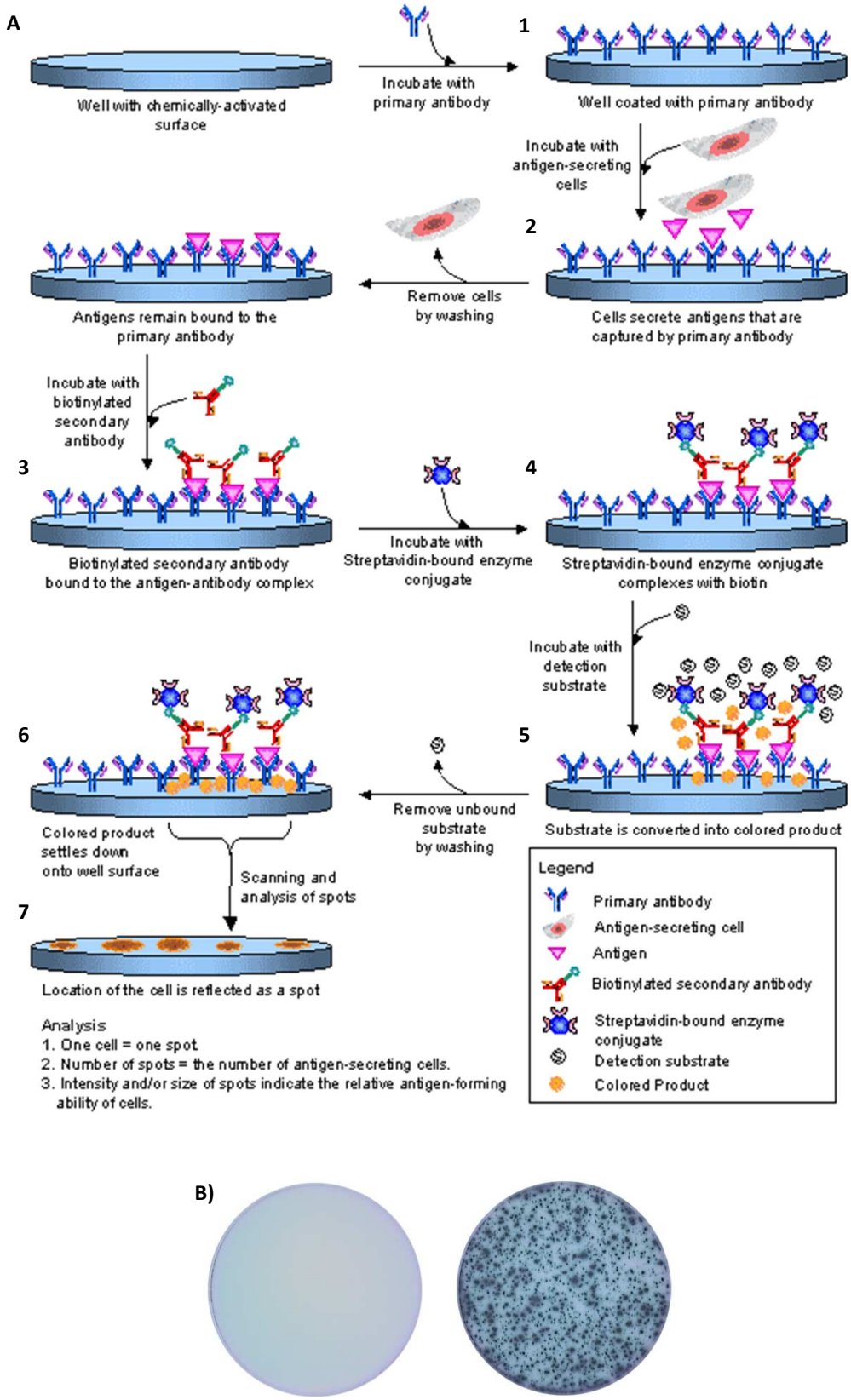

Figure 9 –ELISPOT Assay: A) Generalized Step of the ELISPOT Assay procedure (Sigma-Aldrich s.d.). B) ELISPOT results with or without stimuli. The spots represent each cell that have secreted cytokines

IFN-γ and TNF-α ELISA assays

The enzyme-linked immunosorbent assay (ELISA) is an immunological assay commonly used to measure antibodies, antigens, proteins and glycoproteins in biological samples using a colorimetric reaction which correlates to the amount of analyte present in the original sample (*Figure 10*) (Bidwell and Buck 1976). For this reason is a quantitative test and not only a qualitative test as ELISPOT.

Antigen-specific IFN-γ and TNF-α releasing level were determined by using Human IFN-γ and TNF-α ELISA assays according to the manufacturer's instructions (Thermo Fisher Scientific).

For the Human TNF-α ELISA, the samples and standards were added to anti-human TNF-α pre-coated 96-well strip plates and incubated for 1 hour at room temperature (*Figure 10-1*). After washing step, the biotinylated antibody reagent was added to wells and plates incubated again for 1 hour at room temperature (*Figure 10-2*). To the washed plates were added for 30 minutes the streptavidin-HRP reagent. Color development was performed adding the TMB substrate solution for 30 minutes, and then stopped adding the stop solution (*Figure 10-3*). Measurement of the absorbance was performed with SpectraMax reader (Molecular devices, Sunnyvale, California, USA) set at 450nm and 550nm (*Figure 10-4*).

For the Human IFN-γ ELISA, the samples, the standards and the biotinylated antibody reagent were added to anti-human IFN-γ pre-coated 96-well strip plates and incubated for 2 hours at room temperature. After washing step, the streptavidin-HRP solution was added to wells and plates incubated for 30 minutes at room temperature. To the washed plates were added for 30 minutes TMB substrate solution. The color development was stopped adding the stop solution. Measurement of the absorbance was performed with SpectraMax reader set at 450nm and 550nm.

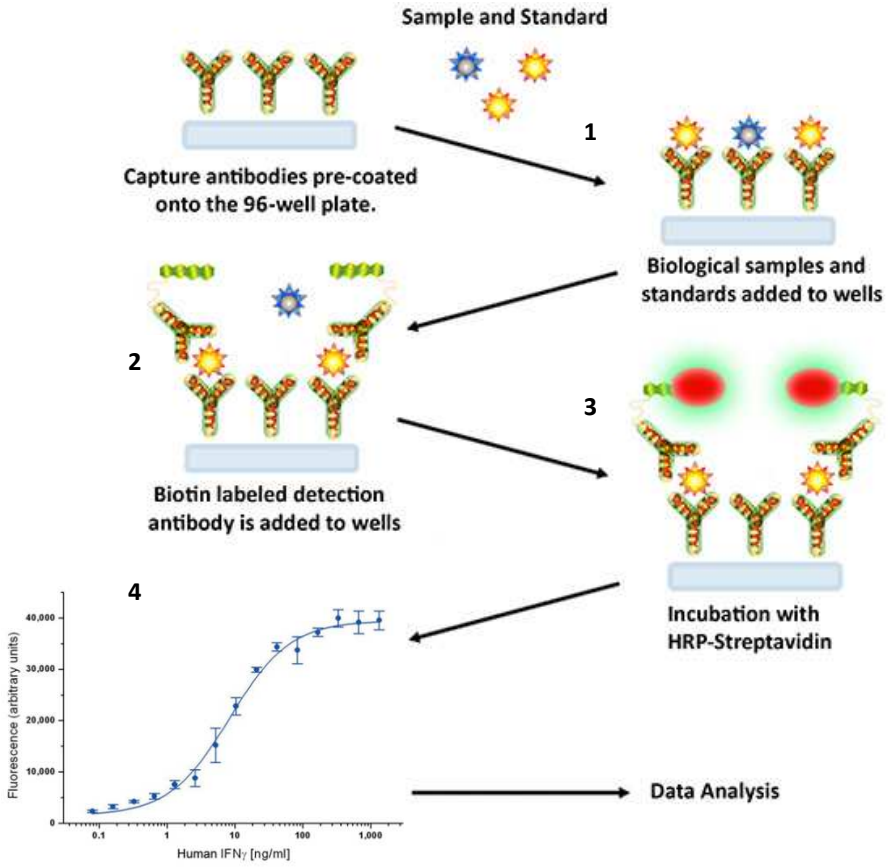

Figure 10 –ELISA Assay procedure (Sigma-Aldrich s.d.)

Flow cytometry

Flow cytometry is a laser based technique to count cells and to monitor a cell population simultaneously for multiple parameters, such as size and morphological complexity. Additionally, a wide range of fluorophores can be used to stain different cell surface and/or intracellular markers (*Figure 11*).

For the staining, the cells were washed with Cell Staining Buffer (BioLegend), counted and seed at $2x10^6$ cells/mL in a 96-well plate. In the same time, also the BD™ CompBeads Set Anti-Mouse Ig, κ (BD Biosciences) for the compensation were prepared following the manufacturer's instructions. After the preparation, the cells and the beads were stained with conjugated antibodies for 20 minutes at 2°-8°C, protected from light. The following antibodies, supplied by BioLegend, were used: APC anti-human CD20, APC/Cy7 anti-human CD20, FITC anti-human CD20, FITC anti-human CD279 (PD-1), PE anti-human CD279 (PD-1), FITC anti-human CD8, PE/Cy7 anti-human CD274 (B7-H1, PD-

L1), APC anti-human CD69, APC anti-human CD366 (Tim-3), PE anti-human CD3, PE/Cy7 anti-human CD4, PerCP anti-human CD45RA, APC/Cy7 anti-human CD197 (CCR7) and 7-AAD Viability Staining Solution. Cells were washed in Cell Staining Buffer and transfer in 5mL FACS tubes. Analysis of cells for the expression of cell surface markers was performed using a FACS Canto II flow cytometer and FACSDiva software (BD Biosciences).

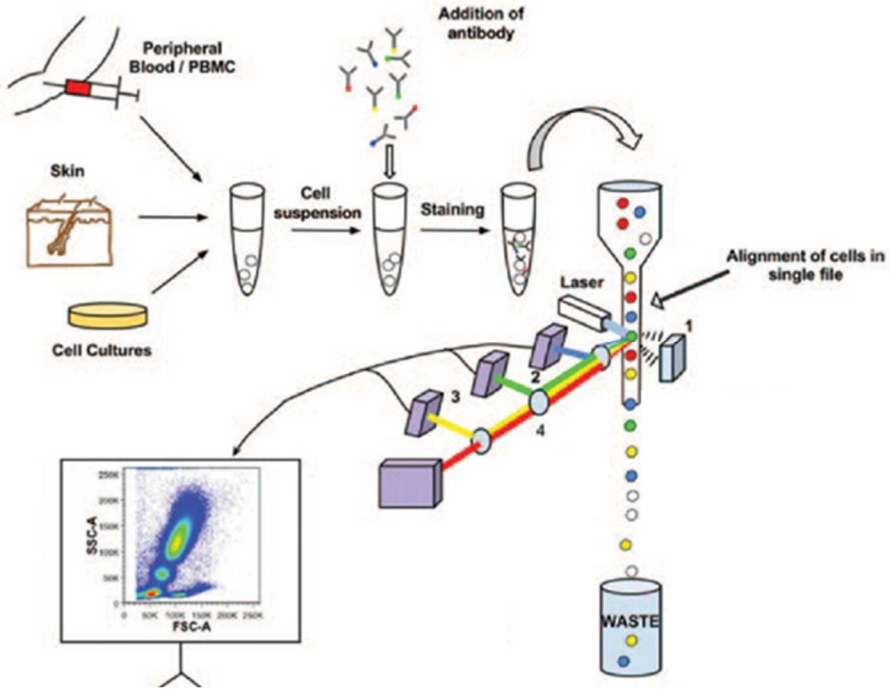

Figure 11 – Flow Cytometry technique: different samples can be staining with fluorophore conjugate antibodies, specific for antigen of interest. The lasers present in the cytometer hit the flow of cell passing through the nozzle, allowing the identification of each fluorophore corresponding to different antigen.

Immortalization of T cell clones

For the immortalization retrovirus and lentivirus containing the sequences of human telomerase were used. Retro-E1/hTERT virus and Lenti-hTERT virus were obtained by Applied Biological Materials (abm, Vancouver, Canada). The CD8+ cell were activated in a 24-well plate coated with 5 µg/ml anti-CD3 and 1 µg/ml anti-CD28 for 3 days. After the activation, the cells were transferred in a coated RetroNectin (Takara BIO Inc., Shiga, Japan) plate, in which hTERT virus had been centrifuged before at 2000 rpm for 2 hours at 30°C. The plate was incubated at 37° ± 1°C in a humidified atmosphere with 5 ± 1% CO_2. After 3 days 250 ng/mL of Puromycin (Merck KGaA) was added the cells to select the positive transfected clones.

Results

T cells Expansion

High numbers of T cells are needed to develop bioassays with T cells, for cancer vaccines or immune checkpoint inhibitors, the best method for the T cells expansion had to be found and optimized.

The first method taken into account was the expansion using Dynabeads® Human T-Activator CD3/CD28/CD137 to activate the T cells. These beads are normally used for *ex-vivo* expansion of lymphocytes, because they are a simple method for activating and expanding T cells that does not require feeder cells or antigens (Rasmussen, et al. 2010). They are inert, magnetic beads, of a size similar to that of APCs and are covalently coupled to anti-CD3, anti-CD28 and anti-137 antibodies, primary and co-stimulatory signals necessary for T cell activation and expansion. Different Beads:Cells ratio (1:1, 1:2, 1:3, 1:5, and 1:10) were tested, and the optimal one was 1:10. With this protocol the cells started to die, after the first stimulation, probably due to an incomplete or inefficient stimulation.

The second method tested was the T cells expansion with feeder cells, EBV-transformed B cells and PBMCs, treated with Mitomycin C (Ponchio, et al. 2000) (Matsushita, et al. 2001). The function of feeder cells is to support the proliferation of T cells. The Mitomycin C treatment inhibit and arrest the growth of feeder cells, which however continue to release growth factor and expose primary and co-stimulatory signals necessary for T cell activation and proliferation (Llames, et al. 2015).
Different time points and concentrations of Mitomycin C treatment were tested and the optimal one was PBMCs and the EBV-transformed B cells treated for 120 minutes and 90 minutes respectively with 25µg/mL of Mitomycin C (*Figure 12*). The T cells expansion was repeated for different stimulation and the number of cells was significant, passing from $1x10^5$ cells to $2x10^6$. However, after different tests, unspecific T cell reactions were noticed. Therefore cells were tested in a FACS analysis, and a consistent amount of B cells or unspecific T cells were founded (*Figure 13A*). This could be related to an inefficient Mitomicyn C treatment which could result in an overgrowth of feeder cells in the T cell clone culture.

The last protocol tested was T cells expansion with γ-irradiated feeder cells. This protocol had not been considered before for practicability reasons. γ-irradiated feeder cells stop to proliferate, but maintain a metabolically active state, allowing continued expression of specific ligand or cytokines. With a continuous stimulation of T cells and with no-overgrowth of feeder cells (*Figure 13B*), the γ-irradiation has resulted as the best methods for T cells expansion.

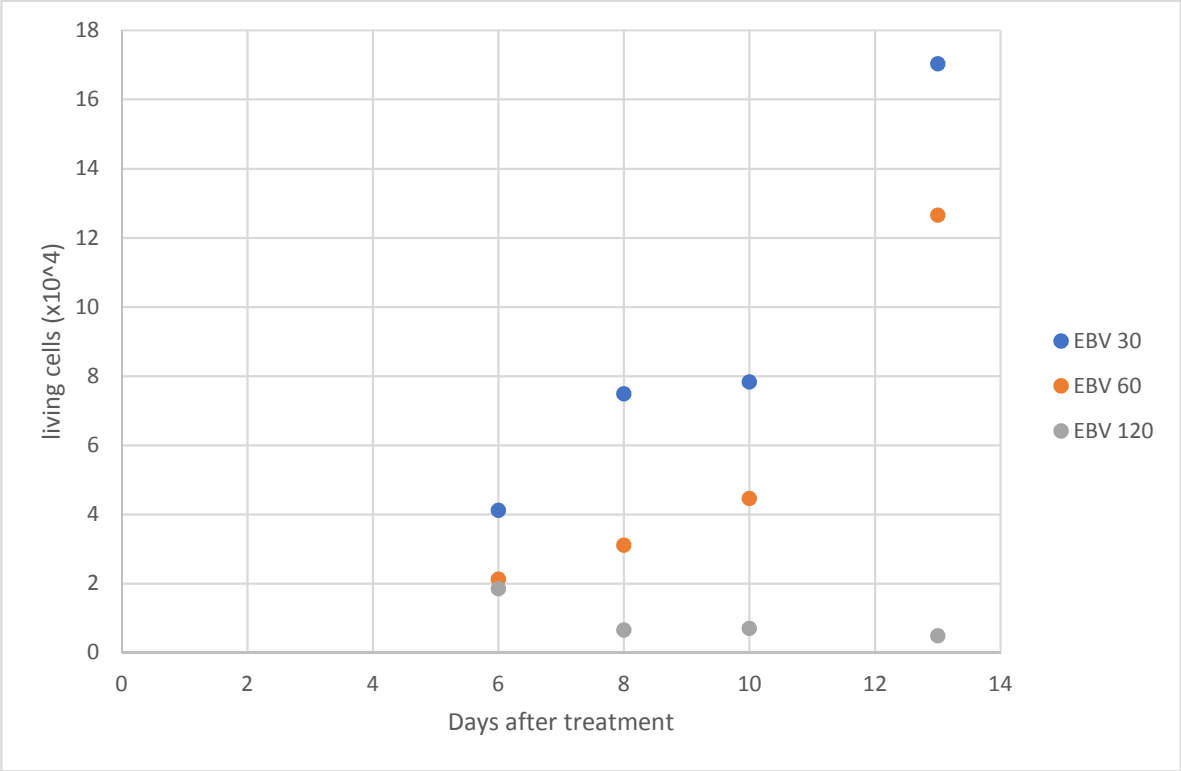

Figure 12 – Mitomicyn C treatment: the graph represents as with a Mitomycin C treatment for 120 minutes, the feeder cell proliferation is stopped

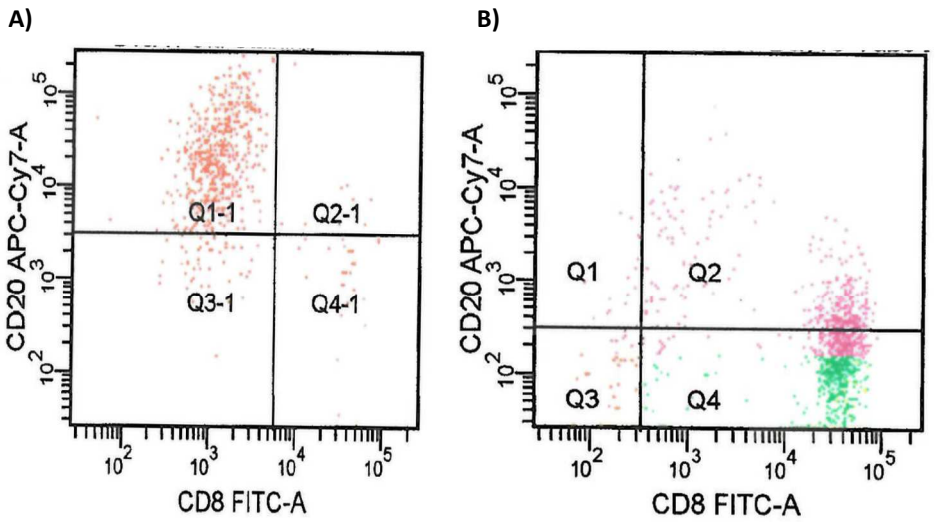

Figure 13 – FACS Analysis: A) T cell expansion using Mitomycin C treated feeder cells. B) T cell expansion using γ-irradiated feeder cells

T cell clone for cancer vaccines bioassay

For the preparation of a T cell clone with the specific TCR, able to recognize the antigen present in the cancer vaccine, two different approaches were used.

First of all, isolation of primary CD8+ with the specific TCR was tried. The pure population of CD8+ were isolated from PBMCs using the Human CD8+ Isolation kit from Miltenyi. The cells were than co-cultured with dendritic cells loaded with the peptide, and a limiting dilution was performed. After some weeks, the cultures were tested for the presence of T cell clones responsive for the specific antigen. Probably due to the rarity of the antigen in human body, no specific T cell clones were found.

The second approach was to transduce a primary CD8+ with the specific TCR. In this case, it was tried to transduce the lymphocytes with a combination of the two chains of the receptor, α and β, or with the two chains separately. After the transduction, the cells were tested in an ELISPOT assay to evaluate the specificity of their receptor. In ELISPOT no signal appear, meaning no CD8+ with the specific TCR were created.

The development of the assay was stopped for company reasons so the assay was no finalized.

T cell clone for immune-checkpoint inhibitor antibodies bioassay

The immune checkpoints are present on all the T cells, independently from the TCR present on the membrane surface. For this reason, we decided to isolate a T cell clone specific for a common antigen, and use it as the test system to analyze all the checkpoint inhibitors developed by ours company.

As happened with the development of the cancer vaccines bioassay, the pure population of CD8+ were isolated from PBMCs using the Human CD8+ Isolation kit from Miltenyi. The cells were than co-cultured with dendritic cells loaded with the common peptide, and a limiting dilution was performed. After some weeks, the cultures were tested for the presence of T cell clones responsive for the specific antigen. As shown in *Figure 14*, many clones were reactive to the peptides. All the clones reactive to both peptides, specific and not specific, and all clones not or low reactive, were eliminated. The best clones strongly and univocally reactive were expanded and tested in parallel.

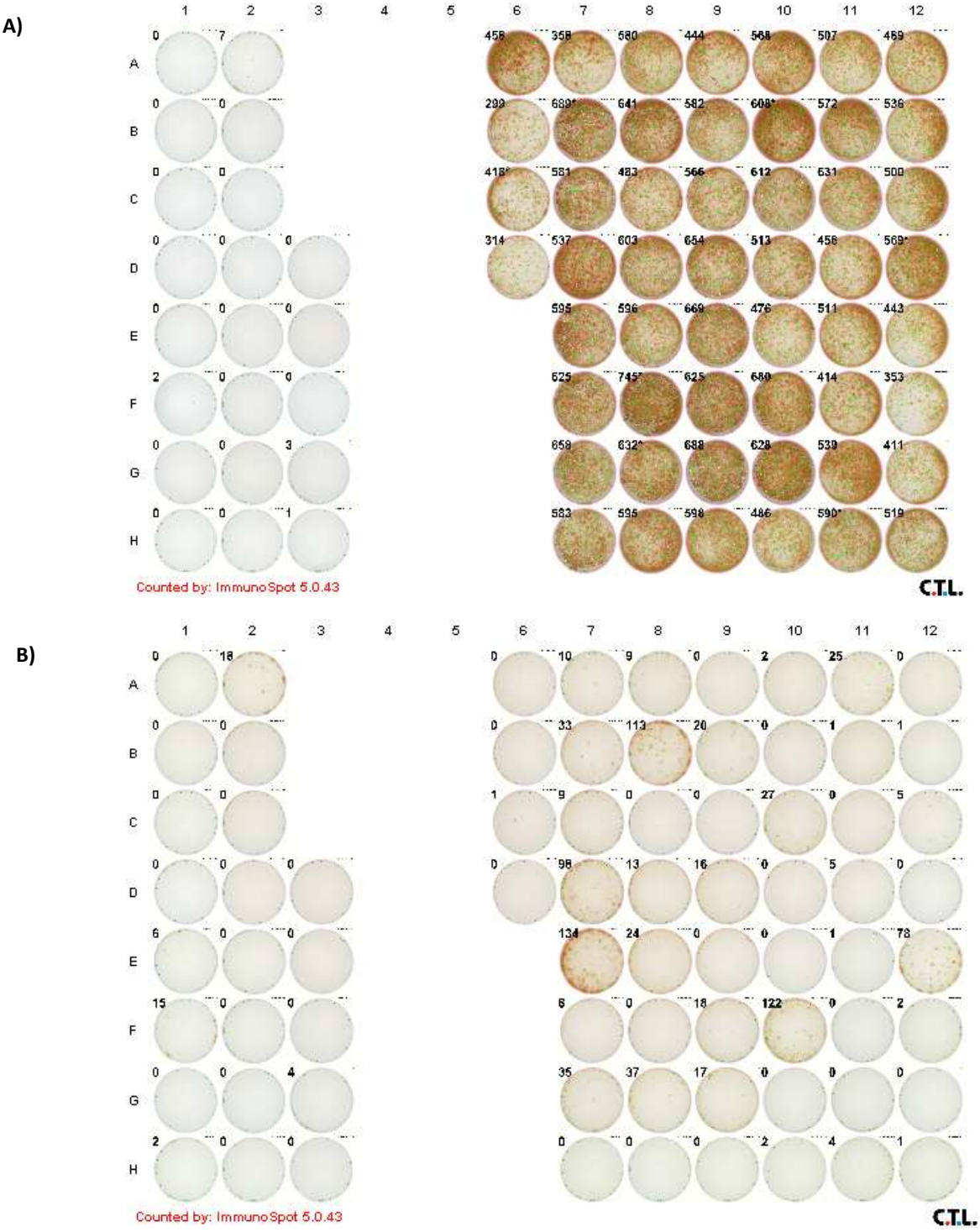

Figure 14 – ELISPOT for T cell clones: an IFN-γ ELISPOT were the T cell clones were tested for the A) specific antigen or B) unspecific antigen.

ELISPOT and ELISA for immune checkpoint inhibitor antibodies drug responses

The T cell clones were tested different time to ensure their specificity by INF-γ and TNF-α ELISPOT assays. In the tests, T cells were co-cultured for 16 hours with HEK293, T2 or dendritic cells, as antigen presenting cells loaded with the specific peptide. The production of cytokines was proportionated to the activation and response of T cells against the antigen (Nordströma e Ferrua 1992) (Fujihashi, et al. 1993).

Once the T cell clone lines were tested and confirmed specific, they were used to set-up the bioassay for the checkpoint inhibitor antibodies.

The immune-checkpoint inhibitor antibodies were used at different concentration to treat the T cell clones before testing them in ELISPOT. As expected, after the treatment with the antibodies an increase in the response of T lymphocytes was detected, confirming the release of T cells to the inhibition (*Figure 15*). Moreover the response was directly correlated with the different doses of immune-checkpoint inhibitor antibodies, increasing at the increasing of doses.

The correlation shown in *Figure 15* is very promising as for the use of the test as a bioassay for the potency drug quantification, a curve of response should be prepared. In addition shows that the correlation can be obtained with different immune-checkpoint inhibitor antibodies.

Because INF-γ and the TNF-α ELISPOT are qualitative assays and not quantitative, transfer of the assays in the INF-γ and in the TNF-α ELISA is necessary.

Preliminary results show as the concentration of immune-checkpoint inhibitor antibodies need to be change to better define the curve of dose-response using the ELISA assays (*Figure 16*).

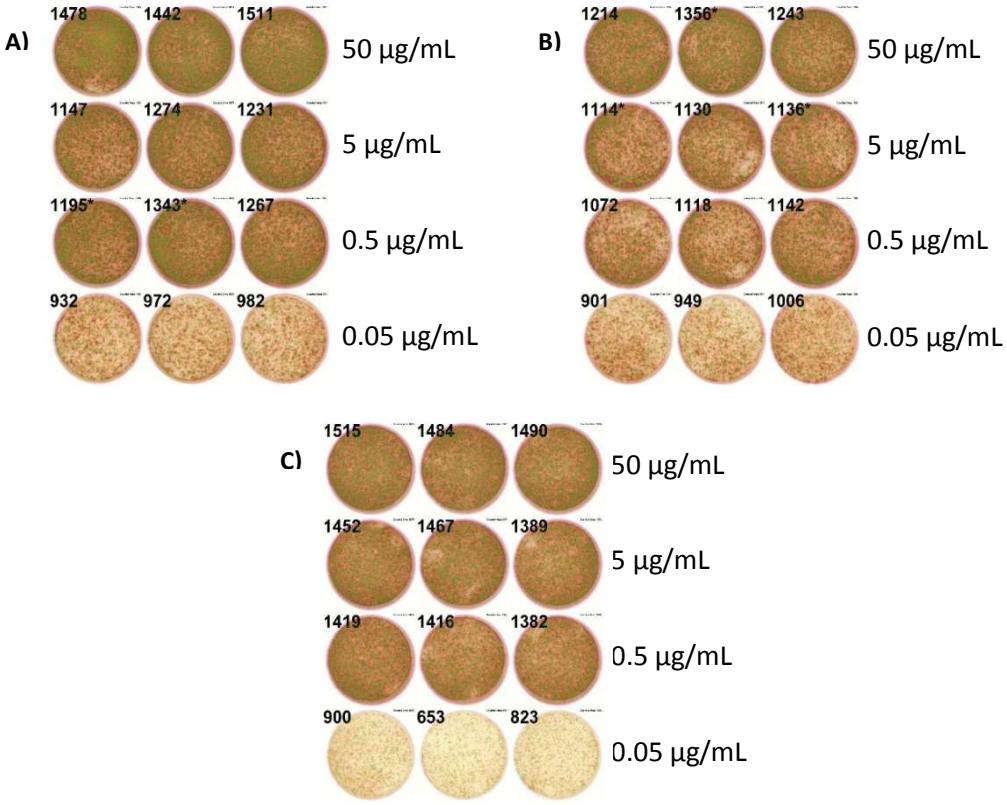

Figure 15 – T cell clones response to immune checkpoint inhibitors: A) and B) represent the same T cell clone with two different immune checkpoint inhibitor antibodies. In C) a different T cell clone response in the same way to another immune checkpoint inhibitor antibody

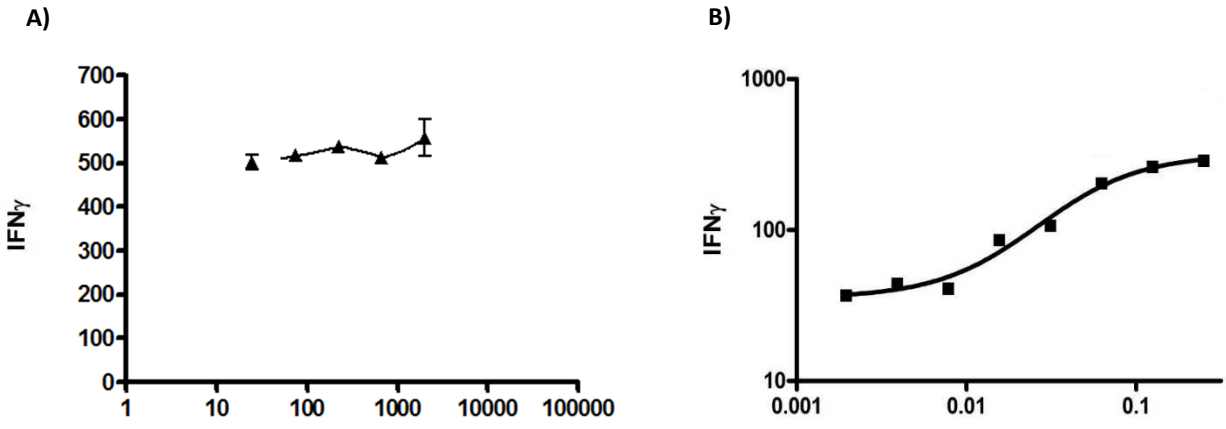

Figure 16 – ELISA results: A) preliminary results using the curve identified with ELISPOT concentration. B) preliminary results setting the curve with ELISA parameters

Immortalization T cell clones

In a bioassay all the components of the test should be maintain under control. For this reason, a cell line as T cell that after several passages went in senescence couldn't be a good test system. To avoid this the immortalization of the cell line was took in account, using viruses containing the vector for the human enzyme telomerase reverse transcriptase (hTERT) (Barsov 2011).

At the beginning, the hTERT retrovirus was used but no results were obtained because all cells died during the culturing. Therefore, the use of hTERT lentivirus was considered for their ability to infect non-replicative cell lines. Also this strategy fell through and no cells survived.

Up to now the T cell clones immortalization with retrovirus and lentivirus is impossible, and new strategies with new technologies are under discussion.

Discussion

The cancer immunotherapy has become a promising area in cancer struggle. More and more publications demonstrate the interest in research improvement, drug development, clinical trials, investigation of new targets and so on (Pasquali, et al. 2017) (Iwamoto, et al. 2016) (Illumina 2016). However, in parallel with the production and use of biologics, the biopharmaceuticals have to be tested with analytical methods that ensure their efficacy and potency, in addiction to their purity for the release on the market according to the guidelines.

Because of their different functional domains, authorities' requests for bioassays that reproduce the mechanism of action of biopharmaceuticals have increased. To keep up with the demands, the non-compendial methods development has become more and more important. Therefore, in ours laboratory new methods for cancer vaccines and immune checkpoint inhibitor antibodies are being set-up.

The cancer vaccines supply immunogenic tumor associated antigens (TAAs), proteins overexpressed in tumor cells but also expressed at low level in normal cells, able to stimulate effector T cells against cancer and drive anti-tumor immunity (Butterfield 2015).

The immune checkpoint inhibitor antibodies are antibodies generated against the immune inhibitor pathways, that tumors use for escape from the immune system attack, inducing the exhaustion of T cells (Pardoll 2012). The use of antibodies releases the T-lymphocytes from the inhibition, driving also in this case anti-tumor immunity (Thallinger, et al. 2017).

In both methods being set-up in our laboratory, the essential element is T cell clone specificity (Baker, et al. 2012). As is well known, each T lymphocyte is able to recognize a unique antigen through the T cell receptor (TCR) present on their surfaces providing their extreme specificity. The sensitive T cell could be used to detect differences in biologic batches, assuring the quality of drugs with a T cell related mode of action.

For the development of methods with T cells as test system, a high number of cells is needed so the best method for the T cell expansion was found and optimized. Generally the expansion of T cells is carried out with a pool of γ-irradiated feeder cells, PBMCs and EBV-transformed B cells. The γ-irradiation inhibits and arrests the growth of feeder cells, which however continue to release growth factor and expose primary and co-stimulatory signals. For technical reasons other methods were tested, as Dynabeads® Human T-Activator CD3/CD28/CD137 and feeder cells treated with Mitomycin C. It has been shown in literature that these two methods are able to activate and expand

T cell *in-vitro* (Rasmussen, et al. 2010) (Ponchio, et al. 2000) (Matsushita, et al. 2001). However in our case the T lymphocyte cell line was expanded best using the irradiated feeder cells. The Dynabeads® are inert, magnetic beads covalently coupled to primary and co-stimulatory signals, but not able to release growth factor. This can be the reason why they don't expand T cells in our case. Instead, the Mitomycin C treatment should have a similar effect to γ-irradiation, nevertheless they are metabolically altered, and consequently less efficient at maintaining cell expansion (Roy, et al. 2004). Moreover, there are cell population in the peripheral blood (PBMCs) of normal individuals which are resistant to Mitomycin C, and so able to overgrowth the T cell line expanded (Fernandez and MacSween 1987). This could confirm that T cell are not expanded so well and over the time overgrown by Mitomycin C resistant feeder cells.

The plan for the method to analyze cancer vaccines was to have a T cell clone specific for a tumor-associated antigen targeted by the vaccine. The isolation of a specific T cell clone was not successful. This can be explained because the TAAs (Vigneron 2015) (Criscitiello 2012) are molecules normally present in the human body. During the lymphocyte development process there is a negative selection, in which all lymphocytes reactive for self-antigen are remove (von Boehmer 1992). For this reason, it is very difficult to find some T cell reactive for these antigens in primary human PBMCs.

For the immune checkpoint inhibitor antibodies another approach was used. As in the case of the immune checkpoint inhibitors (Pardoll 2012), the specificity of the TCR was not important for the isolation of T cell clone specific for a common non-self viral antigen. By this the difficulty to get a TAAs specific T cell clone could be avoided. Different clone were obtained and tested for their specificity (*Figure 14*).

Once the T cell lines were set-up, their response to immune checkpoint inhibitor antibodies was analyzed. As expected, the T cell release from inhibition increased the response of lymphocytes to non-self antigens (*Figure 15*).

In a bioassay the cell line should be immortalized, to keep all the parameter of the test under control. Since after several passages in culture the T lymphocytes undergo in senescence the T cell clone should be immortalized for use it (Adibzadeh, et al. 1995) (Effros 1998). The mechanisms behind the finite replicative life span of cells is now well understood (Allsopp, et al. 1995) (Campisi 2013). It is due to a shortening of the telomeres. These DNA-protein structures cap the ends of linear chromosomes but shorten with each cell division. After a certain number of cell divisions they are too short to protect the chromosomes from degradation. Therefore the insertion of an active human enzyme telomerase reverse transcriptase (hTERT), able to maintain the telomere structure, has

already been used for cell immortalization, including T cell lines (Hooijberg, et al. 2000) (Barsov 2011) (E. V. Barsov 2011).

The transfection with retroviruses and lentiviruses has been taken into account for the long-term transgene expression as their genome integrates into the host DNA (Daly and Chernajovsky 2000) (Skipper and Mikkelsen 2015). Retrovirus and lentivirus transductions were performed but with no results. The negative results obtained with retrovirus can be explained by its ability to infect only dividing cell (Miller, Adam and Miller 1990), and the T cells were replicating only slowly. Therefore the use of lentivirus was investigated for its capability to infect both dividing and non-dividing cell. Also in this case no results were obtained. The explanation could be that primary T lymphocytes are notoriously refractory to genetic modification, so other methodologies and technologies are under evaluation (Zhao, et al. 2006).

The development of the assay was not been finished yet. It is now at the point where the transfer of ELISPOT in ELISA and the immortalization of T cells are performed. Nevertheless the proof of concept of the method efficacy can be stated.

Based on these approaches, new methods can be developed using T cell as test system, to reproduce the drugs mechanism of action in a more realistic way.

General Conclusion

The aim of this thesis was to developed analytical methods for the quality control that are in compliance with authority requests.

The analytical methods include all tests able to characterize a drug substance or drug product. They can be distinguished in compendial or non-compendial methods. The compendial methods are described in the authorities' guidelines, and they are requested for the quality control of each drug batch produced. One example of a compendial method is the adventitious virus identification by cytopathic effect and immunofluorescence assay. Non-compendial methods are novel methods that are being yet developed, not reported in regulations. They use new methodologies or technologies not yet taken into account by authorities.

In this thesis a compendial method was developed using new technology, remaining in compliance with the request of regulators. The use of an automatic image reader allows to speed up and improve the quality of results, meeting the high quality standards.

To maintain high quality standards it is also necessary to develop new methods able to reproduce the mode of action of drugs in the best possible way. For this reason a non-compendial method is being set-up for the testing of new immune checkpoint inhibitor antibodies. Up to now all the developed methods were not able to achieve the purpose. Our approach mimics what normally happens in human bodies, laying the groundwork for a new generation of bioassays.

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