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FRAUNHOFER INSTITUTE FOR INTERFACIAL ENGINEERING AND BIOTECHNOLOGY IGB

CELL-BASED ASSAYS FOR DIAGNOSTICS, DRUG AND TARGET DISCOVERY

CELL-BASED ASSAYS FOR DRUG AND TARGET DISCOVERY

Over the past years, there has been a growing trend toward the use of cell-based assays, particularly for pharmaceutical research and drug development. Cell-based assays refer to the use of living cells as diagnostic tools. This includes a variety of assays that measure cell proliferation, toxicity, motility, production of a measurable product, and morphology. Cell-based assays offer a more accurate representation of the real-life model than non-cell based and moreover offer the possibility of monitoring the behavior of the respective cells.

Insights from these cellular assays have been shown to facilitate drug discovery, saving considerable time and expense. These developments also help to reduce subsequent secondary screening.

Applications

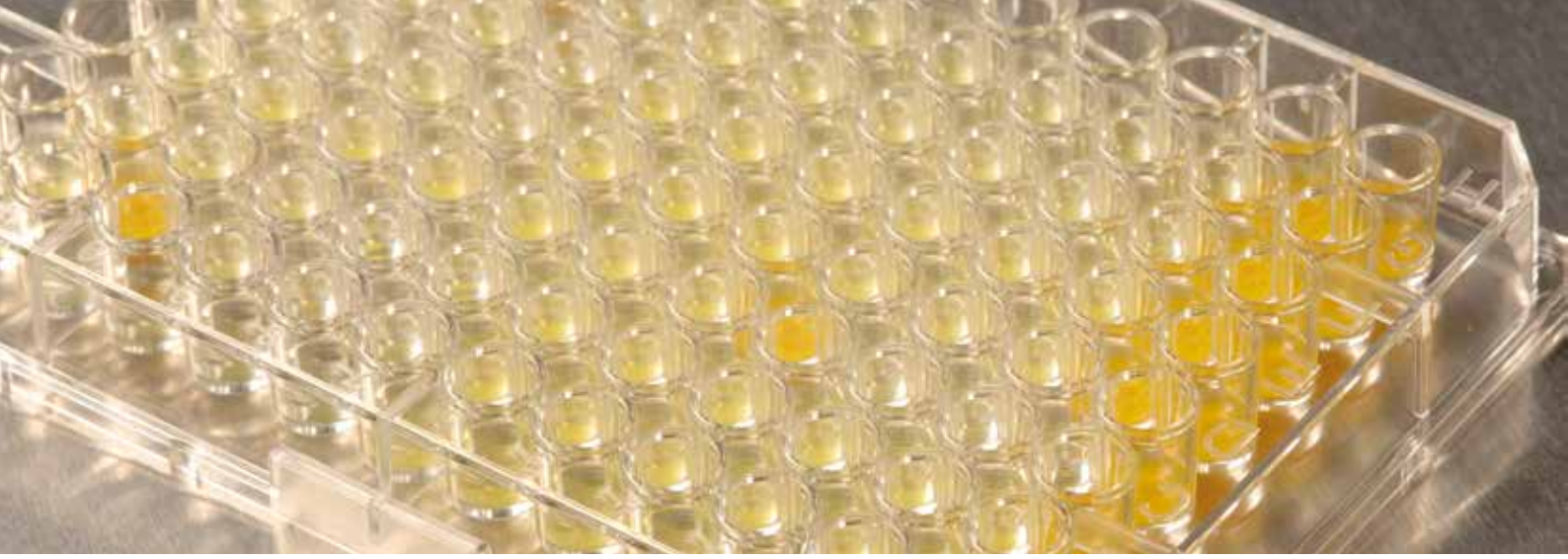
The cell-based assays established by the Fraunhofer IGB offer a broad range of applications:

- Screening for antimicrobial and antiviral compounds
- Evaluating toxic and non-toxic substances
- Detection of infectious viral contaminations
- Detection of pathogen-associated molecular patterns (PAMPs)
- Screening for pathogen-recognition-receptor agonists and antagonists

These assays allow a set of simple as well as more complex read-outs, e.g. visual or photometrical, via qPCR, or via changes in cell morphology.

The Fraunhofer IGB develops cell-based assays as diagnostic tools to be implemented in industrial processes or clinical standards. We also carry out screening assays on behalf of our customers.

Cell-based test system for the detection of pyrogenic residues.



SCREENING FOR NOVEL ANTIMICROBIAL COMPOUNDS

USING A HIGH-THROUGPUT SCREENING (HTS) ACTIVITY-SELECTIVITY ASSAY

The challenge

Efficacy and tolerability of a drug are the key criteria for its successful clinical use. Here, activity-selectivity assay is a highly advantageous method for obtaining selective and active lead molecules. Numerous antimicrobial drugs act only on a narrow range of pathogens or may cause severe side effects. Using a new, automatable screening assay, compound libraries can be screened fast and efficiently for a broad range and better tolerability of antimicrobial agents. The assay is a generally applicable screening test, which covers all potential *in vitro* targets of both the pathogen and the host simultaneously. In this assay, human host cells are incubated in the presence of the respective compounds (drug candidates) with the infectious microbes, such as viruses, bacteria, or fungi. Thus, the test system mimics the smallest unit of a natural infection. Cell survival is determined analogous to animal models (lethal challenge). This assay maximizes the chances for successful discovery of anti-infective drugs. It is sensitive, robust, time- and cost-efficient, and especially effective in optimizing screening hits to lead structures (hit-to-lead optimization) and the development of candidates in the preclinical phase of development.

Process steps

The individual wells of a microplate are initially coated with a layer of living human cells, which are then incubated with the compounds to be tested as well as the respective pathogen. Normally, the pathogen immediately unfolds its pathogenic potential and kills the susceptible human cells.

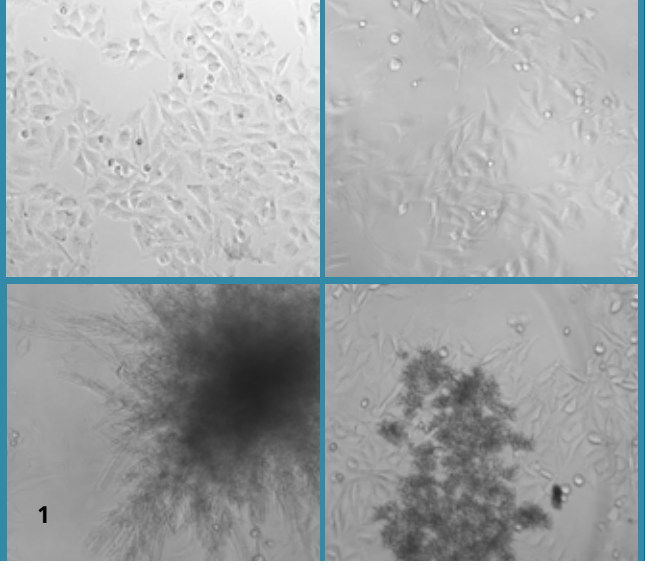
If, however, one of the compounds tested inhibits growth (proliferation) of the pathogen or blocks its virulence mechanisms, the human cells remain vital. Vital human cells can easily be detected photometrically using live dyes. Microscopic control of the individual wells of a microplate allows an additional evaluation of the physiological condition of the human cells. This results in another read-out parameter which ensures that only well-tolerated and biocompatible compounds are selected for further testing. This assay is HTS compatible and has been semi-automated at the Fraunhofer IGB.

Specifications

- Generally applicable in antimicrobial drug discovery; it can also be applied to bacteria and fungi in a standard operation procedure.
- Test system that simultaneously monitors the tolerance and activity of antimicrobial agents in a single assay.
- Approach that analyses the survival of host cells in a cell culture experiment infected with a pathogen, instead of measuring growth retardation, replication of the microbe itself, or inhibition of enzymatic functions thereof.
- Semi-automated screening of compound libraries.

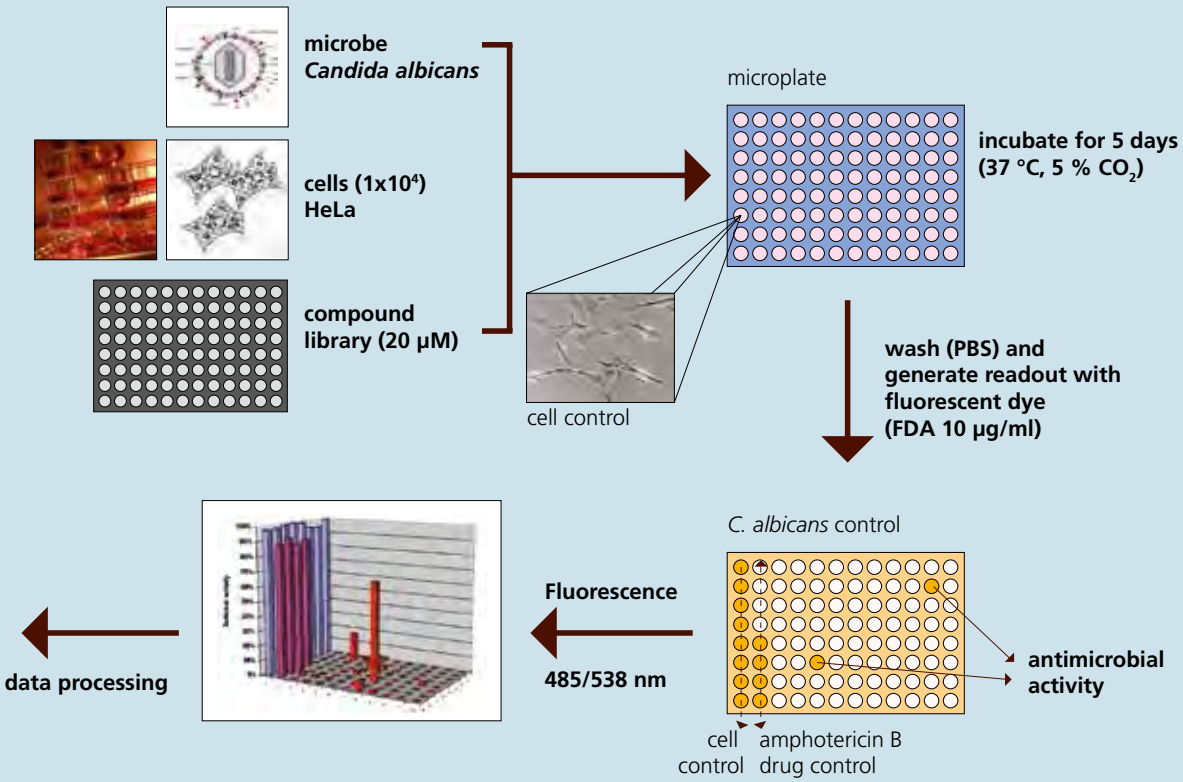
Services

- Transfer of cell-based screening assay to further species (fungi, bacteria, viruses)
- Semi-automated screening of compound libraries
- Target-directed screening using additional reporter-mediated cell-based assays



1 *Example for fungal infections: Phase contrast microscopic images of the HTS test system 24 hours after the infection with Candida albicans.*
A: *Microscopic image of the mammalian test cells (live control).*
B: *Cells infected with C. albicans treated with Amphotericin B (drug control).*
C: *Cells overgrown with C. albicans.*
D: *Cells infected with C. albicans and simultaneous addition of a testing compound identified as a hit: The compound inhibits growth of C. albicans while vital growth of the cells is not affected.*

HTS Screening Assay



Schematic representation of the activity-selectivity assay for the identification of lead structures for anti-infectives.
It mimics the smallest unit of an infection by incubating host cells (human cell line HeLa) with the pathogenic organism in the presence of the compounds to be tested.

CELL-BASED TEST SYSTEM FOR THE DETECTION OF PYROGENIC RESIDUES

The challenge

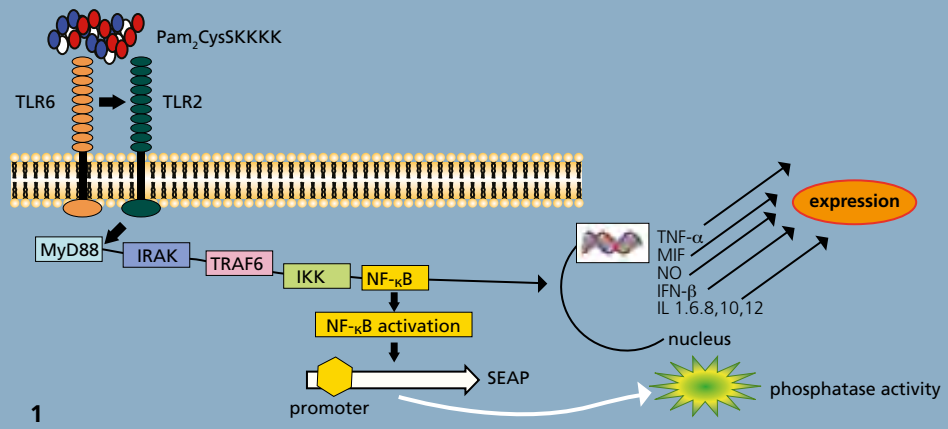
Pyrogens are fever-producing remnants from bacteria, viruses or fungi which can cause sepsis after entering the human bloodstream. Sepsis is considered one of the most severe complications in hospitals, and is caused by the sum of life-threatening symptoms and pathophysiological changes induced by these compounds, also called pathogen-associated molecular patterns (PAMPs). These can be micro-bial remnants, isolated chemical structures such as cell wall components or even entire microorganisms. The body responds by producing endogenous mediators (cytokines), which activate inflammation cascades. In sepsis, hyperstimulation of these cascades leads to a systemic reaction that is no longer under control and may result in multi-organ failure. In order to prevent the transmission of pyrogenic residues into the human bloodstream, surgical instruments, medical equipment and products (implants and medication applied intravenously) must therefore be tested for the absence of pyrogenic residues. There are currently three commercially available methods for the detection of pyrogens (Table 1) but they are either very costly or limited to specific pyrogens. The challenge was to provide a simple universal detection system for pyrogens at low cost.

Process steps

At Fraunhofer IGB we have developed a new, cell-based test system that allows PAMPs to be identified and differentiated via their natural pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), NOD-like receptors (NLRs) or dectins coupled to a reporter gene assay. PRRs are receptors of the human immune system which recognize components of viruses, bacteria or fungi and normally initiate cytokine response. For this assay, the appropriate receptor complex (e.g. TLR2/6, see Fig. 1) was stably transfected and expressed in NIH3T3 fibroblasts. This cell line expresses no other PRR receptors and

Table 1: Overview of the pros and cons of commercially available test systems for the detection of pyrogens.

	Rabbit test	LAL (Limulus amebocyte lysate) test	*In vitro* pyrogen test (IPT)
Test principle	Animal experiment: febrile reaction	Defense reaction of arthropods: coaguation of lysed amebocytes in the arrow tail crab with lipopoly-saccharides from gram-negative bacteria	ELISA test on whole blood: febrile reaction of human cells
Gram-negative microorganisms	+	+	+
Gram-positive microorganisms	+	-	+
Fungi	+	-	+
Viruses	-	-	+



1 *Principle of the cell-based test system. After specific ligand binding to the receptor, an intracellular signaling cascade activates NF-kB and induces the expression of the reporter gene (secreted alkaline phosphatase SEAP).*

contains a reporter gene which is induced by PRR activation. The induction of TLR2/6 for example by a specific ligand, Pam_2CysSK_4, leads to activation of the transcription factor NF-$_k$B. This, in turn, induces the expression of the reporter gene, e.g. a secreted alkaline phosphatase (SEAP). Pyrogens present in the analyte thus can be detected via expression of the reporter gene. Depending on the assay conditions, either formation of an insoluble deep blue precipitate that is easily detected visually or a HTS assay with photometric analysis can be performed.

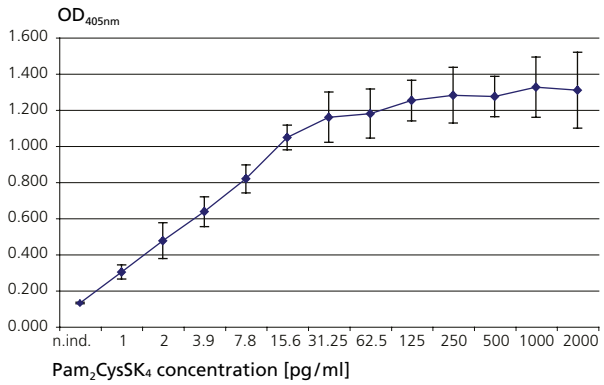
Specifications

- The cell-based test system allows fast and easy qualitative and quantitative detection of pyrogens without standard laboratory equipment.
- It can complement or replace existing tests such as LAL (Limulus amebocyte lysate) and IPT (*in vitro* pyrogen test).
- Pyrogens can be detected on medical equipment, injectable drugs, on implants or instruments as well as in food.
- In addition, the assay enables screening for TLR antagonists which are increasingly used in dermatology in order to suppress immune reactions.

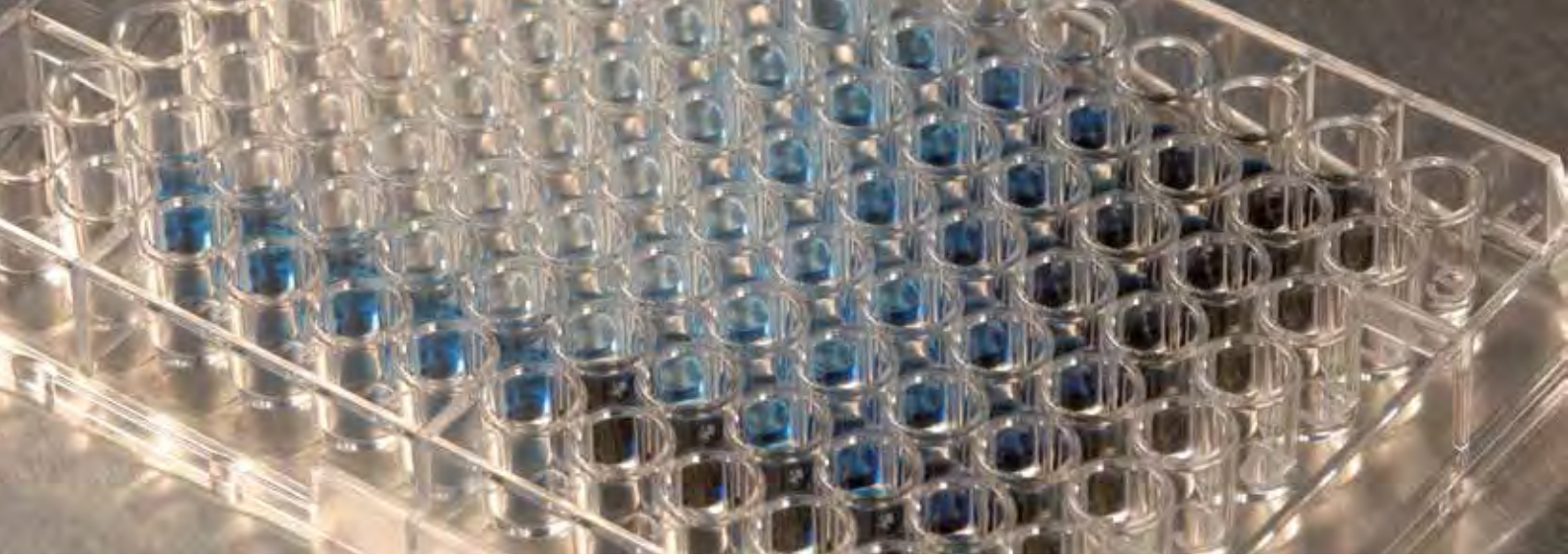
Services

- Adaption and further development of the pyrogen detection test to the specific demands of our customers
- Extension of the pyrogen detection assay to all PRRs, thus reflecting the complete human innate immune system

Photometric analysis of pyrogen test



The TLR2/6 test system is activated with its specific ligand Pam_2CysSK_4 in a dose-dependent manner. The reporter gene product (SEAP) catalyzes the hydrolysis of the substrate to a yellow end product measured photometrically at 405 nm.



VIRUS-PROTECTION ASSAY (ANTIVIRAL ASSAY, AVA)

The challenge

Pharmaceutical proteins produced by using cell cultures and medical devices derived from animal tissues must be checked for virus contaminations according to USP Pharmacopoe Europeae or ISO standards. However, viruses used in these assays pose a risk of human infection. Therefore, these assays have to be performed under strict biosafety standards up to BL2 level.

Testing for antiviral activity can be performed at the Fraunhofer IGB according to GLP (Good Laboratory Practice) standards. The lab is certified for assay types involving "Cell-based assays for the determination of biological parameters". An Antiviral Assay (AVA) is routinely used for measuring the biological activity of interferons (IFN) according to GLP. The determination of the antiviral activity of interferons is based on the induction of cellular responses in cell cultures, suppressing the cytopathic effect of the infectious virus. This can be detected quantitatively using a simple and robust photometric assay. Additionally, other viral assays such as the Tissue Culture Infectious Dose$_{50}$ (TCID$_{50}$) and the Plaque Assay are carried out according to GLP.

Process steps

Based on the extent of cytopathic effect (CPE) in the lung epithelial cell line A549, the sample activity was compared with the dose-response curve of the standard. A quantified virus titer was added to all assays. Cell controls received only cells and medium, while virus controls received virus but no test compound. Plates were incubated until the viral CPE in the virus control wells reached 100 % (mostly in 24 hours). The dilutions at which 50 % of the maximal cytopathic effect could be observed were compared and revealed the relative activity of the sample.

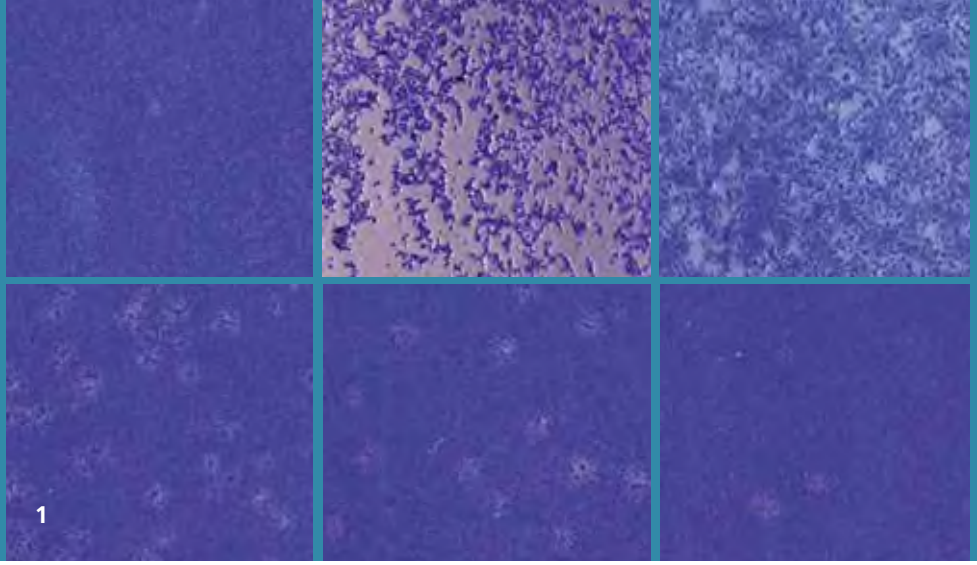
The results were expressed as EC$_{50}$ values defined as the concentration of compound achieving 50 % inhibition of the virus-reduced dye signals as compared with the uninfected cell control. The signal-to-noise ratio of an assay is the ratio between the mean dye signals of the cell controls and the virus controls. The dynamic range is defined as the ratio between the signals at the last (maximal signal) and first point in the linear range of the dose-response curve.

Specifications

- Photometrical determination of the cytopathic effect of the respective virus
- GLP certification

Services

- Testing of recombinant proteins for virus contaminations
- Determination of the titer of cytopathogenic viruses in samples under contract for customers
- Implementation of virus tests in the production process



1 *Plaque assay of Herpes simplex virus-1 (HSV-1) infected Vero B cells (staining with Coomassie).*

The Tissue Culture Infectious Dose$_{50}$ (TCID$_{50}$) and the Plaque Assay

Besides the AVA described above, we perform a variety of assays to determine the titer of cytopathogenic viruses in various samples. Quality System Assay capabilities to suit the different development and regulatory needs are available, from R&D level to certified GLP standards.

The Plaque Assay

Cytopathogenic viruses can be quantified by the number of plaques or pocks they cause on susceptible cell monolayers. Using this assay, we can screen drug compounds for plaque inhibition.

The Tissue Culture Infectious Dose$_{50}$ (TCID$_{50}$) Assay

Viruses which have cytopathic effect (CPE) can be quantitated using the TCID$_{50}$ Assay. Endpoint techniques are used for viruses which do not grow in culture, when 'Lethal Dose$_{50}$' (LD$_{50}$) or 'Infectious Dose$_{50}$' (ID$_{50}$) values must be calculated. They are also used in the case of viruses which are not cytopathic or do not produce plaques. We use several statistical methods for analyzing the data generated, e.g. Spearman-Karber analysis.

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