Joint EDQM-EPAA Event

The future of pyrogenicity testing: phasing out the rabbit pyrogen test Training

16 February 2023

Qualification of Peripheral Blood Mononuclear Cells (PBMCs)

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GSK, Rosia

The future of pyrogenicity testing - Training session
February 16th , 2023

This work is sponsored by GlaxoSmithKline Biologicals SA. Liliana Alleri is employed by the GSK group of companies

gsk.com

Agenda

- Pharmacopoeial requirements for donors' qualification
- Overview of the donors' qualification procedure in GSK:
  I. for method A and B
  II. for method C

Qualification of blood donors is necessary to guarantuee cells' responsiveness to pyrogens in the test

❖ Two main requirements in Ph.Eur. Chapter 2.6.30:

1. Cells (derived from single donation or pools of them) need to be stimulated with at least 4 concentrations of Reference Standard Endotoxin. The dose-response curve has to meet the following acceptance criteria:
   - The regression of responses (appropriately transformed if necessary) on log10 dose shall be statistically significant ($p < 0.01$);
   - The regression of responses on log10 dose must not deviate significantly from linearity ($p > 0.05$).
2. The chosen test system has to detect, in addition to bacterial endotoxin, non-endotoxin pro-inflammatory or pyrogenic contaminants. At least 2 non-endotoxin ligands for toll-like receptors should be included to validate the system.

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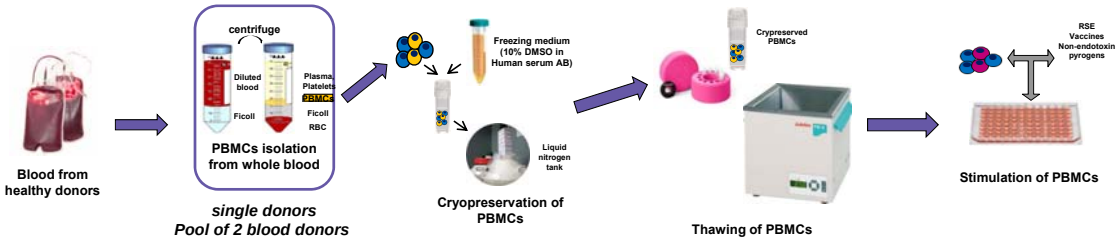
Human Peripheral Blood Mononuclear Cells (PBMC) are used in GSK



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The donors' qualification procedure was set up based on the MAT method applied to the product

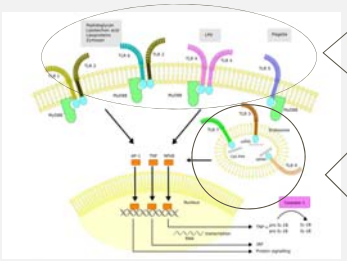
Method	Principle of method	Stimuli used to qualify donors
Quantitative test (METHOD A)	• Comparison of the tested preparation with a dose-response curve of standard endotoxin (used as Reference)	• RSE and 2 non-endotoxin pyrogens.
Semi-quantitative test (METHOD B)	• Comparison of the tested preparation with standard endotoxin (used as Reference)	
Reference lot comparison test (METHOD C)	• Comparison of the tested preparation with a validated reference lot of that preparation	• Validated Reference lot of the preparation used in the test

Donors' qualification procedure for method A and B

RSE, FSL-1 and R848 stimuli were selected for the donor qualification procedure of MAT method A and B

**InvivoGen product*

- PBMCs' response assessed on the following Endotoxin and non-endotoxin stimuli:
  - **RSE** (Reference Standard Endotoxin), agonist for TLR4
  - **FSL-1*** synthetic diacylated lipoprotein derived from Mycoplasma salivarium, agonist for TLR2/TLR6 heterodimer.
  - **R848*** a small molecular weight imidazoquinoline compound, agonist for TLR7/8



Toll-like receptors		Ligands
TLR4	RSE	LPS
TLR-2, -6, -1 Heterodimer complexes	FSL-1	LTA, lipoproteins, mycobacteria, spirochetes, fungal, viral proteins
TLR5		Flagellin
TLR3		Double stranded (ds) RNA
TLR -7, -8	R848	Single stranded (ss) RNA
TLR9		DNA containing unmethylated CpG motifs

Surface located

Intracellular

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Procedure for donor qualification: PBMCs' response to RSE, R848 and FSL-1 is assessed

Stimulation of cells - Day 1

- PBMC are stimulated preparing RSE, FSL-1 and R848 dose response curves of 8 points (3 repetitions per dilution point), 2-fold steps, starting from a RSE, FSL-1 and R848 concentration of 0,448 EU/ml, 0,32 ng/ml and 0,896 µg/ml, respectively.

Plate layout

	RSE			FSL-1			R848					
	1	2	3	4	5	6	7	8	9	10	11	12
A	0,64 EU/ml			0,32 ng/ml			0,896 µg/ml			B		
B	0,32 EU/ml			0,16 ng/ml			0,448 µg/ml					
C	0,16 EU/ml			0,08 ng/ml			0,224 µg/ml					
D	0,08 EU/ml			0,04 ng/ml			0,112 µg/ml					
E	0,04 EU/ml			0,02 ng/ml			0,056 µg/ml			b		
F	0,02 EU/ml			0,01 ng/ml			0,028 µg/ml					
G	0,01 EU/ml			0,005 ng/ml			0,014 µg/ml					
H	0,005 EU/ml			0,0025 ng/ml			0,007 µg/ml					

Dose-response curves for each stimulus

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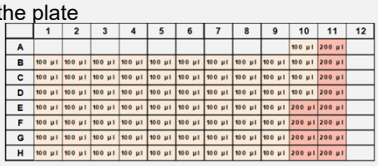
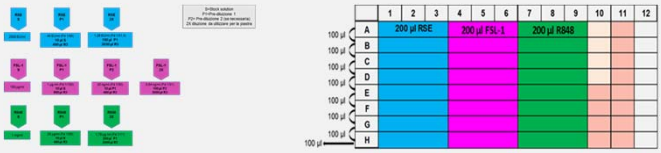
Procedure for donor qualification: PBMCs' response to RSE, R848 and FSL-1 is assessed

Stimulation of cells - Day 1

- Dispensation of growth medium onto the plate



- Preparation of stimuli (2X concentration), dispensation of that stimuli onto the plate and execution of serial dilutions.



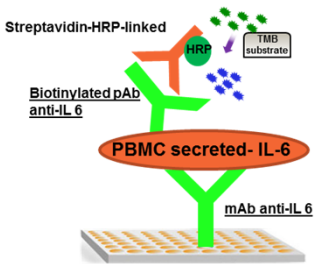
- Thawing of the cells, cells counting and dispensation of the cell suspension (100 µL equal to 1.0 x 10^5 cells/well, 1x 10^6 /ml cell suspension) onto the plate
- Incubation of the stimulation plate at +37°C ± 2°C (or ± 1°C) and CO2 5% ± 2% (or ± 1% or 0.5%) for 22 ± 1 hours

Procedure for donor qualification: IL-6 specific ELISA

IL-6 specific ELISA – Day 2

1. Coating and saturation of ELISA plates
2. Incubation of cell supernatants
3. Incubation with biotin-conjugated goat anti-Human IL-6 polyclonal antibody
4. Incubation with horse radish peroxidase-conjugated Streptavidin.

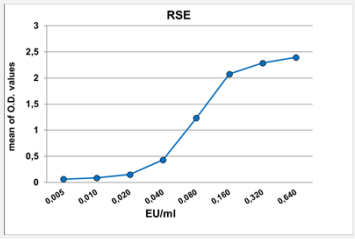
An IL-6 curve is prepared the day of the ELISA assay (positive control).
Concentration range: from 40.000 to 9.766 pg/ml

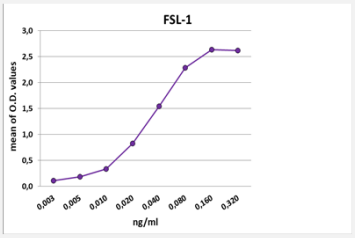


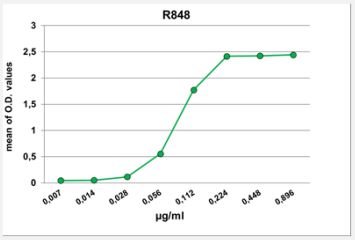
IL6 detection in PBMCs supernatants by sandwich ELISA

PBMCs respond to both endotoxin and non-endotoxin pyrogens

- Comparable response observed in single donors and pool of 8 donors
- Limit of Detection (LOD) calculated as concentration corresponding to *cut-off value**
  - **RSE (Reference Standard Endotoxin) LOD: 0.01 EU/ml**
  - **FSL-1 (mycoplasmal lipopeptide) LOD: 0.005 ng/ml**
  - **R848 (imidazoquinoline) LOD: 0.014 µg/ml**







**cut-off value* was calculated by applying the following formula: x + 3*s where x is the mean of the 4 replicates for the responses to the cell blank, S is the correlated standard deviation

Procedure for donor qualification: parameter and acceptance criteria

Parameter	Acceptance criteria
Negative control (i.e. Cells only w/o stimuli)	Mean response of O.D. (Optical Density) values corresponding to R0 replicates (cells stimulated only with culture medium) needs to be ≤ 0.2;
Positive control (IL-6 curve)	Mean response of O.D. values corresponding to the replicates of the first point of the IL-6 curve needs to be ≥ 1.0
Goodness of the RSE dose response curve	1. Coefficient correlation of the 4-PL mathematical model (R2) has to be higher than 0.950; 2. At least three dilution points of the full dose-response curve has to be in the linearity part of the curve.
Reactivity to non-endotoxin pyrogens (i.e. FSL-1 and R848)	The donor (or pool of donors) needs to respond to stimuli of non-endotoxin pyrogens

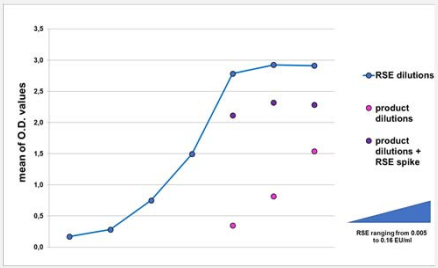
***For method B, only the reactivity of the cells to RSE is assessed**

The parameters assessed for RSE in the qualification procedure rely on the type of RSE dose-response curve used in the method final layout

Quantitative and Semi-Quantitative Tests: final layout

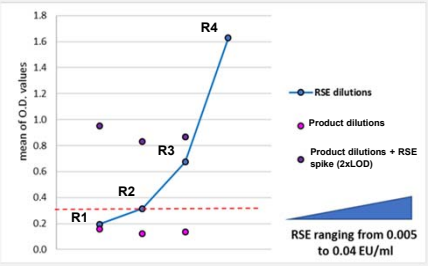
Quantitative Test

- ✓ 8 RSE concentrations
- ✓ 3 Product dilutions: optimum dilution* (Sol.A), ½ Sol.A (Sol. B), ½ Sol B
- ✓ RSE spikes at a concentration corresponding to ½ dose of RSE curve



Semi-Quantitative Test

- ✓ 4 RSE concentrations
- ✓ 3 Product dilutions: optimum dilution*, ½ MVD, MVD
- ✓ RSE spikes at a concentration corresponding to 2xLOD



Optimum dilution = first dilution of the product for which the endotoxin recovery (in the test for interfering factor) is centered within the validity range 50-200%

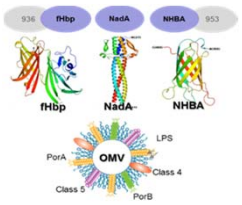
Donors' qualification procedure for method C: The example of Bexsero*

** Bexsero is a trademark owned by the GSK group of companies.*

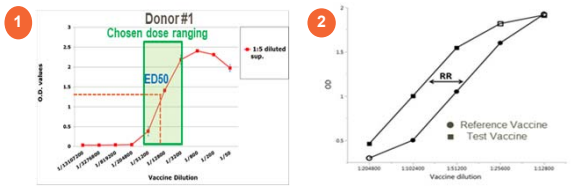
Procedure for donors' qualification in Bexsero: a dual scope

❖ Main objectives of the donors' qualification procedure in MAT applied to Bexsero are:

1. Assess responsiveness of the cells to the Bexsero
2. Select the optimal dilution range and cell supernatant condition for each donor to be used in the final test layout.



BEXSERO is constituted by three recombinant protein antigens and the Outer Membrane Vesicles (OMV) from serogroup B N. meningitidis adsorbed onto aluminum hydroxide.



1. Qualification step. A full dose-response curve of Bexsero is generated and analysed for each donor.
2. MAT release assay: Five two-fold serial dilutions of the Bexsero Reference and Test lots are assessed in the final layout.

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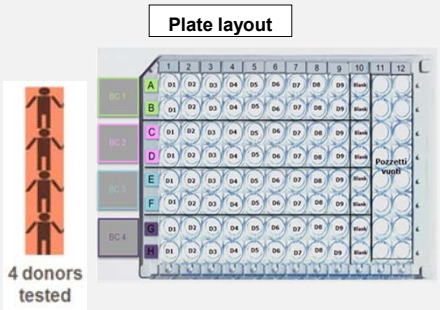
Procedure for donors' qualification: PBMCs' response to a Bexsero qualified lot (Reference lot for the assay) is assessed

Stimulation of cells - Day 1

❖ Each PBMCs' donor is stimulated with a Bexsero dose-response curve of 9 points (2 repetitions per dilution point), 4-fold steps, starting from 1/50 dilution of the vaccine.



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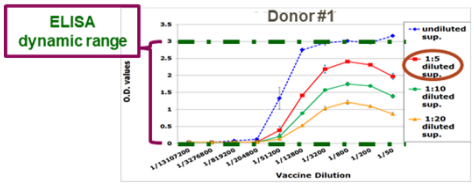
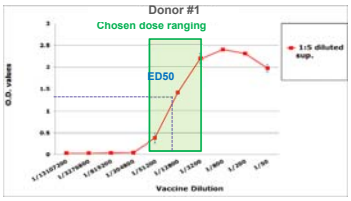
Procedure for donor qualification: results elaboration

- The ELISA assay is performed on undiluted, 1:5 diluted, 1:10 diluted and 1:20 diluted cellular supernatants to select the dose-response curve which cover, in terms of O.D. values, the dynamic range of the ELISA test.



- The selected dose-response curve for the donor is assessed by means of a 4-parameter logistic model with fixed upper asymptote.
- Once ED50 is estimated, 2 dilutions before the estimated ED50 and 3 dilutions (2-fold step) after it are selected to be used in the final assay format.



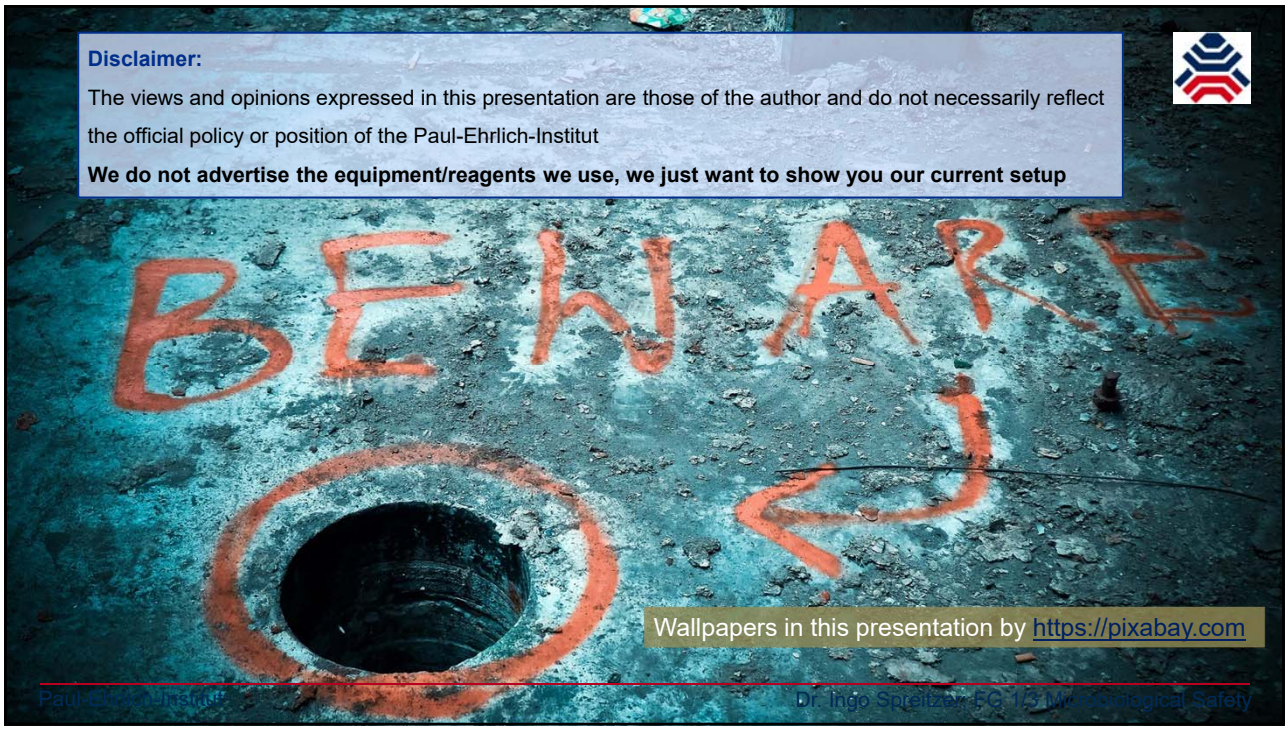
Estimated ED50: dilution 1/15896.7
Determined dose ranging for the donor: 1/3200 – 1/51200

Procedure for donor qualification: parameter and acceptance criteria

Parameter	Acceptance criteria
Negative control (i.e. Cells only w/o stimuli)	Mean response of O.D. (Optical Density) values corresponding to R0 replicates (cells stimulated only with culture medium) needs to be ≤ 0.2;
Positive control (IL-6 curve)	Mean response of O.D. values corresponding to the replicates of the first point of the IL-6 curve needs to be ≥ 1.0
Goodness of the Bexsero dose response curve	the analysis on the 4-PL model of the curve is conducted

Conclusions

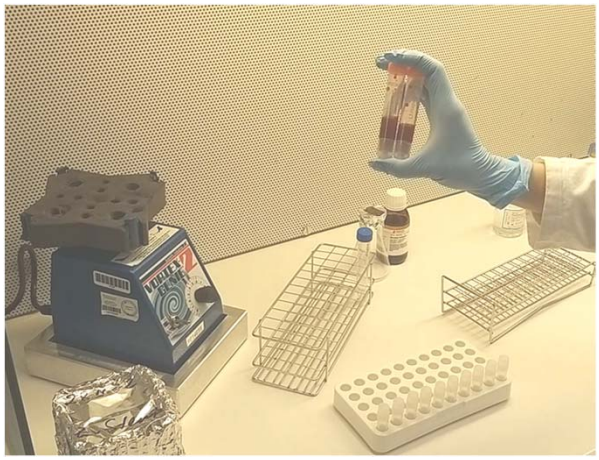
- A Donors' qualification procedure method-dependent was set up in GSK
  1. In Method A and B cells are qualified assessing the responsivness of the cells to:
     - ✓ RSE -> criteria are in force for method A on the 4-PL curve while only the reactivity of the cells to RSE is assessed in Method B;
     - ✓ FSL-1 and R848 -> reactivity of the cells to both non-endotoxin stimuli is assessed.
  2. In Method C, the cells are qualified assessing the responsiveness of the cells to a validated Reference lot of the preparation used in the test. A dual scope for this qualification procedure:
     - ✓ Assess responsiveness of the cells to the preparation used in the test
     - ✓ Select the optimal dilution range and cell supernatant condition for each donor to be used in the final test layout



Cryopreservation of human whole blood PEI

- **Blood donation: Legal, ethical and safety regulations!**

- Blood withdrawal system:
- Sarstedt: S-Monovette® Plasma/**Li-Heparin** 9 ml
  no Citrate, no EDTA!
- Invert Monovette gently 5 times after blood collection (for better anticoagulation)



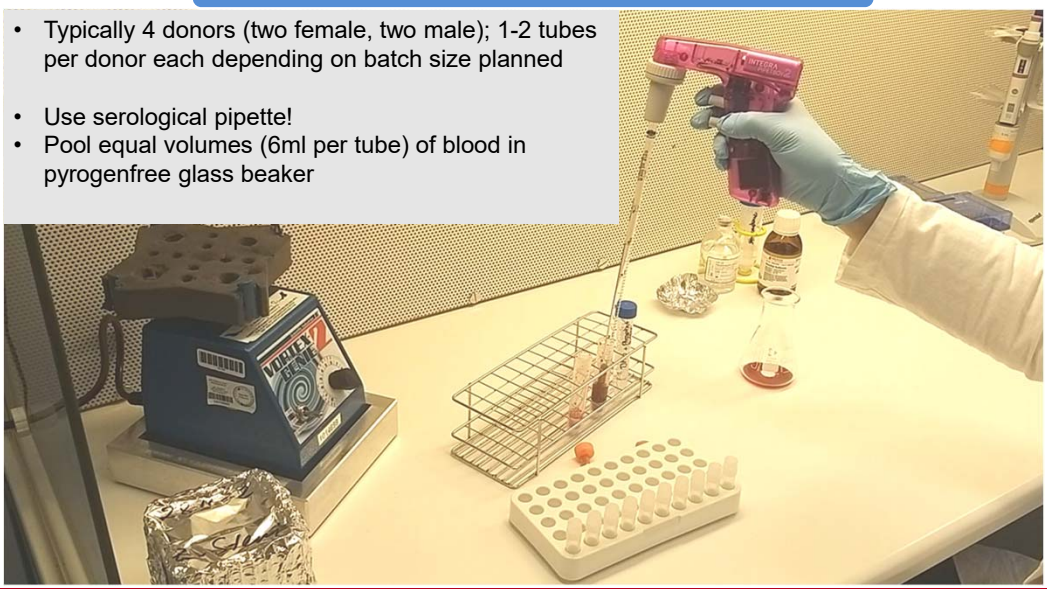
Use serological pipettes until freezing!

- At this stage the use of other pipettes / steppers / tips / Combitips with small inner tip Diameter can **damage** the cells by shear forces

Cryopreservation of human whole blood PEI

- Typically 4 donors (two female, two male); 1-2 tubes per donor each depending on batch size planned

- Use serological pipette!
- Pool equal volumes (6ml per tube) of blood in pyrogenfree glass beaker

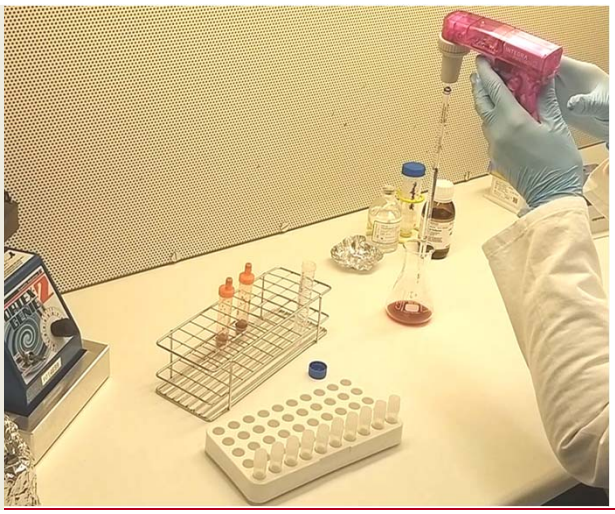


Cryopreservation of human whole blood PEI

- Prepare Cryoprotective solution: **all solutions at oom temperature!**, 1:5 dilution of DMSO
  1 part DMSO cell culture grade
  4 parts Soerensen phosphate buffer pH 7,0 (Endotoxin <0,01 IU/ml)
  = 20% DMSO (Cryoprotectant solution)

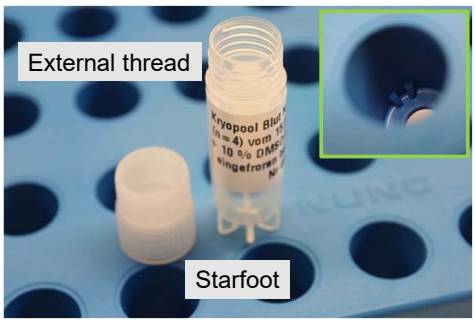
- use a cell culture serological pipette to add **SLOWLY** the same volume of the 20% Cryoprotectant solution (rinse down inner wall of glass beaker) under gentle swirling of the blood (1:2 dilution of blood in 10% DMSO)

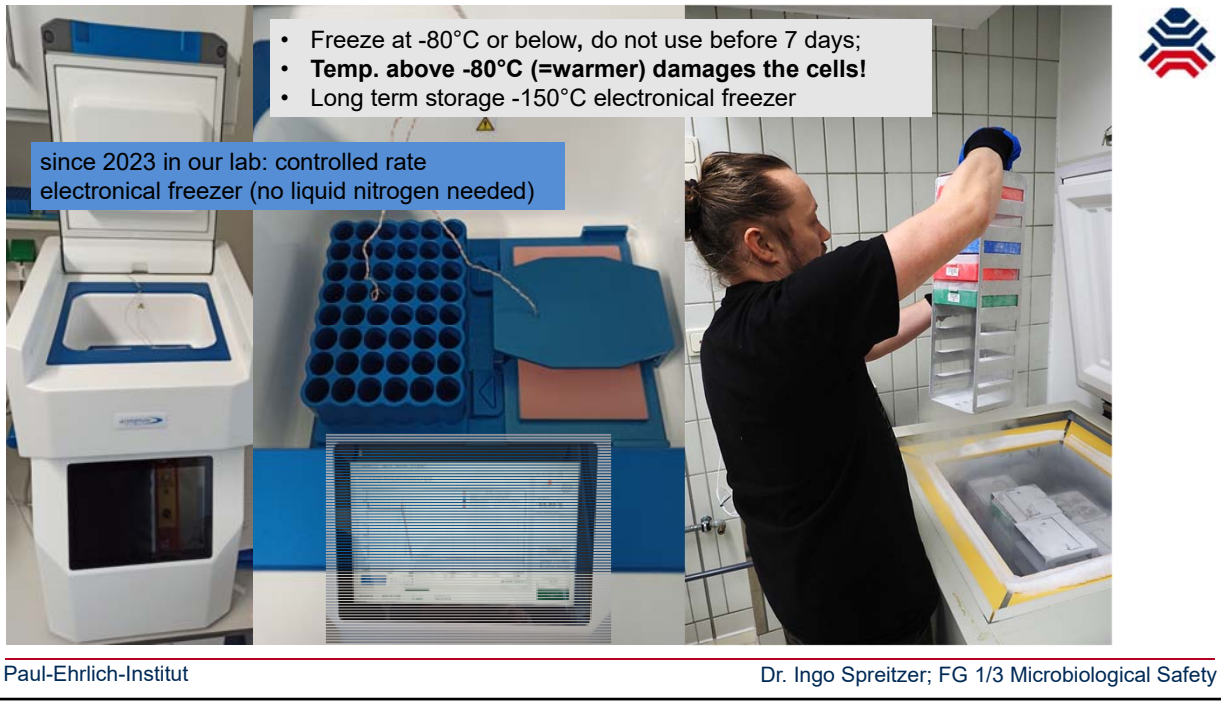
- Let for **1h** at room temperature



Cryopreservation of human whole blood PEI

- In between prepare cryovials (2ml, external thread, starfoot (will not twist in starfoot stand while opening and closing); labeling with preprinted Cryotags (they stick even at ultra low temperatutes)
- After 1h, open vials, aliquot with serological pipette 1ml per Cryovial, close vials





- Freeze at -80°C or below, do not use before 7 days;
- **Temp. above -80°C (=warmer) damages the cells!**
- Long term storage -150°C electronical freezer

since 2023 in our lab: controlled rate electronical freezer (no liquid nitrogen needed)

DEPARTMENT
INFECTIOUS DISEASES

Freezing and Thawing of PBMC

Marilena P. Etna

EDQM-EPAA MAT Training Session
Training session organised by the EDQM, Council of Europe, and the European Partnership for Alternative Approaches to Animal Testing (EPAA), European Commission

16 February 2023, Brussels, Belgium

Why should you freeze PBMC?

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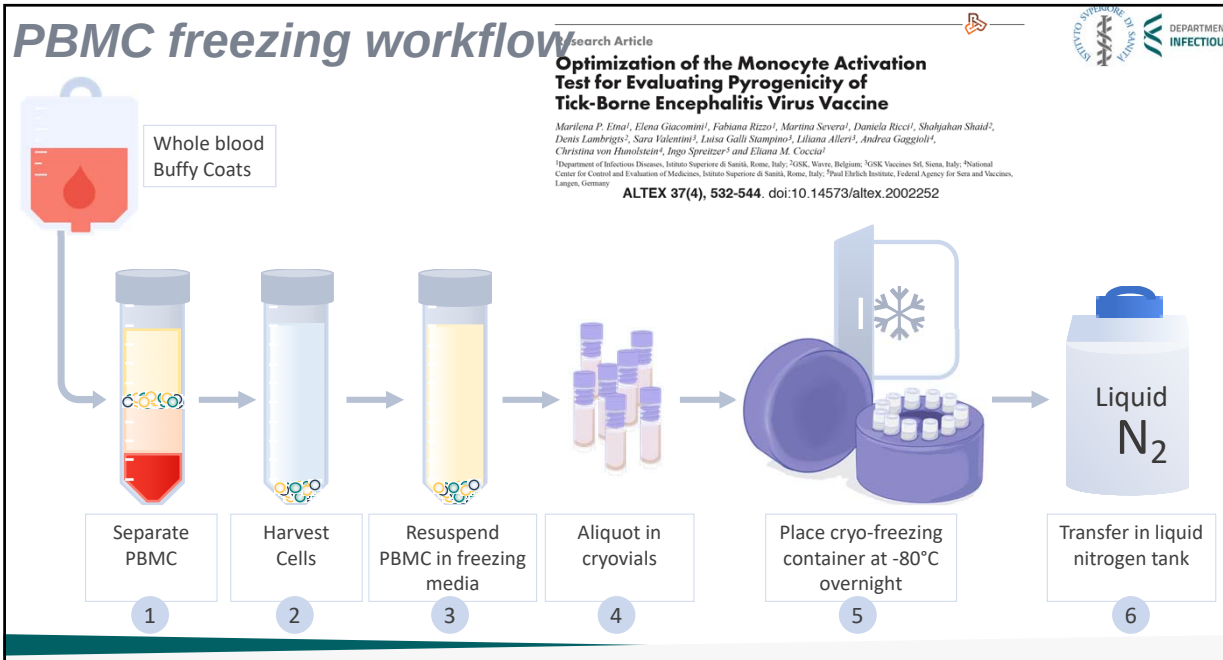
1. **To preserve qualified cells**
2. **To ensure the long-term use of a cell source with robust results**
3. **To provide a backup in case of issues during cell manipulation**

Creation of cell banks

Before starting…

1. **Ensure aseptic conditions while freezing cells**
2. **Define the optimal number of cells per vial to avoid low cell viability as well as unwanted cell clump (for PBMC 5 - 20 x 10^6 cells/mL)**

PBMC freezing workflow



Research Article

Optimization of the Monocyte Activation Test for Evaluating Pyrogenicity of Tick-Borne Encephalitis Virus Vaccine

Marilena P. Etna[1], Elena Giacomini[1], Fabiana Rizzo[1], Martina Severa[1], Daniela Ricci[1], Shahjahan Shaid[2], Denis Lambrigts[2], Sara Valentini[3], Luisa Galli Stampino[3], Liliana Alleri[3], Andrea Gaggioli[4], Christina von Hunolstein[4], Ingo Spreitzer[5] and Eliana M. Coccia[1]

[1]Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy; [2]GSK, Wavre, Belgium; [3]GSK Vaccines Srl, Siena, Italy; [4]National Center for Control and Evaluation of Medicines, Istituto Superiore di Sanità, Rome, Italy; [5]Paul Ehrlich Institute, Federal Agency for Sera and Vaccines, Langen, Germany

ALTEX 37(4), 532-544. doi:10.14573/altex.2002252

Tips & Tricks [I]

DEPARTMENT INFECTIOUS DISEASES

Home-made

⇨ Fetal Bovine Serum (FBS) + Cryoprotectant agent* (DMSO)

⇨ Human Serum (HS) + Cryoprotectant agent* (DMSO) [Animal-free]

*avoids: ice crystal formation/ osmotic stress/ membrane damage

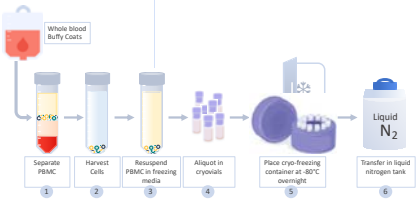
3

Resuspend PBMC in freezing media

Commercially ready-to-use

⇨ With Fetal Bovine Serum (FBS)

⇨ Synthetic [Animal-free]



Tips & Tricks [II]

DEPARTMENT INFECTIOUS DISEASES

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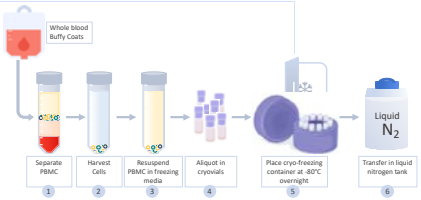
Place cryo-freezing container at -80°C overnight

Controlled freezing rate

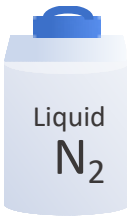
Slow freezing is critical to ensure cell viability and integrity

⇨ Controlled-rate freezer

⇨ Isopropanol freezing container



Tips & Tricks [III]



Transfer in liquid nitrogen tank

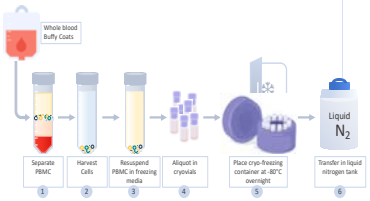
6

Storage & Recording

⇨ Very low temperature (-135°C to -196°C) for long-term storage

⇨ Alcohol/liquid nitrogen-resistant markers or printed cryo-labels

⇨ Inventory of cell bank



Reagents & Equipment [critical]

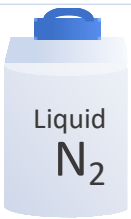
Cryopreservation Medium

Serum (FBS or HS) Cryoprotectant

Ready-to-use freezing media

Cryovials resistant to very low temperature

Freezing containers



Liquid nitrogen tank

PBMC Thawing: before starting…

1. **Ensure aseptic conditions while thawing cells**
2. **Warm thawing medium of choice in a 37°C water bath**
3. **Put warmed thawing medium in a 15 ml conic tube (sufficient for one vial of PBMC)**

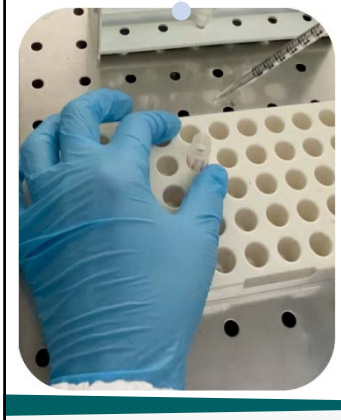
Rapid thawing helps to minimize any impact on cell recovery

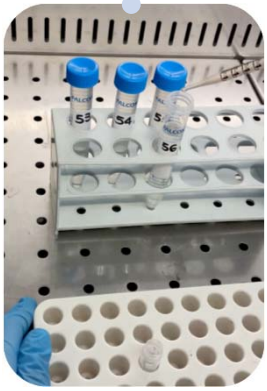
PBMC thawing workflow [I]

1. Remove the cryovial from liquid nitrogen storage tank and place it in dry ice (no exposure to room temperature until thawing)
2. Place cryovial in 37°C water bath and thaw cells by swirling it until a little piece of ice remains
3. Wipe the outside of the cryovial with 70% ethanol or isopropanol before transferring into the biosafety hood

PBMC thawing workflow [II]

4 Add 1 ml of thawing medium (warm) **dropwise** directly into the cryovial then slowly transfer cell suspension in the conic tube pre-filled with warm thawing medium

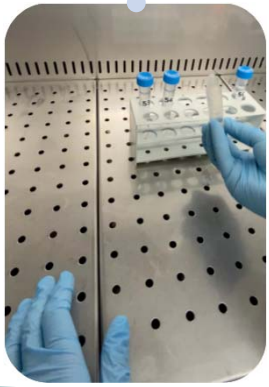




5 Wash the cryovial with an additional 1 ml of thawing medium and add it to the conic tube

PBMC thawing workflow [III]

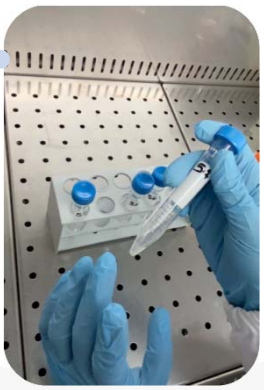
6 Mix by inverting the tube several times and then centrifuge at room temperature at 300 g for 10 minutes



7 Discard the supernatants and leave a small amount of medium to ensure the cell pellet is not disturbed

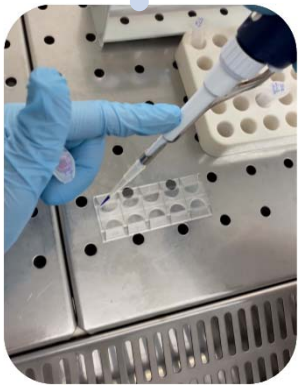
8 Resuspend the pellet by gently flicking the tube and then add thawing medium and repeat the washing step

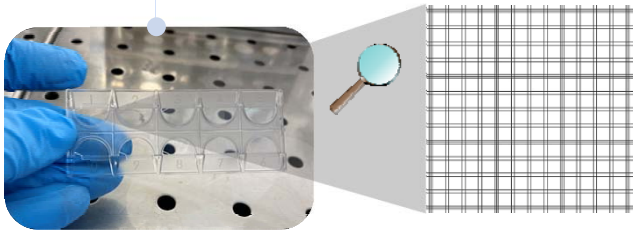
9 Repeat action n°7 and then add an established volume of cell culture medium. Take a small aliquot for cell viability count



PBMC thawing workflow [IV]

10 Dilute cell suspension with trypan blue exclusion dye and, by using a hemocytometer, count 3 small squares of the chamber to calculate the cell number





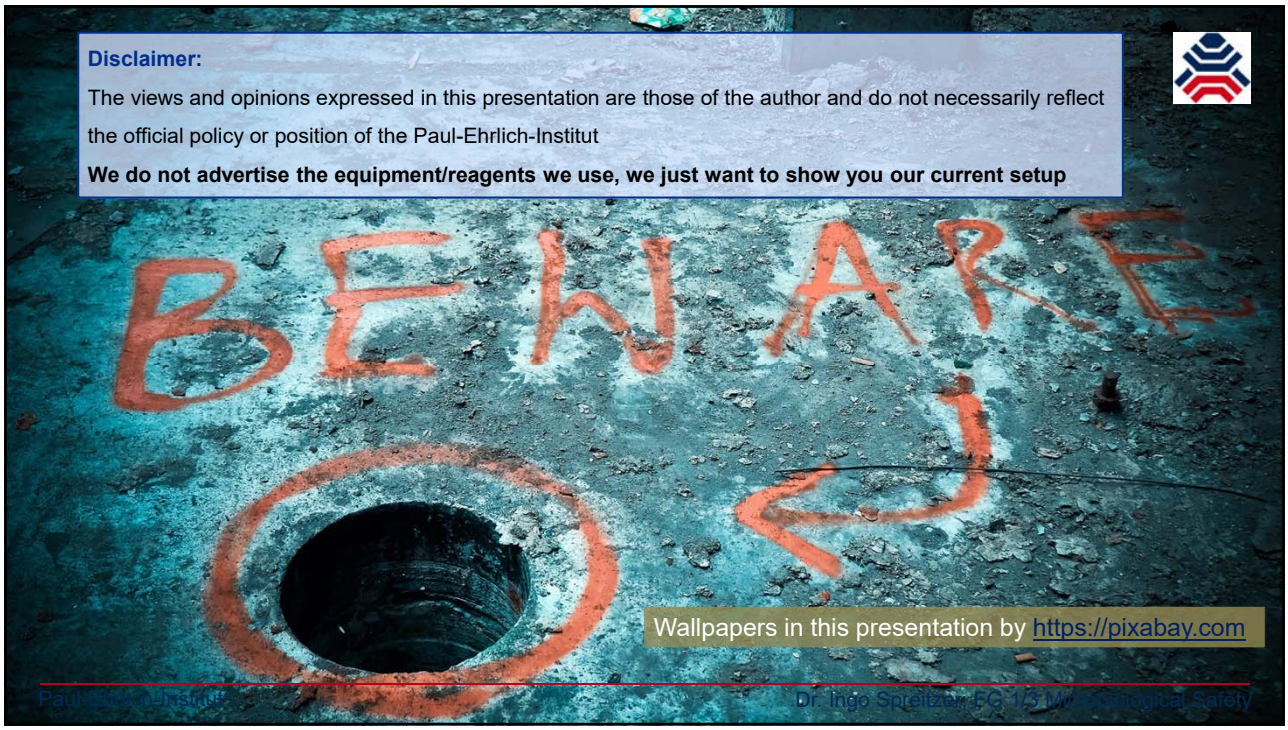
Counting Chamber

Counting Grid

12 Adjust cell volume to the desired cell concentration with cell culture medium

Cells are now ready for use in downstream applications

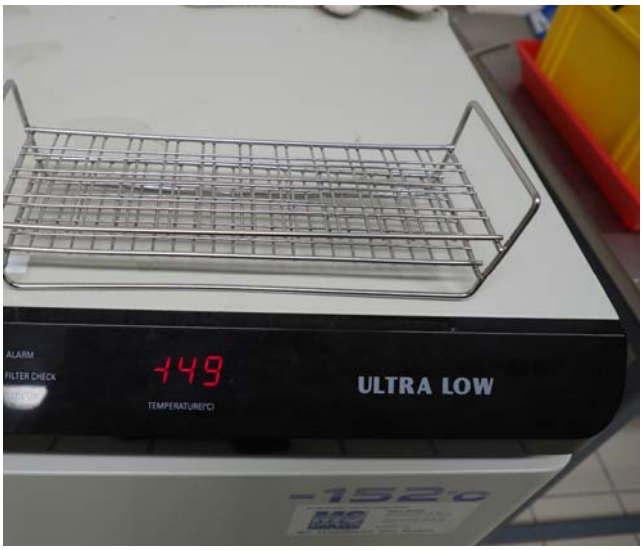
Questions?

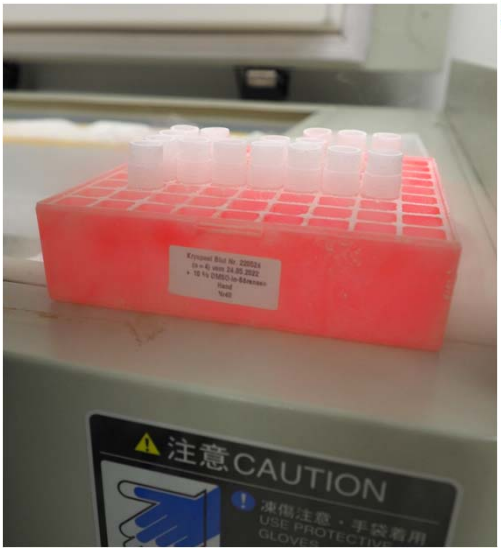


Thawing procedure Cryoblood PEI

- **Critical step!**
- The 10% DMSO is needed for cryoprotection, but toxic after thawing. After thawing you have to dilute as soon as possible (<= 5 min) to a DMSO-concentration below 1%
- Therefore you have to **preprepare the incubation plate** (cell-culture media in the wells of the 96well-plate) **before**, to be able to add the Cryoblood (still 10% DMSO!) immedeately. The PEI-setup (1:13 dilution of the Cryoblood) results in a final DMSO-concentration of 0.77%.

  Other MAT-manuals propose a direct dilution of cryopreserved cells (typically 10% DMSO too) with Cell-culture media in a pyrogen-free beaker (or Falcon-tube) before pipetting on the plate, or a washing step.
- Take number of vials needed from the freezer
- For our 2ml Cryovials filled with 1ml Cryoblood the thawing time in a 37°C-Incubator is exactly 12 minutes in a thin metal wire tube rack (use wide open racks for good temperature transport).
  We do not recommend water bathes, they are a frequent source of contaminations
- After 12 minutes take rack out of the 37°C Incubator, and inverse rack (fix vials with hand) gently several times upside down to remix the vial content



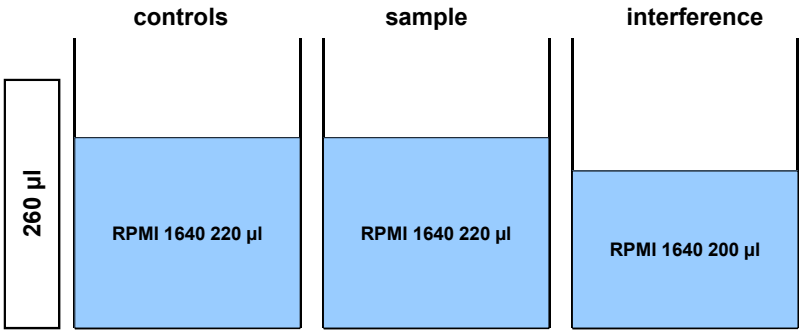


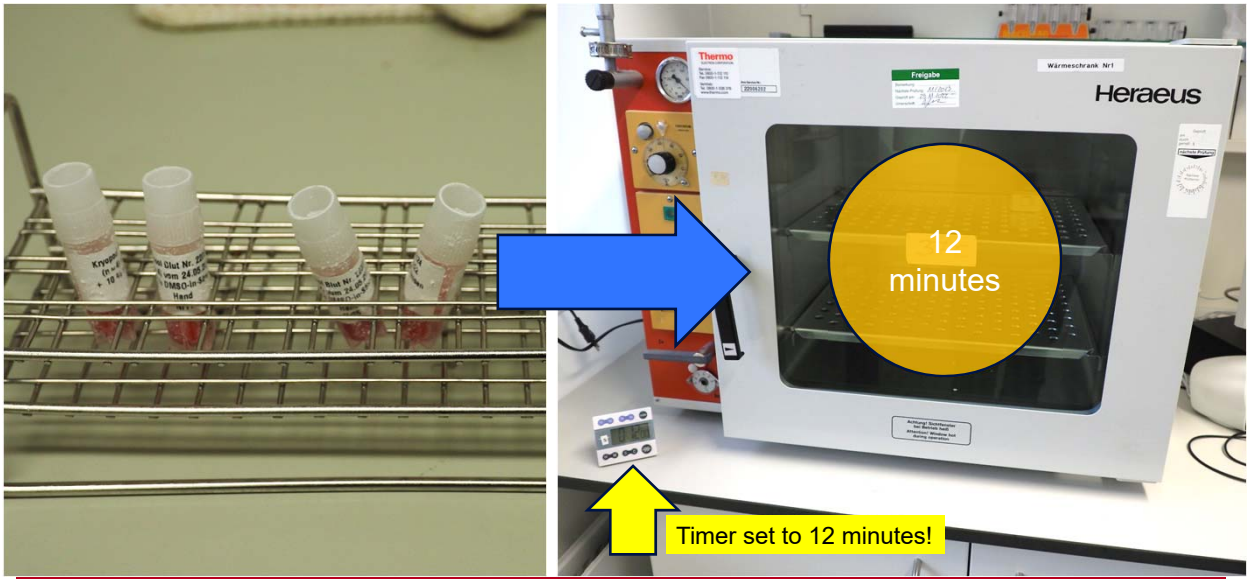
Before or during thawing add cell culture medium to incubation plate

RPMI 1640,
220µl (sample well)
or 200 µl
(spiked sample well)

Prepare incubation plate

1. **RPMI 1640**
2. **Cryoblood (now DMSO 10% is diluted below 1%, time critical!)**
3. **control or sample** (resulting 1/13 sample dilution avoids monocyte damage
4. **Spike (PPC)** (Spike latest diminishes sample/spike interference)



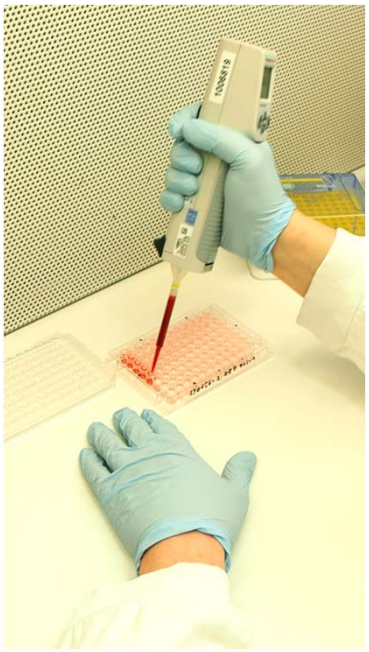


Exactly after 12 min

Inverting!

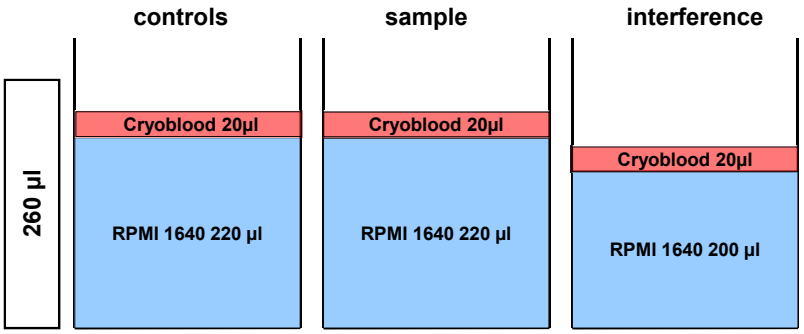
Add thawed cryoblood within 5 minutes to RPMI

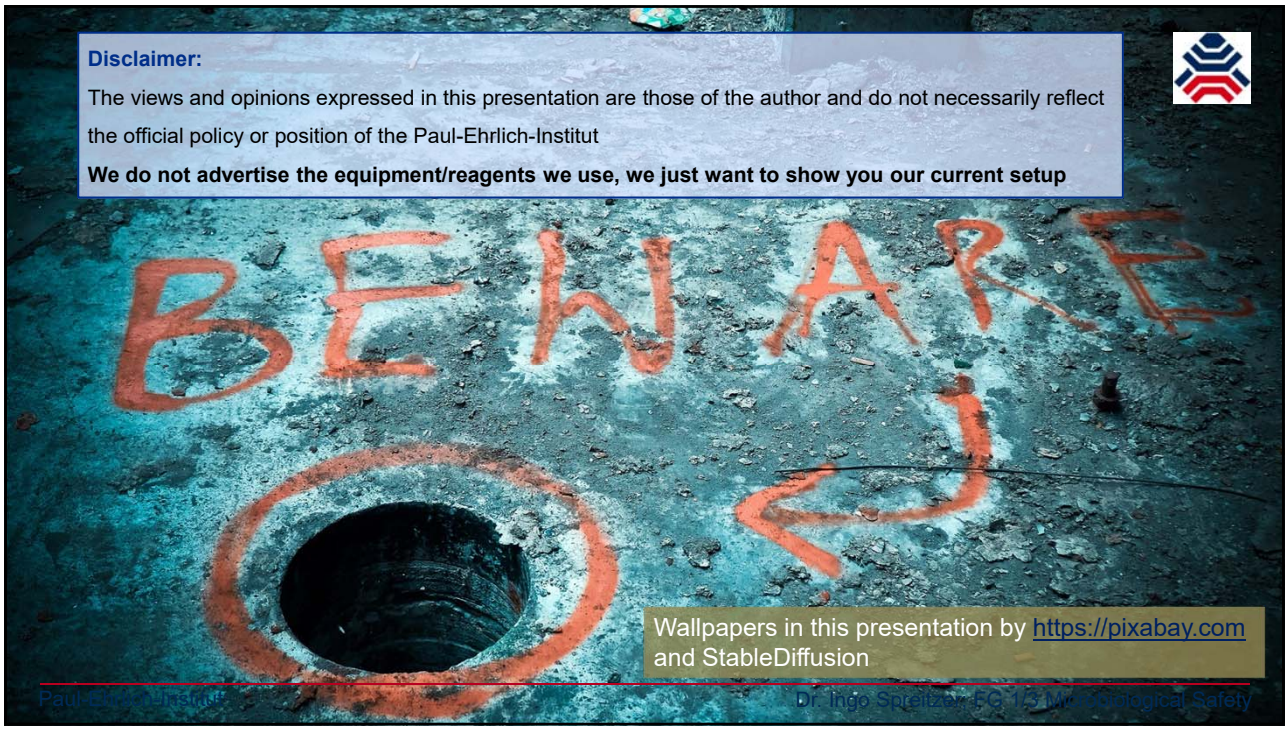
- From now you can handle the Cryoblood with all kinds of Pipettes / tips of suitable quality



Cryoblood diluted in plate, ready for main assay

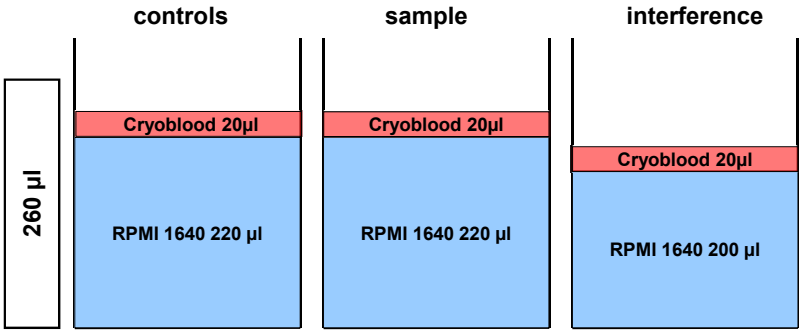
1. **RPMI 1640**
2. **Cryoblood** (now DMSO 10% is diluted below 1%, time critical!)
3. **control or sample** (resulting 1/13 sample dilution avoids monocyte damage
4. **Spike (PPC)** (Spike latest diminishes sample/spike interference)





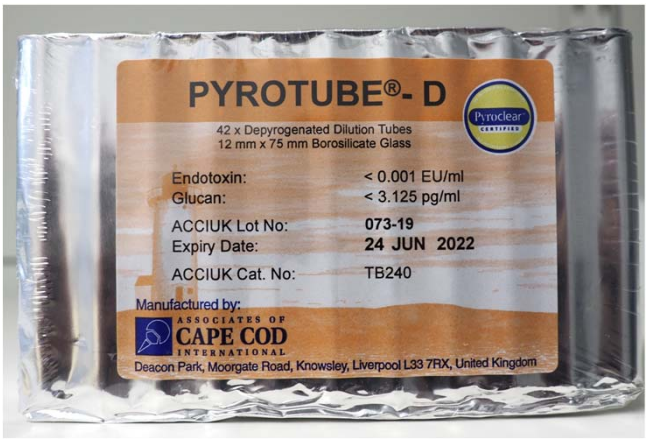
Preparation of the assay PEI
Steps 1 and 2 finished

1. **RPMI 1640**
2. **Cryoblood** (now DMSO 10% is diluted below 1%, time critical!)
3. **control or sample** (resulting 1/13 sample dilution avoids monocyte damage
4. **Spike (PPC)** (Spike latest diminishes sample/spike interference)



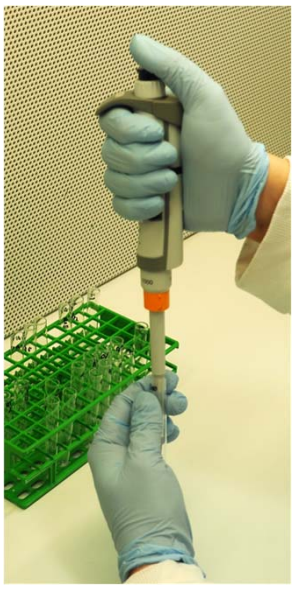
Preparation of LPS, NEP and samples

LPS dilutions in endotoxin-free Borosilicate tubes
(BET consumables)



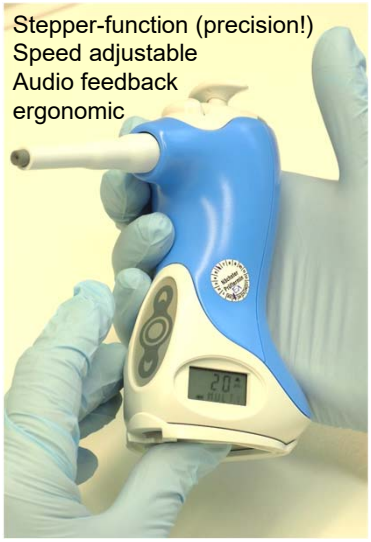
Preparation of Endotoxin, NEP and samples

Endotoxin and most other stimuli need vortexing, Flagellin not!

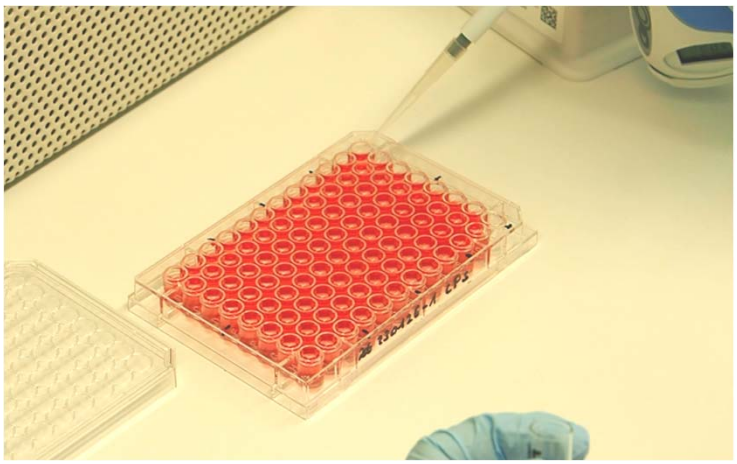


Benefits of semi-automation

Stepper-function (precision!)
Speed adjustable
Audio feedback
ergonomic

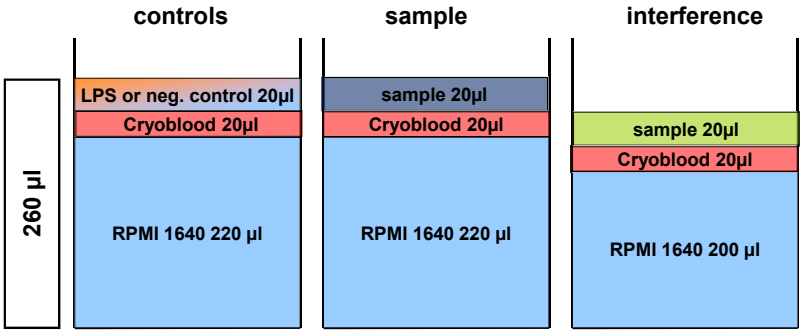


Addition of 20µl control / sample (4 wells each)

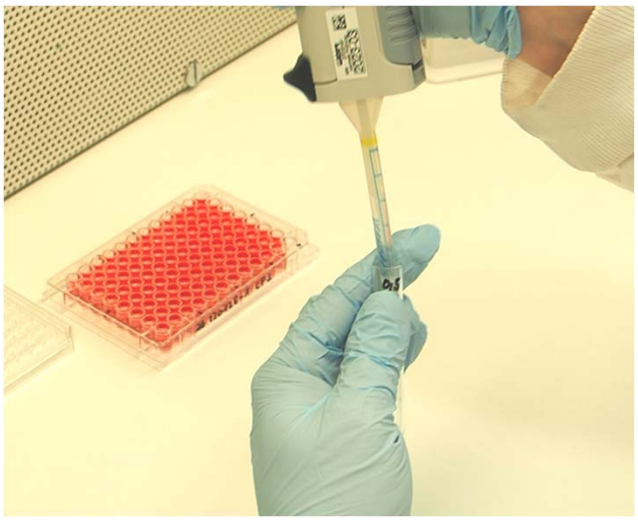


Preparation of the assay PEI
Steps 1, 2 and 3 finished

1. **RPMI 1640**
2. **Cryoblood** (now DMSO 10% is diluted below 1%, time critical!)
3. **control or sample** (resulting 1/13 sample dilution avoids monocyte damage)
4. **Spike (PPC)** (Spike latest diminishes sample/spike interference)



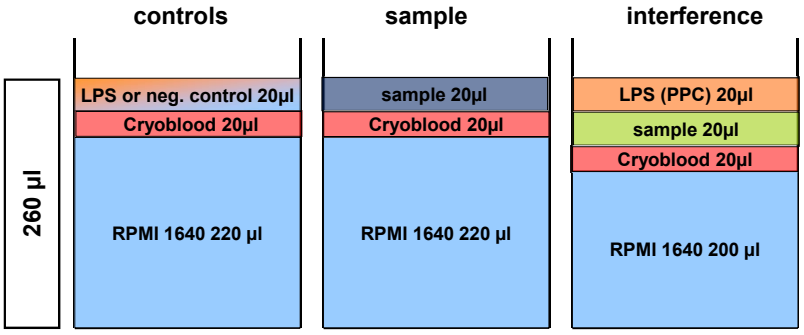
Addition of 20µl Spike (PPC); stepper for multiple wells



Preparation of the assay PEI

Steps 1-4 finished, but added volume of steps 2-4 too small for mixing -> mixing needed!

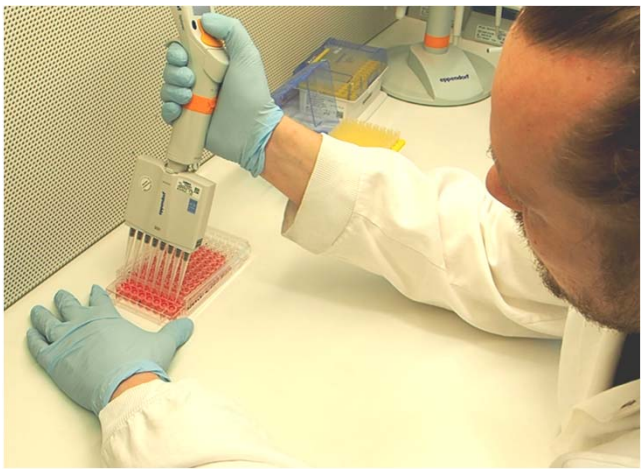
1. **RPMI 1640**
2. **Cryoblood** (now DMSO 10% is diluted below 1%, time critical!)
3. **control or sample** (resulting 1/13 sample dilution avoids monocyte damage
4. **Spike (PPC)** (Spike latest diminishes sample/spike interference)



Steps 1-4 finished, but added volume of steps 2-4 too small for mixing -> mixing needed!

Automated 8 Channel-Pipette, 5 x mixing of 96 wells at defined speed

Save the Thumb of your technician!

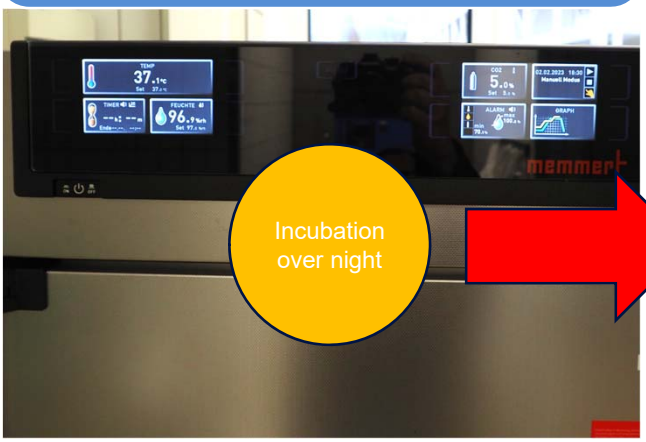


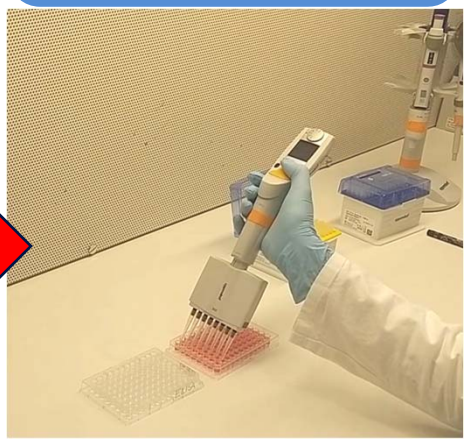
Put the incubation plate into a cell culture breeder over night

37°C 5% CO_2 (if not HEPES-buffered)

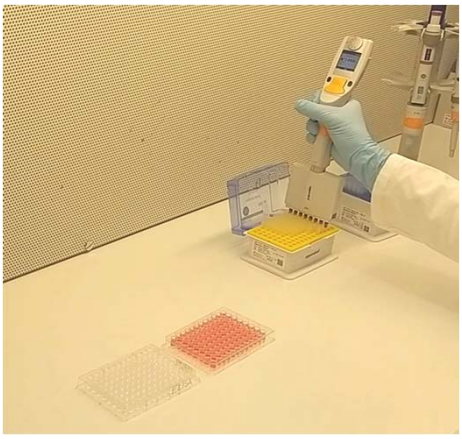
Mix supernatants 5x before transfer to ELISA!

save the Thumb…





Transfer mixed supernatant to ELISA-Plate



Perform ELISA and measurement

im possible

Acknowledgment:
The PEI
Prof. Isabelle Bekeredjian-Ding
Dr. Oleg Krut
Dr. Josephine Hubloher
Björn Becker
Anna Grothaus

The BET-Working Party
The EDQM
The EU
The EPAA

Prof. Thomas Hartung

DEPARTMENT
INFECTIOUS DISEASES

Handling of cells in the MAT assay

Mix with Reagent

Marilena P. Etna

EDQM-EPAA MAT Training Session

Training session organised by the EDQM, Council of Europe, and the European Partnership for Alternative Approaches to Animal Testing (EPAA), European Commission

16 February 2023, Brussels, Belgium

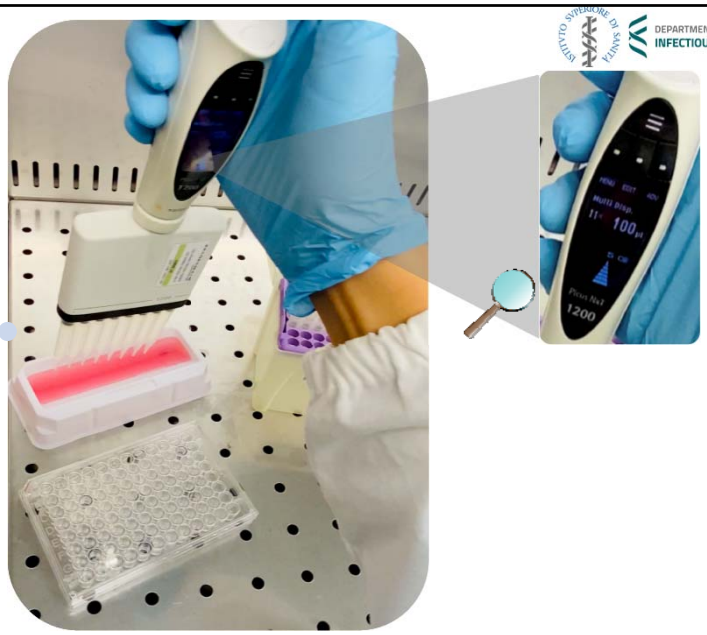
Useful equipment

Electronic Pipettes

Mechanical Multi-channel Pipettes

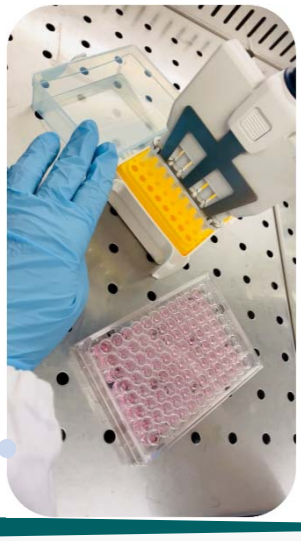
Use of multichannel pipettes for the preparation of stimuli [I]

Electronic multi-channel pipette with **Multi-dispensing mode**

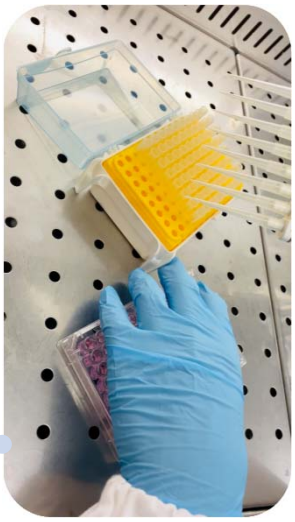


Use of multichannel pipettes for the preparation of stimuli [II]

Tip fitting



Mixing

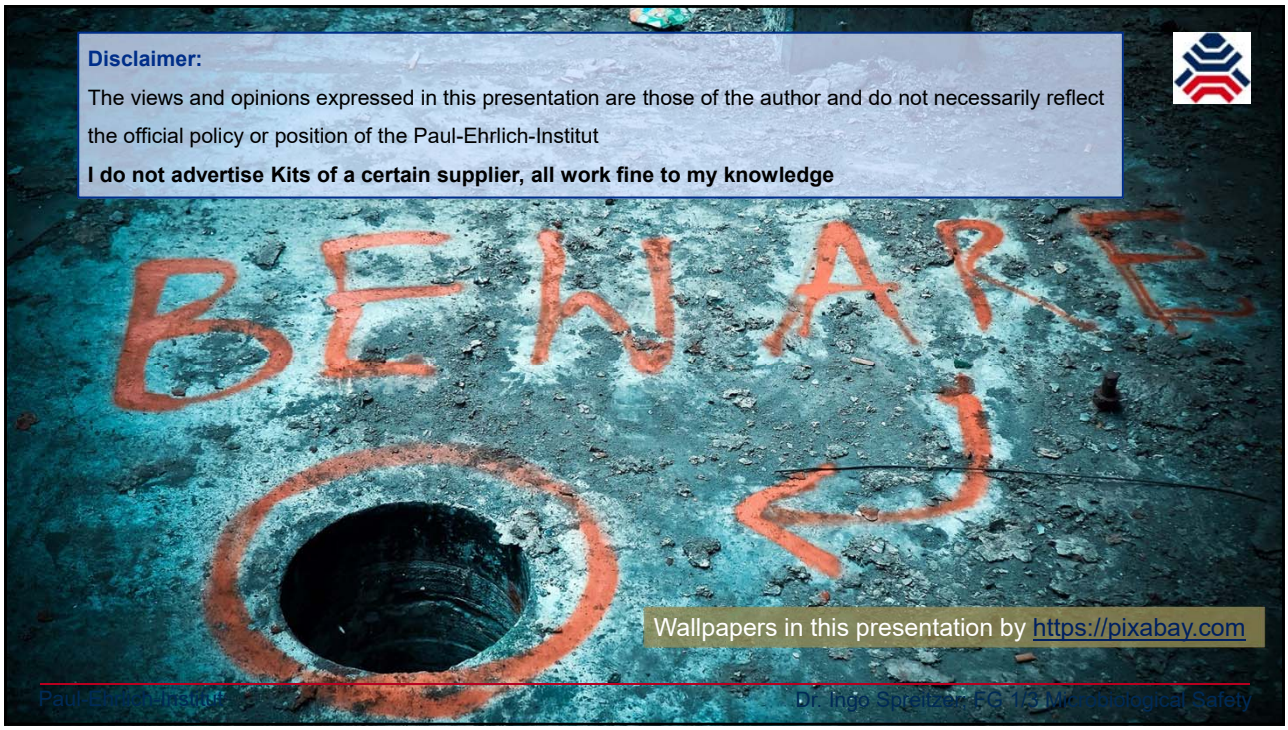


Serial Dilution

PBMC seeding

Use of a mechanical multi-channel pipette for **PBMC seeding**

Questions?


Disclaimer:
The views and opinions expressed in this presentation are those of the author and do not necessarily reflect the official policy or position of the Paul-Ehrlich-Institut
I do not advertise Kits of a certain supplier, all work fine to my knowledge
BEWARE
Wallpapers in this presentation by https://pixabay.com
Paul-Ehrlich-Institut
Dr. Ingo Spreitzer; FG 1/3 Microbiological Safety


Which MAT to choose?
Paul-Ehrlich-Institut
Dr. Ingo Spreitzer; FG 1/3 Microbiological Safety

Some thoughts…

- which cells
- which readout / readout system
- upcoming technologies

which cells

- Until now these cell types have been in use for a longer time:

- Human whole blood (fresh or frozen)
- PBMC (fresh or frozen)

- Monocytic cell lines: MonoMac6, THP-1

- *tlr-transfected HEK-cells for specific NEP-detection*

For research purposes fresh cells might be useful,
for QC you need a cell batch of reasonable size

Readout options

1. detection of secreted cytokines (mainly IL-6, some do IL-1ß)
typically end-point assays; new: digital PCR for cytokine-mRNA

- mostly used:
  - **ELISA**
    - **Sandwich (common for MAT-Kits);** 1 incubation plate, supernatants transferred to ELISA-plate
    - One-plate assays (incubation on the ELISA-plate) were described
    - HTRF (Heterogeneous time resolved fluorescence), AlphaLISA; less pipetting
  - Multiplexing:
    - Luminex
    - MSD
    - Ella
    - Bead-arrays
    - *New: dPCR NAT-MAT: mRNA of IL-1ß, IL-6, TNF-α; IL-8,*

Readout options

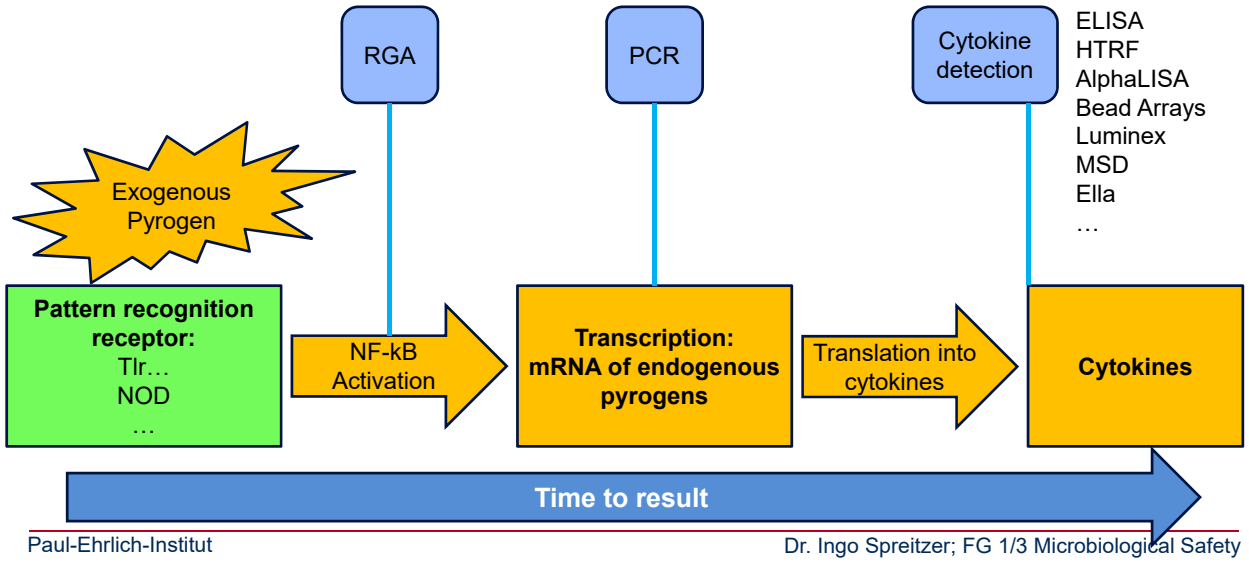
2. Reporter gene assays RGA (until now NF-κB)
Faster access on information possible;
mRNA detection (mentioned before)

- Some publications on modified HEK, THP-1
- The chinese colleagues seem to implement RGA into ChPh in the near future

MAT-suppliers (to my best knowledge)

Company	Cells	Readout	Kit	Cells separate available
Sanquin / Lonza	PBMC	IL-6	yes, 2 Versions (FCS; HSA)	yes
MAT-BioTech	PBMC	IL-6	yes	yes
MAT-Research	PBMC	IL-6	yes	yes
Haemochrom	PBMC	IL-6	yes	?
Merck Millipore	MM6	IL-6	yes	yes
	Cryoblood	IL-1ß	yes	yes
Minerva biolabs (announced Sept. 2022)	THP-1	mRNA Il-1ß, IL-6, TNFα, IL-8	yes	?

Current MAT readout options



Upcoming technologies

- Robotic platforms for MAT: standardisation, ease of use, transferability
- HTRF, AlphaLISA, one-plate ….: less pipetting, less variation

- Engineered cells
  - sensitivity: e.g. tlr-overexpression
  - specificity: only single tlr
  - speed: RGA and mRNA should be faster
    iPSC-derived macrophages seem to react faster too
  - reproducibility: iPSC-derived macrophages theoretically infinite access to one healthy donor / donation

Training Session

Dr. Ruth Röder

EDQM-EPAA Pyrogenicity Event: The future of pyrogenicity testing: phasing out the rabbit pyrogen test
Brussels, Belgium, 16-Feb-2023

Microcoat Biotechnologie GmbH | D-82347 Bernried | Phone: +49 8158 998 10 | info@microcoat.de | www.microcoat.de

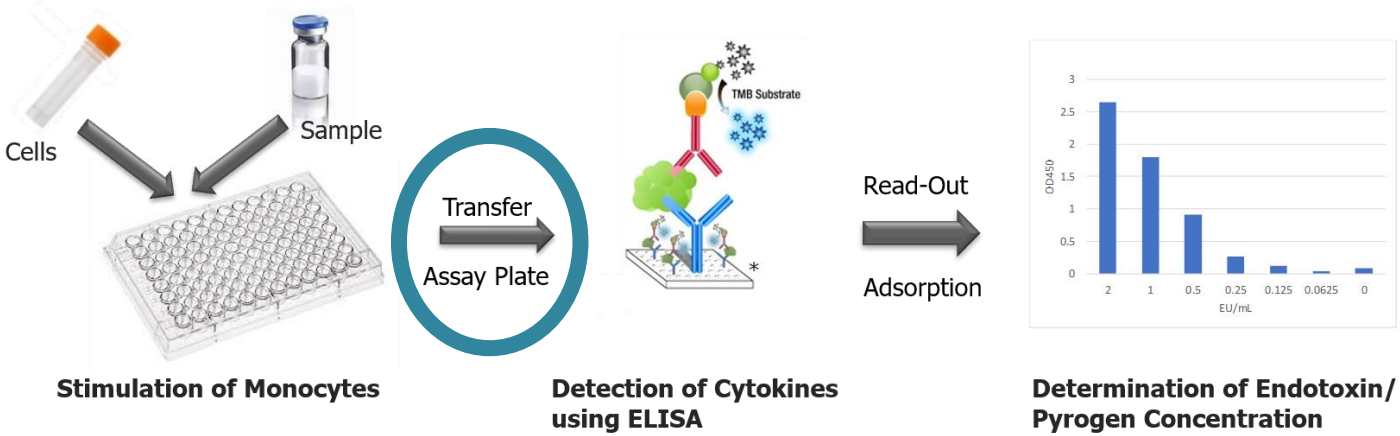
Outline

- Supernatant Handling
- Experience on technical equipment

Supernatant Handling

Pre-condition

- Control cell viability before performance of ELISA
- Freezing of cell suspension after stimulation



** http://www.origene.com.cn/ELISA/default.aspx?alpha=P*

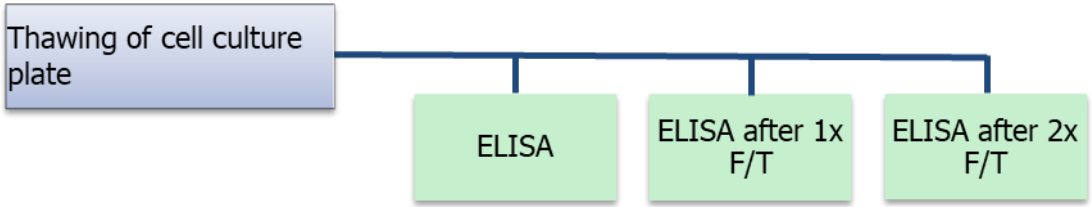
Supernatant Handling

Pre-condition

- Control cell viability before performance of ELISA
- Freezing of cell suspension after stimulation

  → *definition of freeze-thaw cycles*

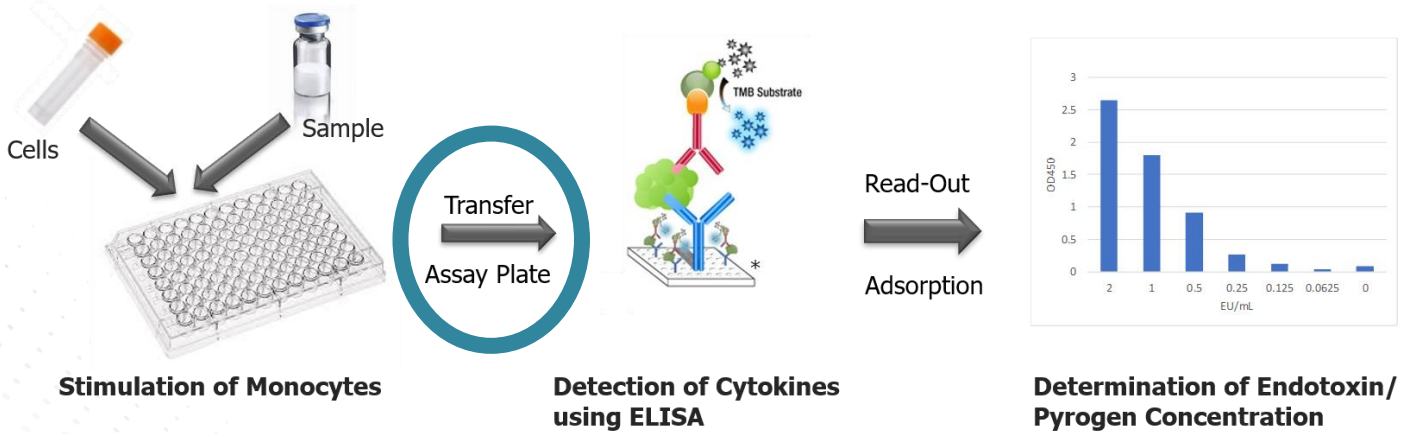
	1	2	3	4	5	6	7	8	9	10	11	12
A	ET Std 1											
B	ET Std 2											
C	ET Std 3											
D	ET Std 4											
E	ET Std 5											
F	Medium = Blank											
G	NEP 1 Control		NEP 2 Control									
H	NEP 3 Control		NEP 4 Control									



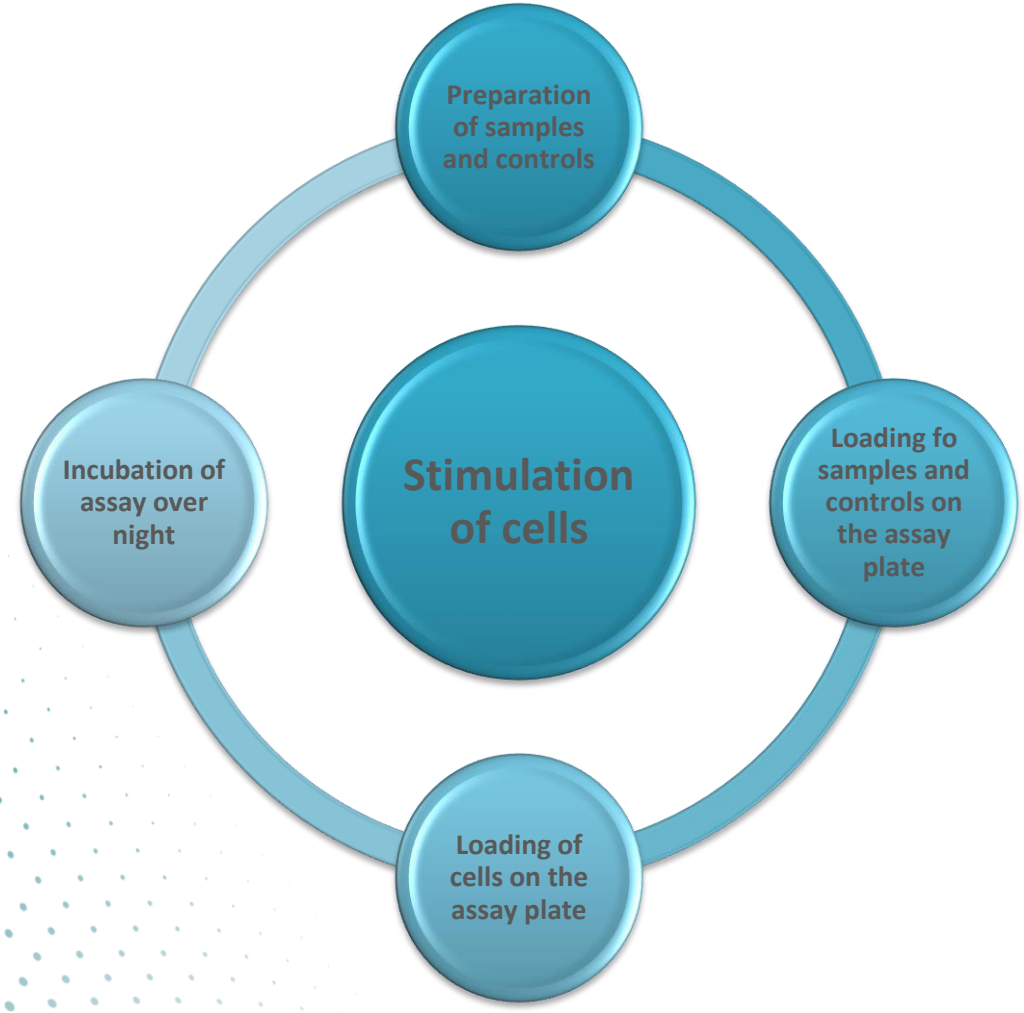
- Evaluation general run acceptance criteria (e.g.)
  - **Standard curve:** ET Std 1 > ET Std 2 > ET Std 3 > ET Std 4 > ET Std 5
  - **Blank:** OD(Blank) ≤ 0.10
  - **NEP Controls:** OD (NEP Controls) > Std 3
  - **Coefficient of Variation:**
    - CV(Standard) ≤ 30 %
    - CV(Controls) ≤ 30 %

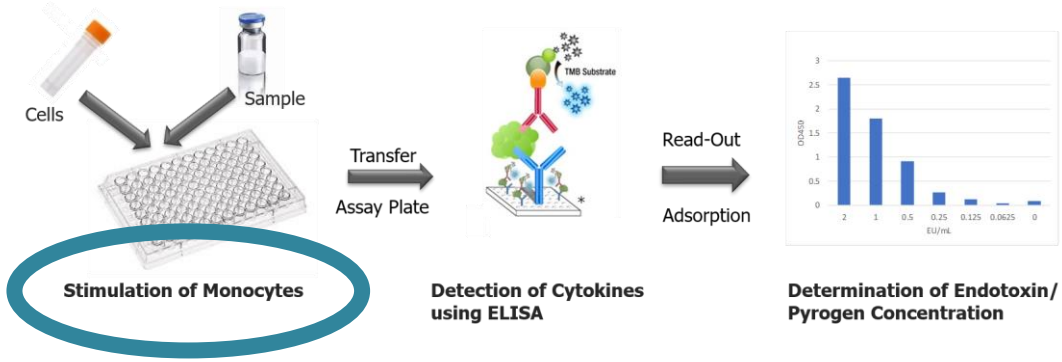
Supernatant Handling

- Control cell viability before performance of ELISA
- Freezing of cell suspension after stimulation
- Use fresh cell suspension
- Centrifugation of cell suspension
- Mixing of cell suspension before transferring on the assay plate
- Usage of single pipette or multichannel

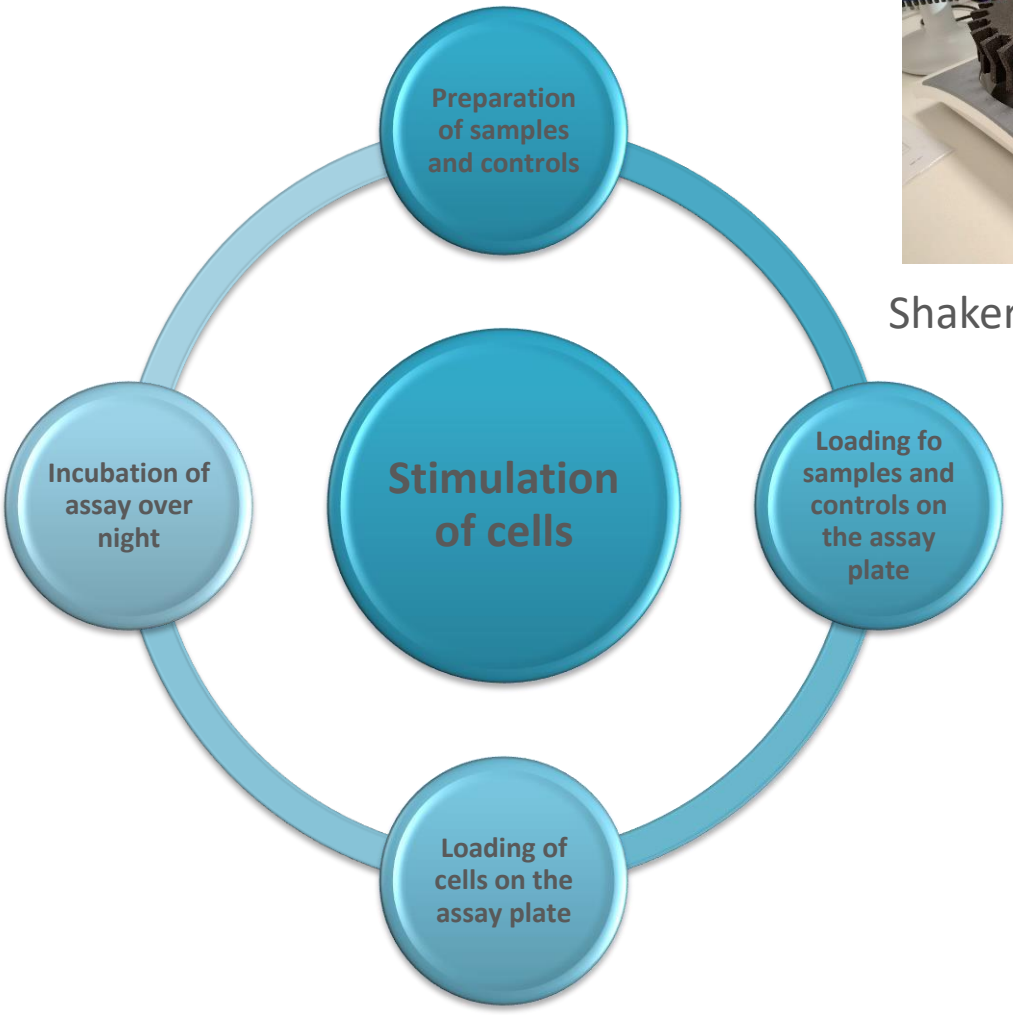


Experience with technical equipment





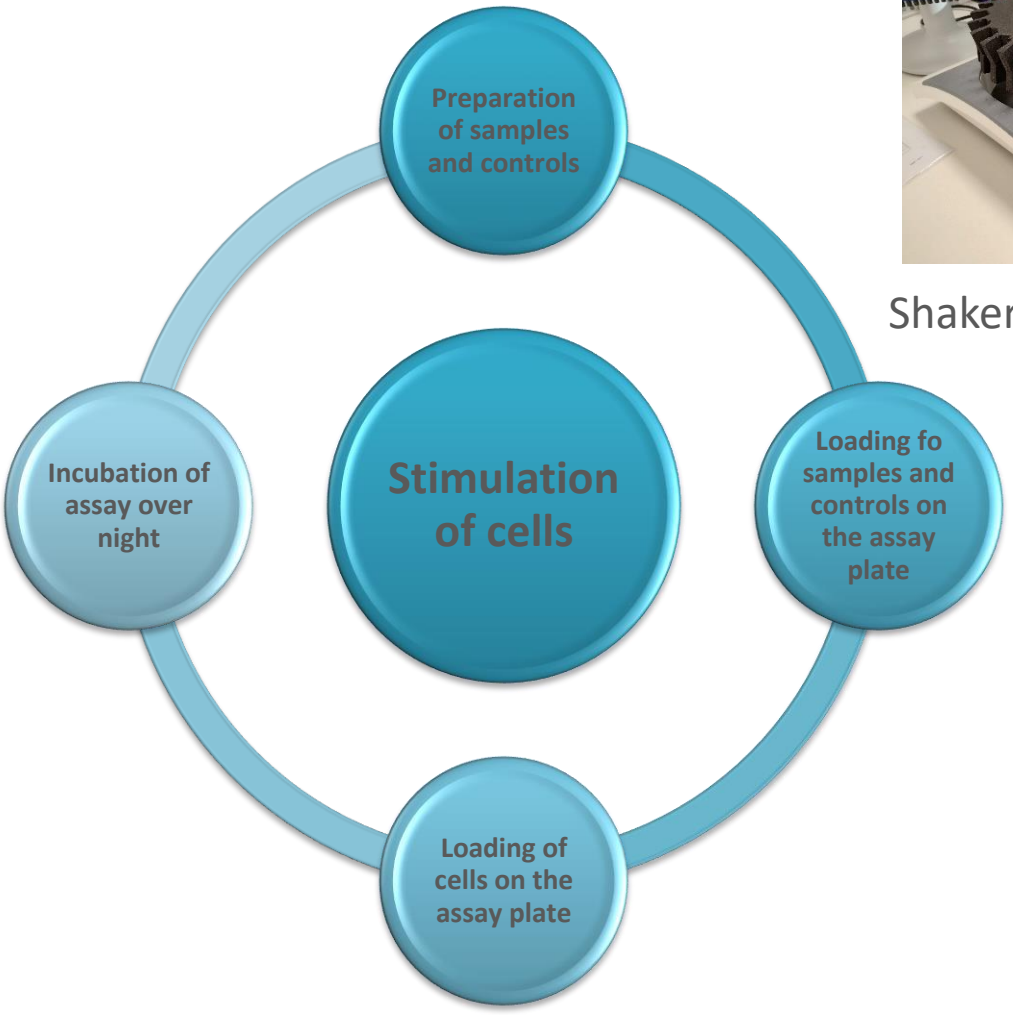
Experience with technical equipment



Shaker for glass test tubes

- *Vortexer or Horizontal shaker can be used*
- *For sensitive samples only pipette should be used*
- *Homogenization of samples is of great importance*

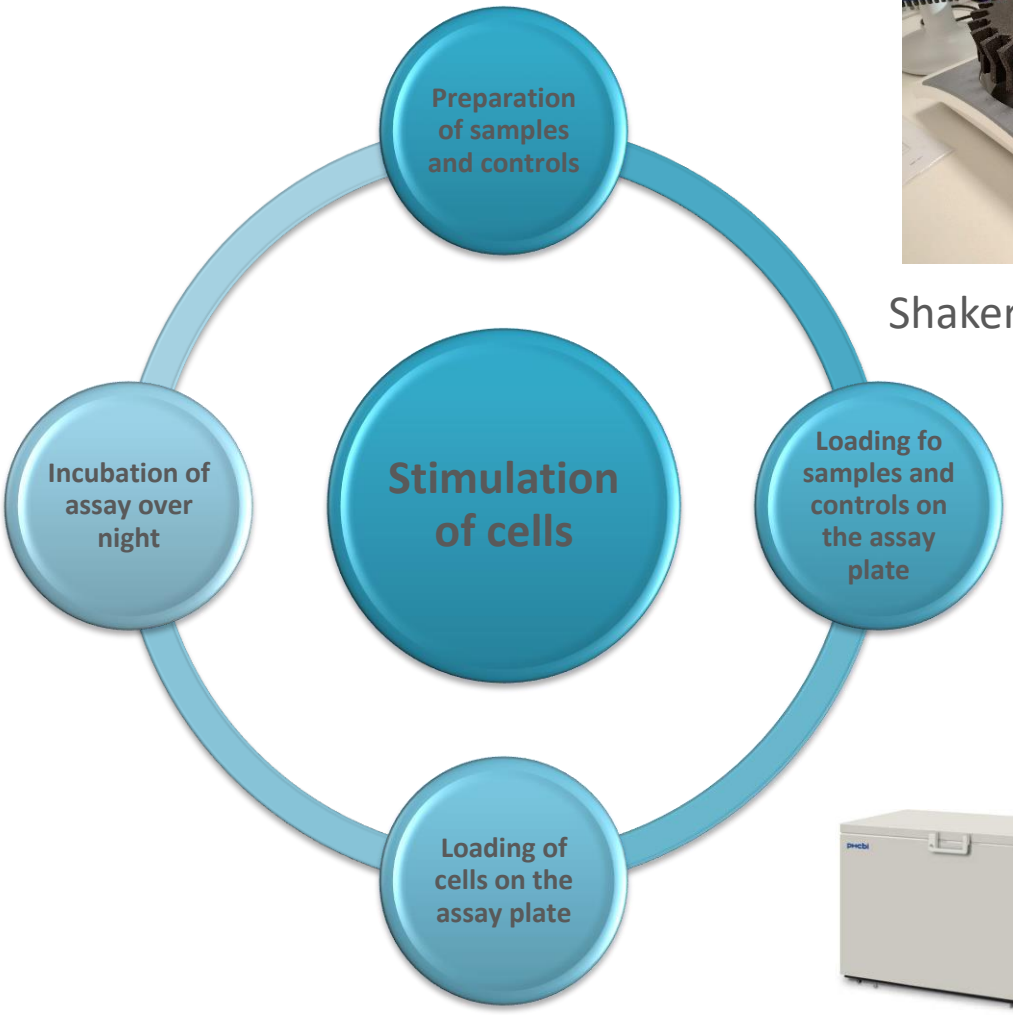
Experience with technical equipment



Shaker for glass test tubes

Multichannel pipette or Stepper

Experience with technical equipment



Shaker for glass test tubes

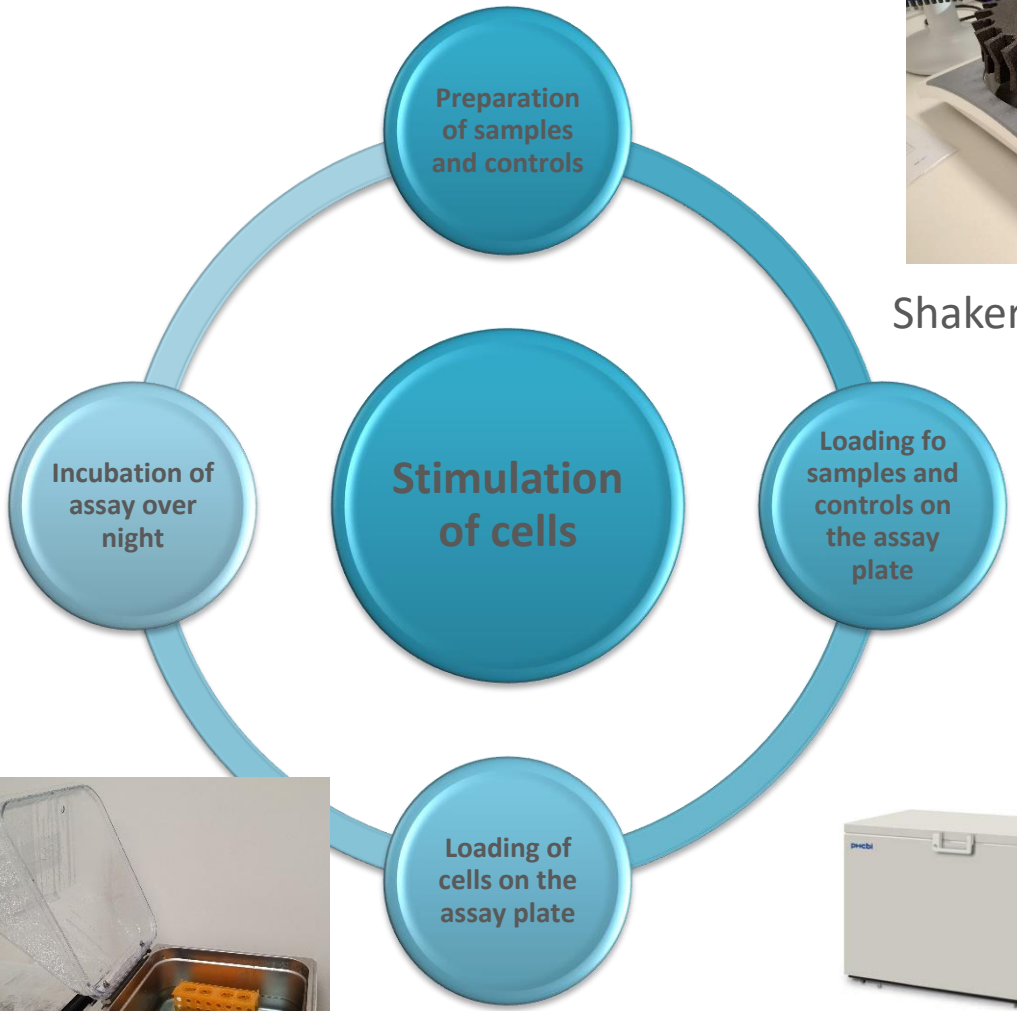
Multichannel pipette or Stepper

Storage of cells*

→ *Ultra deep freezer, Liquid N_2 or -80 °C*

*https://daiscientific.com/product/phcbi-mdf-dc700vxc-pa-ultra-low-chest-freezers/

Experience with technical equipment



Shaker for glass test tubes

Multichannel pipette or Stepper

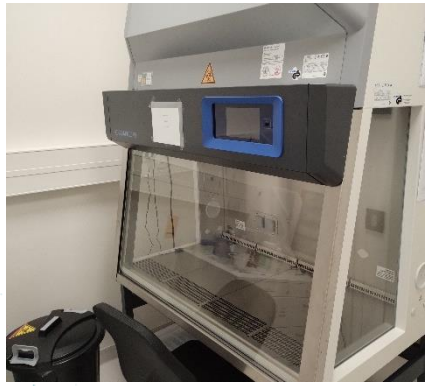
Storage of cells*

Waterbath → *Thawing of cells*

*https://daiscientific.com/product/phcbi-mdf-dc700vxc-pa-ultra-low-chest-freezers/

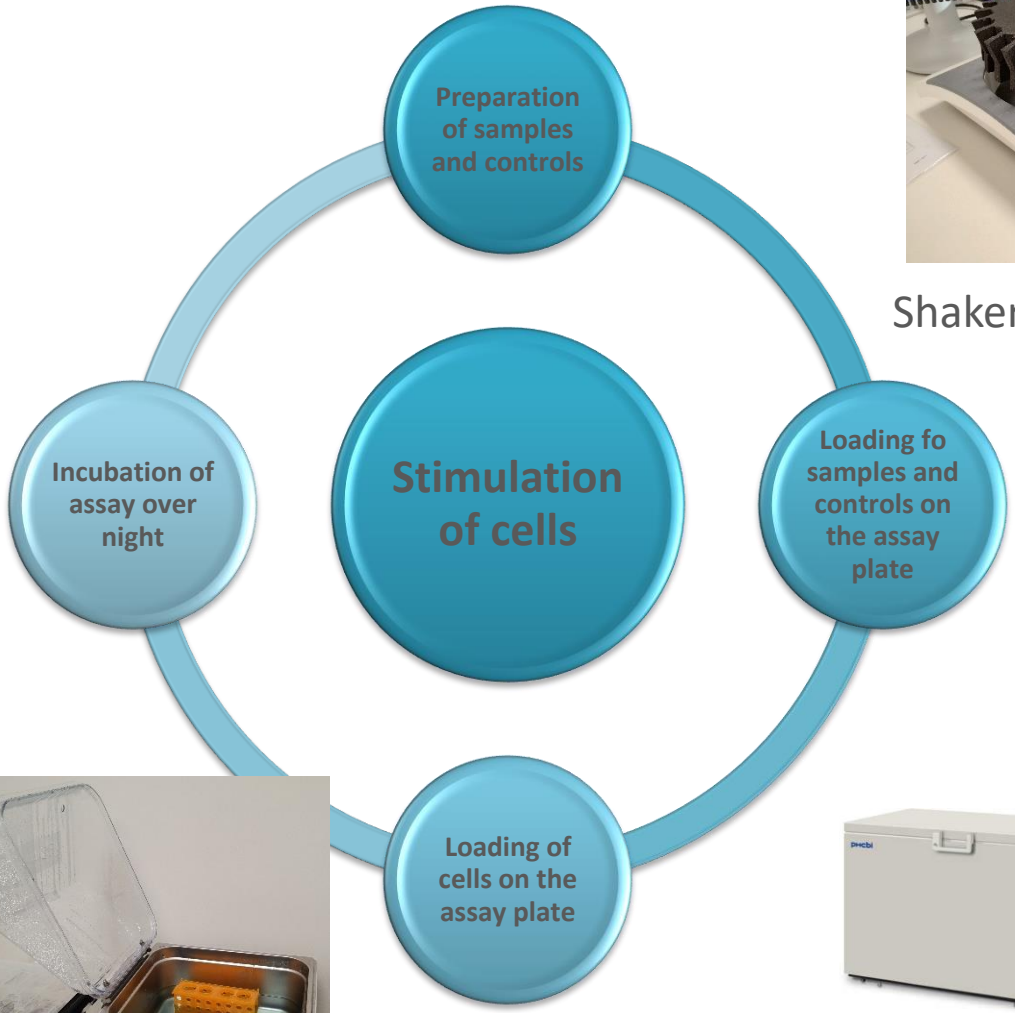
Experience with technical equipment

Shaker for glass test tubes



Cell culture hood

→ *preparation of media, cells etc.*



Multichannel pipette or Stepper

Waterbath

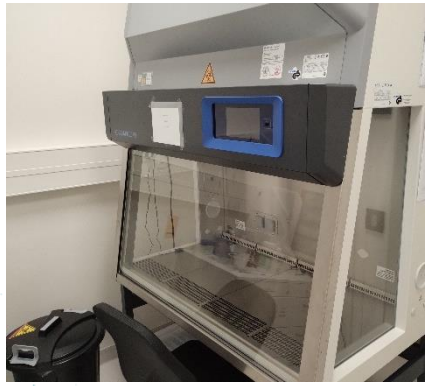
Storage of cells*

*https://daiscientific.com/product/phcbi-mdf-dc700vxc-pa-ultra-low-chest-freezers/

Experience with technical equipment

CO_2 Incubator

- *Incubation @ 37 °C, 5 % CO_2,*
- *Humified atmosphere*
- *Incubation O/N*



Cell culture hood

Waterbath

Shaker for glass test tubes

Multichannel pipette or Stepper

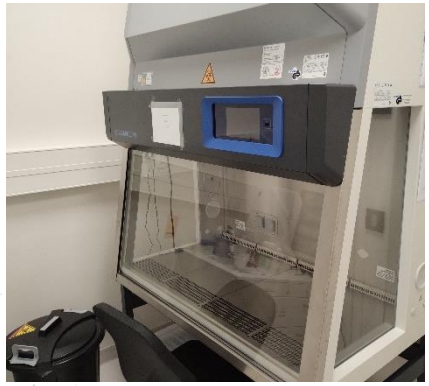
Storage of cells*

*https://daiscientific.com/product/phcbi-mdf-dc700vxc-pa-ultra-low-chest-freezers/

Experience with technical equipment

CO_2 Incubator

Shaker for glass test tubes



Cell culture hood

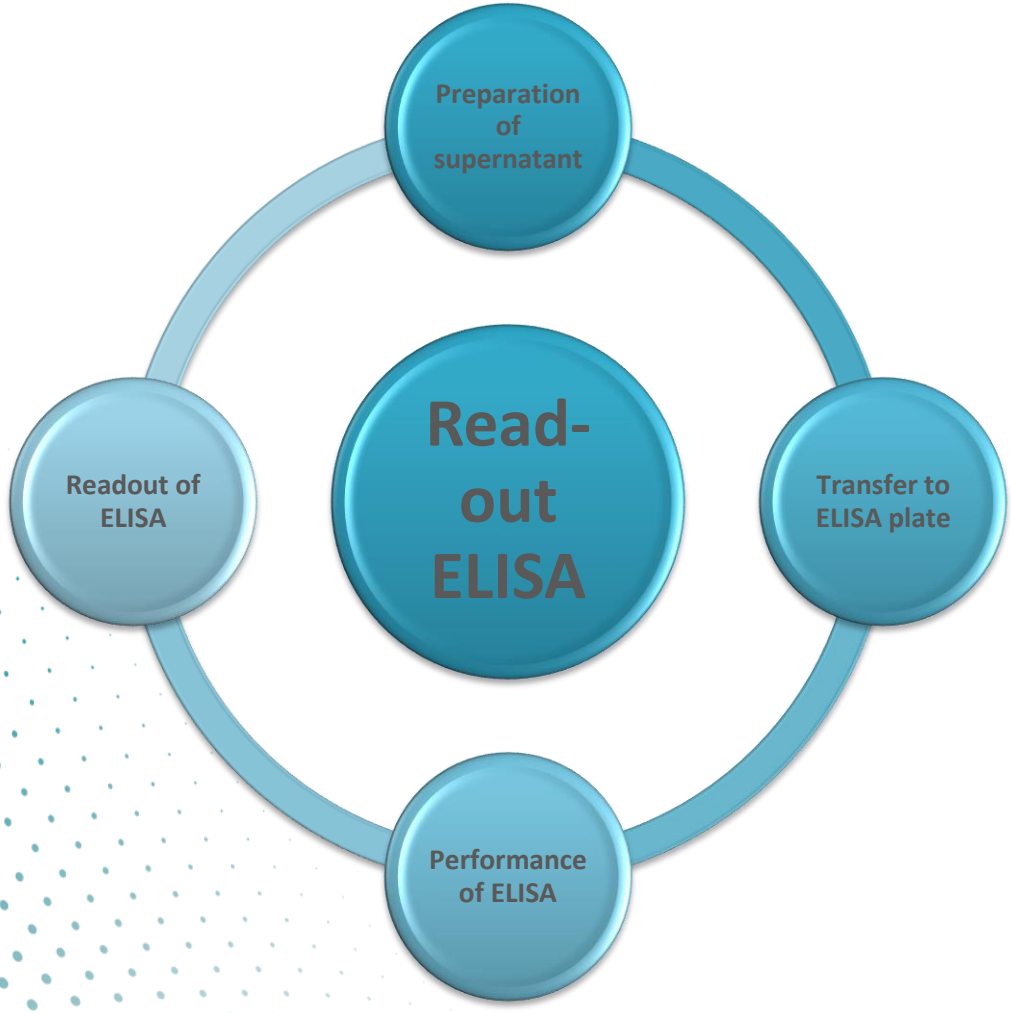
Multichannel pipette or Stepper

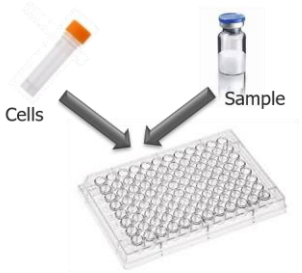
Waterbath

Storage of cells*

*https://daiscientific.com/product/phcbi-mdf-dc700vxc-pa-ultra-low-chest-freezers/

Experience with technical equipment

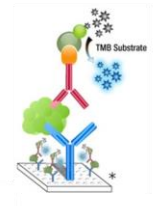




Stimulation of Monocytes

Transfer

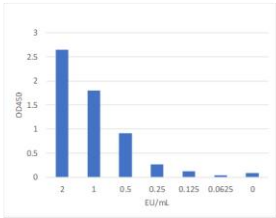
Assay Plate



Detection of Cytokines using ELISA

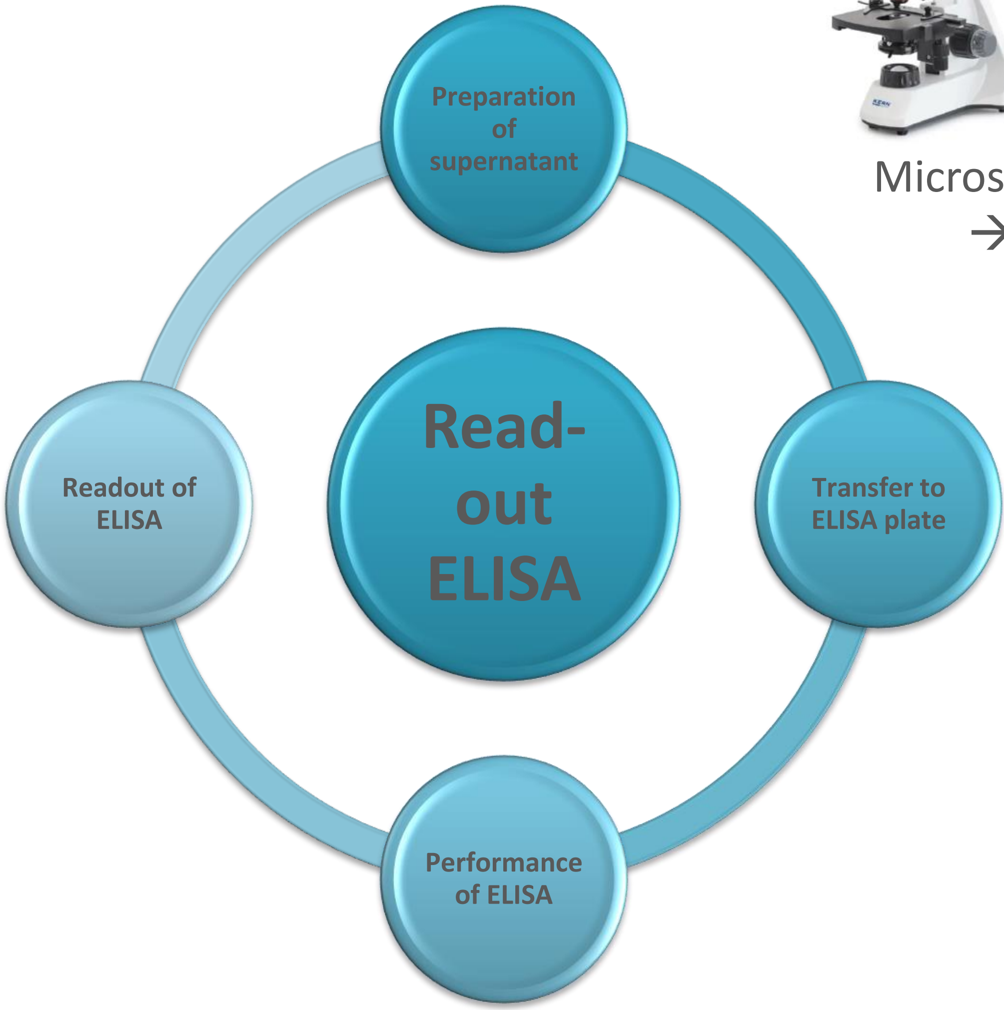
Read-Out

Adsorption



Determination of Endotoxin/ Pyrogen Concentration

Experience with technical equipment

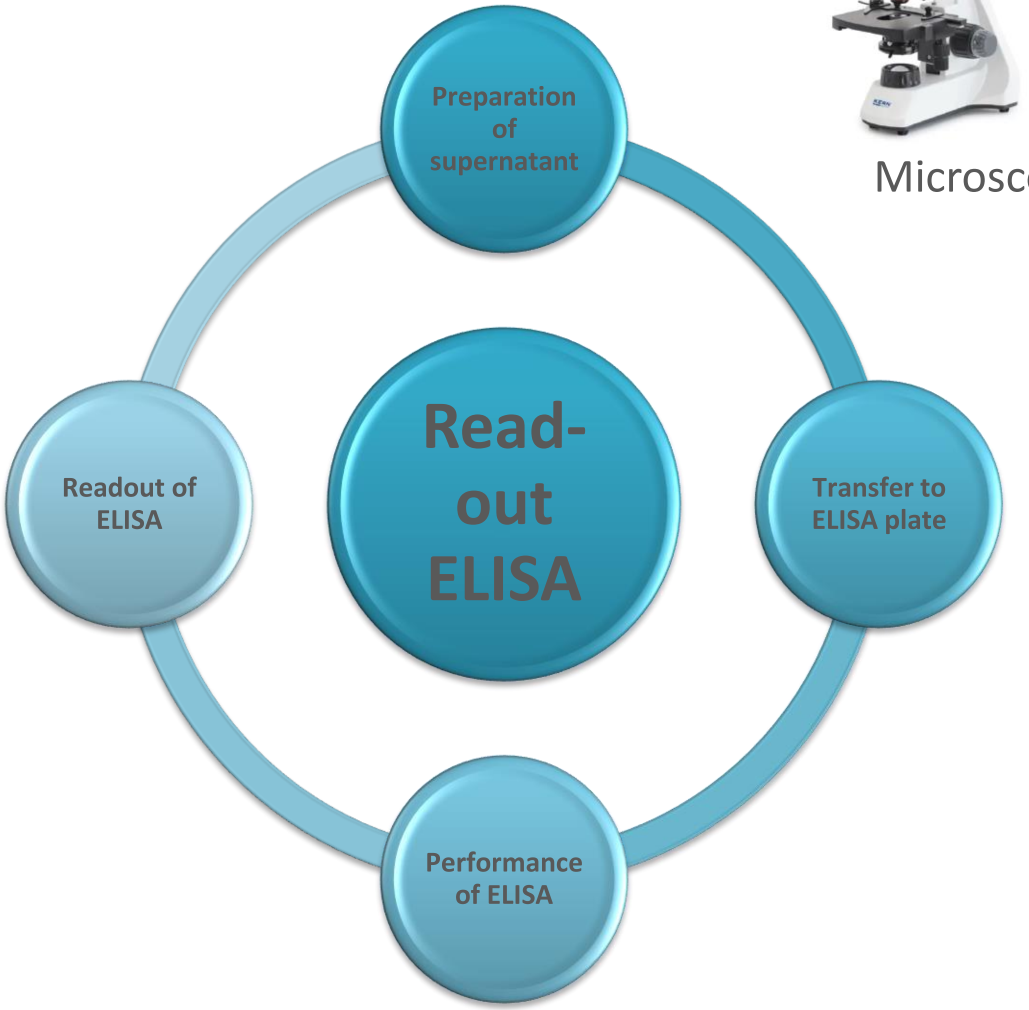


Microscope*

→ *Investigation of cell viability*

* https://de.rs-online.com/web/p/mikroskope/2059608?cm_mmc=DE-PLA-DS3A-_-google-_-CSS_DE_DE_Mess-_und_Pr%C3%BCftechnik_Whoop-_-(DE:Whoop!)+Mikroskope-_-2059608&matchtype=&pla-328086222840&gclid=CjwKCAjA85efBhBbEiwAD7oLQByju25YStuoSvC1yozhEnwmeCFkDEZcdu7siVPiqgui9C4BMR487BoCHsMQAvD_BwE&gclsrc=aw.ds

Experience with technical equipment

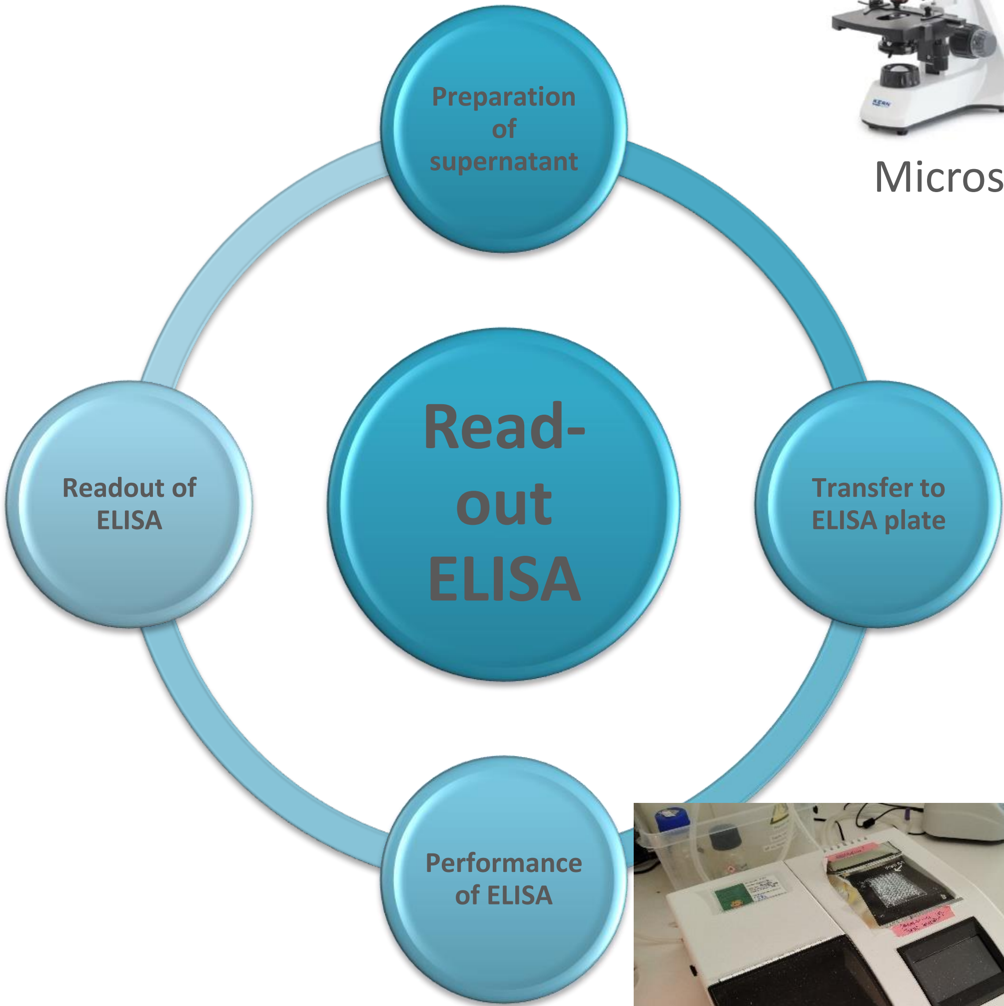


Microscope*

96- well plate shaker

* https://de.rs-online.com/web/p/mikroskope/2059608?cm_mmc=DE-PLA-DS3A-_-google-_-CSS_DE_DE_Mess-_und_Pr%C3%BCftechnik_Whoop-_-(DE:Whoop!)+Mikroskope-_-2059608&matchtype=&pla-328086222840&gclid=CjwKCAiA85efBhBbEiwAD7oLQByiu25YStuoSyC1yozhEnwmeCFkDE7cdu7siVPiqqui9C4BMR487BoCHsMQAvD_BwE&gclsrc=aw.ds

Experience with technical equipment



Microscope*

96- well plate shaker

Washer

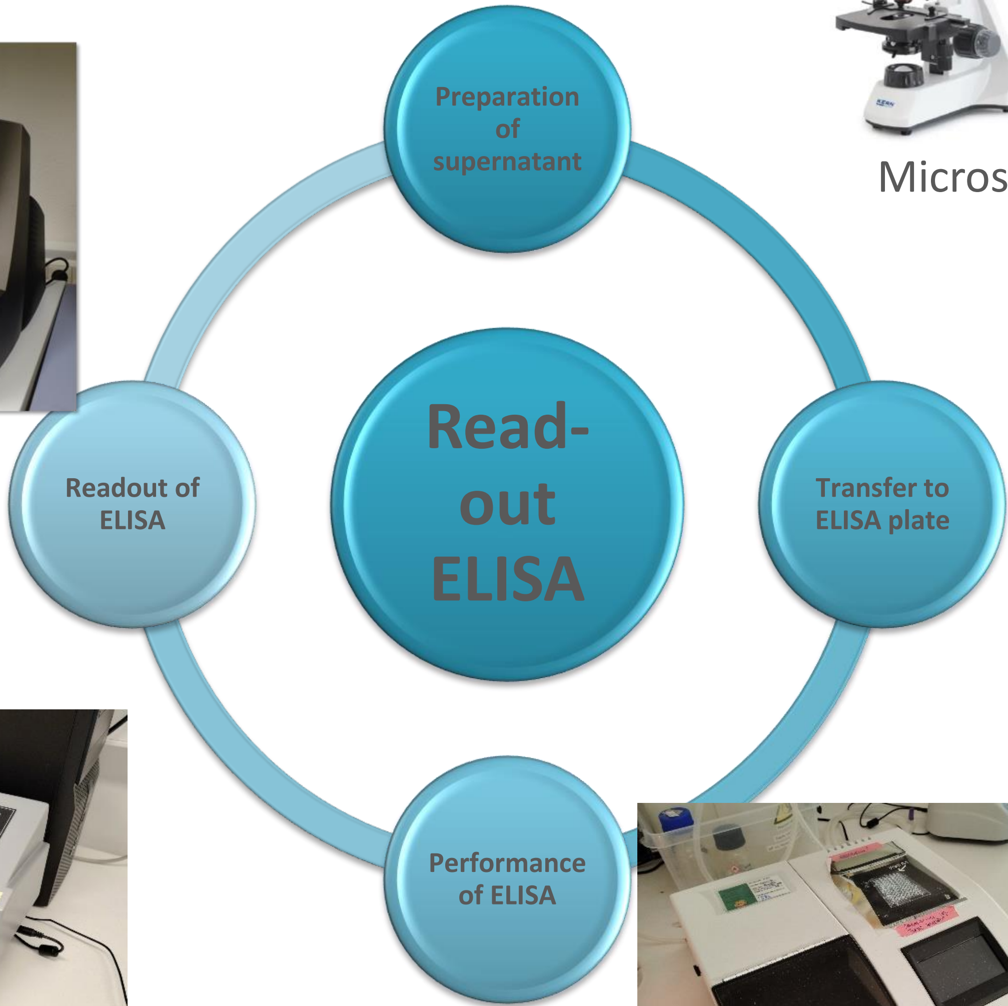
Washing of ELISA plate

- *Washer or multichannel pipette*
- *Automatization of process*

** https://de.rs-online.com/web/p/mikroskope/2059608?cm_mmc=DE-PLA-DS3A-_-google-_-CSS_DE_DE_Mess-_und_Pr%C3%BCftechnik_Whoop-_-(DE:Whoop!)+Mikroskope-_-2059608&matchtype=&pla-328086222840&gclid=CjwKCAjA85efBhBbEiwAD7oLQByju25YStuoSyC1yozhEnwmeCFkDEZcdu7siVPiqqui9C4BMR48ZBoCHsMQAvD_BwE&gclsrc=aw.ds*

Experience with technical equipment

Multiplexing



Microscope*

96- well plate shaker

OD Reader

Washer

* https://de.rs-online.com/web/p/mikroskope/2059608?cm_mmc=DE-PLA-DS3A-_-google-_-CSS_DE_DE_Mess-_und_Pr%C3%BCftechnik_Whoop-_-(DE:Whoop!)+Mikroskope-_-2059608&matchtype=&pla-328086222840&gclid=CjwKCAjA85efBhBbEiwAD7oLQBvju25YStuoSvC1vozhEnwmeCFkDEZcdu7siVPiqgui9C4BMR48ZBoCHsMQAvD_BwE&gclsrc=aw.ds

Next training session @ Microcoat:

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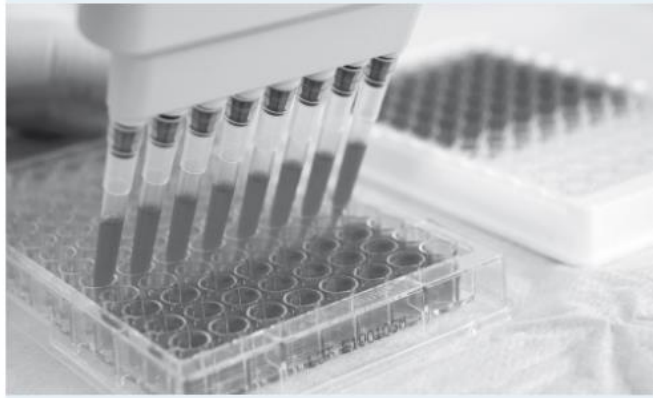
Your GMP/GDP Information Source

GMP Certification Programme
Certified Microbiological Laboratory Manager

Monocyte Activation Test (MAT)

Hands-on Laboratory Training Course

09/10 March 2023 | Munich/Bernried, Germany



Highlights

- Explanation of the MAT principle
- Understanding pharmacopoeial requirements
- Discussion of case studies
- Hands-on experience in performing the MAT in the laboratory

Speakers


Jacqueline Dünisch
Labor LS


Dr Anja Fritsch
Confarma


Maria Gajewi
Microcoat


Dr Andreas Karst
Haemochrom Diagnostica


Dr Koen Marijt
MAT Research


Katrin Pauls
Lonza


Stéphanie Richard
Sanofi Pasteur


Dr Ruth Röder
Microcoat


Shabnam Solati
CTL MAT


Dr Ingo Spreitzer
Paul-Ehrlich-Institut


Dr Sandra Stoppelkamp
Universität Tübingen and South Westphalia University of Applied Sciences

ECA Academy
Your GMP/GDP Information Source

GMP Certification Programme
Certified Microbiological Laboratory Manager

Low Endotoxin Recovery/Masking

Hands-on Laboratory Training Course

07/08 March 2023 | Munich/Bernried, Germany

Highlights

- Interpretation of interference during Endotoxin detection
- Understanding Low Endotoxin Recovery (LER)
- Setup of hold-time studies
- Techniques for demasking Endotoxin

Speakers


Aoife Barron
Lonza Biologics


Jacqueline Dünisch
Labor L+S


Dr Christian Faderl
bioMérieux Deutschland


Dr Bernhard Illes
Microcoat


Dr Andreas Karst
Haemochrom Diagnostica

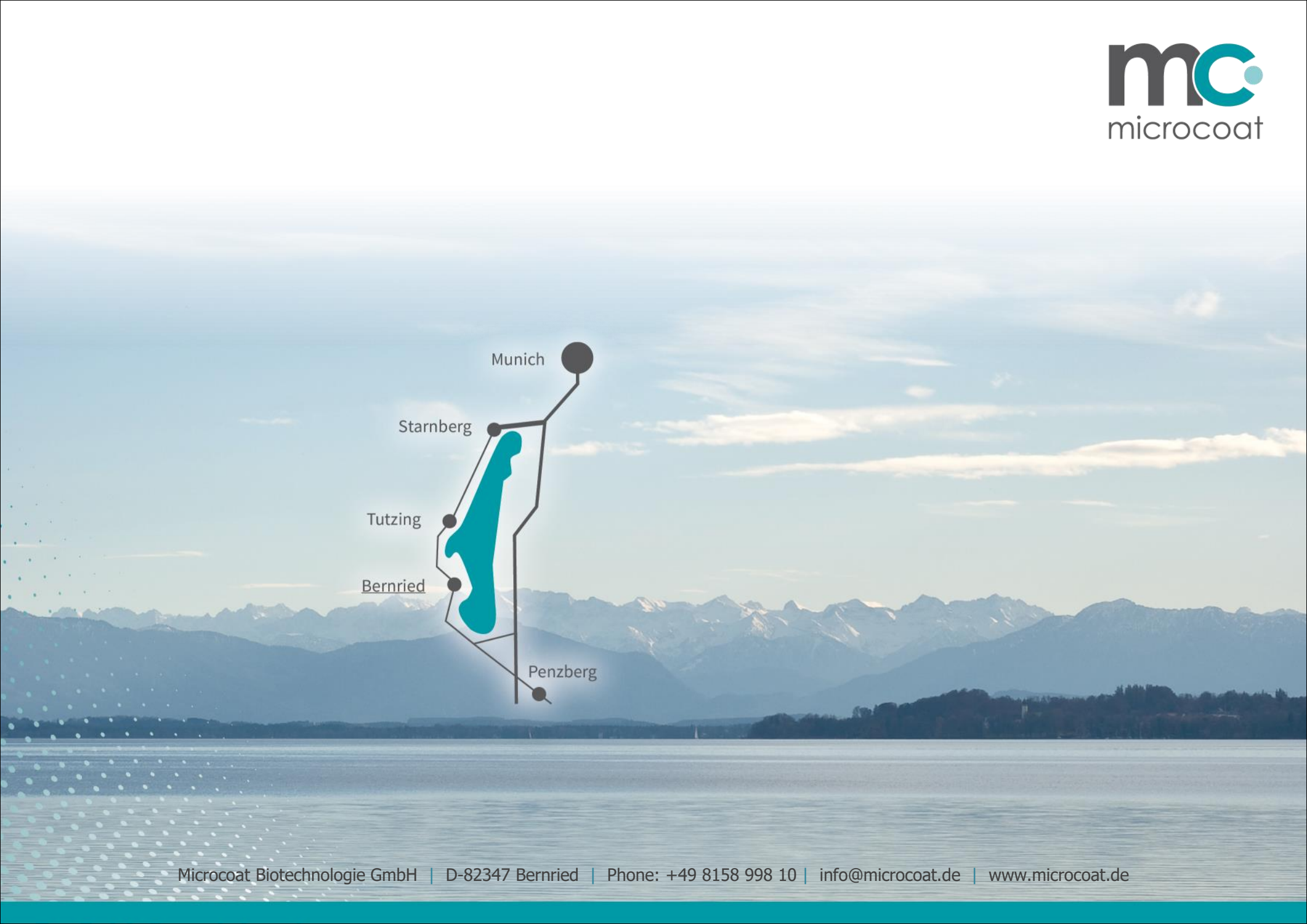

Dr Holger Kavermann
Roche


Dr Michael Kracklauer
Microcoat


Veronika Wills
ACC

Acknowledgment- Endotoxin Service

Feel free to contact me @ r.roeder@microcoat.de



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