PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

Triple culture of the inflamed-like intestine

With optional use of butyric acid as microbial metabolite

This is a SOP recommended for external use by PATROLS

Adapted from the NanoImpactNet SOP, Clift *et al* (Deliverable 5.4 under the European Commission's 7th Framework Programme, Grant Agreement 218539).

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Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	04/05/2020	Draft 1	Angela Kämpfer
1.1	02/06/2020	ELISA protocol added	Angela Kämpfer
2.0	08/04/2021	Migration to new format	Angela Kämpfer

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1 Introduction:

Domain: Human Toxicology

As the importance of intestinal health for the whole organism becomes more apparent, an increasing amount of research investigates the impact of substances, e.g. particles, on the intestine in health and disease. Oral ingestion of micro- and nanoscale particles, both intentional (Peters et al., 2014, Lim et al., 2015) and accidental (von Goetz et al., 2013), has long been identified as a likely route of human exposure to particles.

In the context of the 3Rs principles – to Refine, Reduce, and ultimately Replace animal experiments – the development of novel alternative models (e.g. *in vitro* and *in silico*) is necessary. To date, the availability of relevant 3D intestinal *in vitro* co-culture models is limited but a few, mainly cell line-based, approaches have been established (Susewind et al., 2016, Georgantzopoulou et al., 2016, Leonard et al., 2010, Kämpfer et al., 2017, Lehner et al., 2020). However, a general lack of diseased models to study a potentially increased risk of susceptible sub-groups (e.g. individuals diagnosed with inflammatory bowel diseases (IBD)) is recognised (Lefebvre et al., 2015). So far, only two models have been established solely based on human cell lines that (1) incorporate at least one immunocompetent cell type and (2) can mimic both healthy and inflamed-like conditions (Kämpfer et al., 2017, Susewind et al., 2016). Both models, however, lack the presence of mucus, which can heavily impact the fate and effect of (nano)particles in the intestine (Crater and Carrier, 2010). Furthermore, the available models do not consider the impact of the gut microbiota, which recent studies have demonstrated to be crucial for both physical and mental health (Lloyd-Price et al., 2019, Sampson et al., 2016). It cannot be excluded that the incorporation of microbiota or at least microbial products influence (1) the co-culture and (2) exposure-induced effects.

1.1 Scope and limits of the protocol

Scope: The set-up of a triple co-culture of human cell lines representing enterocytes (Caco-2), goblet cells (HT29-MTX-E12, hereinafter E12) and macrophages (differentiated (d)THP-1) to mimic the human small intestine in a diseased, inflamed-like state. The model can be used to study the toxicity and inflammatory potential of

particles as well as chemicals in the context of ongoing inflammatory processes. In a second or additional step, the influence of microbial metabolites – here butyric acid is used as model compound – on the inflammatory processes and exposure effects can be studied. Endpoints include, but are not limited to: barrier integrity (e.g. by Transepithelial electrical resistance (TEER), passive transport assays like Lucifer Yellow, immunocytochemistry), cytotoxicity (e.g. lactate dehydrogenase (LDH) assay, immunocytochemistry, gene expression, DNA damage, mucus secretion, and cytokine release.

Limitations: It is unclear, to which extent this model can be used to detect a further cytokine-/inflammation-enhancing impact. The damage induced by the ongoing inflammatory processes might be too extensive already to detect further particle- or chemical-induced effects unequivocally. Independent experiments in LPS/IFNγ activated THP-1 monocultures have, however, shown a further increase in cytokine-release after the exposure to silver nanoparticles (Ag-PVP ENM; not shown).

It is unclear to which extent live microbial components can be included in the current model set-up, as the static culture conditions might lead to a rapid overgrowth by bacteria. Also, the use of live bacterial cultures might not be feasible in every laboratory. The use of microbial metabolites might, therefore, be the more favourable alternative.

1.2 Validation state of protocol

Level of advancement towards standardization	**Level reached** (please mark only one with "X")
Stage 1: Internal laboratory method under development	
Stage 2: Validated internal laboratory method	X
Stage 3: Interlaboratory tested method	
Stage 4: Method validated by Round Robin testing	
Standardisation plans	
Is the method considered for standardisation (OECD SPSF or similar)?	No

Has the method been submitted for standardisation (to OECD, CEN, ISO,…) in its own right or as part of another standardisation project?	No
Is the method included in an existing standard (or ongoing standardisation work)	No
If yes, specify	[standard reference number, eg. EN 17199-4]

2 Terms and Definitions:

Differentiated epithelial cells

Caco-2/E12 layers cultured for 18-21 days on transwell filter inserts

Pre-warmed

Warmed to 37°C in a waterbath

Activated THP-1 cells

PMA-differentiated THP-1 cells treated with 10 ng mL^{-1} LPS and IFN-γ

Inflamed-like triple culture

Triple culture of differentiated epithelial cells and activated PMA-differentiated THP-1 cells

Stable triple culture

Triple culture of differentiated epithelial cells and non-activated PMA-differentiated THP-1 cells

3 Abbreviations:

AB	Alcian Blue
AP	Apical
BA	Butyric acid
BL	Basolateral
CCM	Cell culture medium
E12	HT29-MTX-E12 cell line
EtOH	Ethanol
FBS	Foetal Bovine Serum
IFNγ	Interferon gamma
IL	Interleukin
LPS	Lipopolysaccharides
MUC	mucin
NEAA	Non-essential amino acids
PAS	Periodic acid-Schiff's
PBS	Phosphate Buffered Saline
Pen/Strep	Penicillin / Streptomycin
PMA	Phorbol-12-myristat-13-acetat
RT	Room temperature
TEER	Transepithelial Electrical Resistance
TNFα	Tumor necrosis factor alpha
ZO-1	Zonula Occludens 1

4 Principle of the Method:

In vitro co-cultures based on (human) cell lines are used to develop physiologically relevant models with the aim to reduce and ultimately replace *in vivo* testing. A single isolated cell type is usually not capable of adequately reflecting the heterogeneous composition of organs or the organism as a whole. More specifically, cell monocultures lack the communication that generally regulates the interplay and homeostasis between different cell types *in vivo*. Therefore, multi-cellular cultures have been established to better reflect the complexity of an *in vivo* situation. To further advance the previously developed homeostatic intestinal triple culture, the induction of an inflammation-like status was modelled to allow studying the impact of impaired health

on ENM-induced effects. To establish the inflammation-like state, the same process as previously described by Kämpfer et al. (2017) was used. Briefly, a 'priming' effect of IFNγ described by Wang et al. (2005) was exploited, which induces an up-regulation of TNFα receptor (TNFR) 1 and 2 in Caco-2 cells, both located at the BL cell membrane (Wang et al., 2006). Binding of TNFα to TNFR2 leads to an increased expression of myosin light chain kinase (MLCK) (Blumberg, 2009), which results in an amplified phosphorylation of MLC (Zhou et al., 2005). Phosphorylation of MLC, leading to contraction of the actomyosin ring, has been demonstrated to regulate intestinal epithelial permeability by re-organising tight junction proteins. This re-organisation causes an increased paracellular permeability of the barrier and a strong reduction in transepithelial electrical resistance (TEER) (Turner et al., 1997, Wang et al., 2005, Zhou et al., 2005).

To consider the impact of microbial metabolites on the culture and exposure-induced effects, butyric acid (BA) was included as model compound. The short-chain fatty acid is a major fermentation-derived metabolite in the intestine and has been shown to be of importance for the regulation of intestinal immune homeostasis and to positively affect intestinal inflammatory conditions (Borycka-Kiciak et al., 2017).

The procedure can be divided into 4 parts:

1. Culture and maintenance of the 3 individual cell lines (Section 5.7.1)
2. Seeding and maintenance of the epithelial transwell co-culture (Section 5.7.2)
3. PMA-differentiation and activation of THP-1 cells (Section 5.7.3)
4. Establishment and maintenance of the inflamed triple culture (Section 5.7.4)
    a. Standard
    b. Including butyric acid

5 Description of the Method:

5.1 Biological setting & test system used:

- set-up of a cell line-based triple culture model under sterile lab-based work conditions to mimic the intestine in a healthy state for the investigation of toxicity endpoints
- Human derived cell lines used:
    - Caco-2 (human colon adenocarcinoma): DSMZ, ACC169
    - HT29-MTX-E12: sub-clone E12 of the methotrexate-differentiated cell line HT29 (human colon adenocarcinoma), Merck/ECACC, 12040401
    - THP-1 (human monocytic leukemia): ATCC, TIB-202

5.2 Chemicals and reagents used:

Reagent	Supplier / Cat N°	CAS
MEM (NEAA)	Thermo Fisher Scientific, #10370-021	NA
DMEM (high glucose, L-Glutamine)	e.g. Thermo Fisher Scientific, #41965-039	NA
RPMI (HEPES, L-Glutamine)	e.g. Thermo Fisher Scientific, #52400-041	NA
PMA	Merck, #P1585	16561-29-8
Penicillin / Streptomycin	e.g. Thermo Fisher Scientific, #15140122	69-57-8, 3810-74-0
L-Glutamine	e.g. Thermo Fisher Scientific, #35050038	NA
D-Glucose	e.g. Sigma, #G8769	50-99-7
Sodium pyruvate	e.g. Thermo Fisher Scientific, #11360070	113-24-6
2-Mercaptoethanol (50 mM)	e.g. Thermo Fisher Scientific, 31350010	60-24-2
Trypsin	e.g. Sigma T4049	NA

FBS	Sigma, F7524	NA
NEAA	e.g. Sigma, M7145	NA
Accutase	e.g. Merck, A6964	NA
LPS	Sigma, L4391	93572-42-0
IFNγ	Sigma, SRP3058	NA
Sodium butyrate	Sigma, B5887	156-54-7
Diclofenac sodium salt	Sigma, D6899	15307-79-6
PBS	Sigma	NA

5.3 Apparatus and equipment used:

Transwell insert and companion plates

Transwell inserts, PET, 1µm pore size[1]	Cat. N° 353103
Companion plates	Cat. N° 353503

Voltohmmeter

e.g. World Precision Instruments

Model 'EVOM' or newer with chopstick electrode STX2

Absorbance reader

e.g. Thermo Scientific, Multiskan Go

[1] *In principle, other pore sizes can be used. It is, however, possible that the cell growth and TEER development will be influenced by both the pore size and material used.*

5.4 Reporting of protected elements:

This SOP does not have any associated patent restrictions, specific licenses, material transfer agreements or commercial purchase requirements required to perform the protocol described.

5.5 Health and safety precautions:

Prior to any use of this SOP a full risk assessment should be completed, considering all potential risks associated with chemicals equipment and use, in compliance with national regulation. Training of personnel should be completed before any person is working with the SOP.

5.6 Reagent preparation:

Heat inactivation of FBS

- Ta ke a bottle of FBS from -20°C and let thaw over night
- Place the bottle of thawed FBS in the cold waterbath and switch on; set the temperature control to 56°C
- Once the waterbath reached 56°C, keep the bottle in the waterbath for another 30 min
- In the meantime, label an adequate amount of 50 mL tubes, including your initials and the date
- After 30 min, take the bottle from the waterbath, dry and generously spray with 70% EtOh before transferring it under a laminar flow cabinet
- Carefully open the bottle and aliquot into the prepared 50 mL tubes using a Pippette Boy and serological pipettes (25 or 50 mL)
- Let the serum cool for 30 min before transferring the tubes to -20°C

Reconstitution of lyophilized PMA

- Carefully open the glass vial and add 162 µL pure ethanol to reconstitute the lyophilized powder

- Stock 1: 10 mM
- Store in 10 µL aliquots at -20°C

- Stock 2: add 10 µL of stock 1 to 990 µL sterile PBS
  - 100 µM
  - Prepare working aliquots of 25-50 µL each
- When handling PMA: PMA IS LIGHT AND TEMPERATURE SENSITIVE! Keep protected from light! Do not keep PMA at room temperature for more than 10 min!

Reconstitution of lyophilized IFNγ

- Carefully open the vial and add 100 µL 0.1% BSA/PBS to reconstitute the content, resulting in a stock solution of **1 mg mL^{-1}**
  - **Do not vortex to reconstitute!**
  - Only proceed once no solid residue remains
  - Store original stock in 10 µL aliquots at -80°C
- Dilute 1:100 in 0.1% BSA/PBS to obtain the working stock of 10 µg mL^{-1}
  - Aliquot working stock in 50 µL, store at -20°C (check quality after 3 months), better -80°C

Reconstitution of lyophilized LPS

NB: dried LPS is very light, residues might be attached at the bottom of the lid and may be lost if the vial is not opened carefully

- Before reconstitution, the powder should be stored at 2-8°C
- Stock 1: 1 mg mL^{-1} stock solution
  - add 1 mL of sterile PBS to the vial
  - Reconstitute well until no solid powder remains
  - Prepare 100 µL aliquots of stock 1 and store at -20°C
- Stock 2: 10 µg mL^{-1}
  - Prepare a 1:100 dilution by adding 10 µL of stock 1 to 990 µL sterile PBS

- Prepare 50 µL working aliquots of stock 2 and freeze at -20°C

- Avoid repeated thaw-freeze cycles!

5.7 Procedure:

5.7.1 Cell culture

5.7.1.1 Caco-2

Cell culture medium (CCM)

- MEM medium (500 mL)
- 20 % heat inactivated fetal bovine serum (100 mL)
- 5 mL of glutamine (Invitrogen 35050)
- 5 mL Pen/Strep

Culture / Maintenance

- Cells are seeded at $7.5x10^5$ cells in 75 cm² flasks and maintained in the above listed Caco-2 cell culture medium
- Sub-culture cells at the latest when they reach 80% confluence; generally splitting the cells on Monday and Friday is recommended
- Discard the medium and wash 1x with 5mL pre-warmed PBS
- Discard PBS, add 5 mL fresh pre-warmed PBS and place the flask at 37°C, 5% CO_2 for 5 min
- Discard the PBS and add 3 mL pre-warmed trypsin; place the flask at 37°C, 5% CO_2 for 5 min
- To de-activate the trypsin, add 7 mL pre-warmed Caco-2 cell culture medium to the flask; pipette the cell suspension up and down several times in the flask (to help detach more cells and break up cell clumps)
- Transfer cell suspension to a 15 mL tube and perform a cell count
    - The cell count can be performed on the undiluted suspension; the count should be usually be between $0.8\text{-}1.1x10^6$ cells mL^{-1}
    - Ideally cell counts should be performed using an automated cell counter. Alternatively a manual count can be performed using a haemocytometer following the protocol given in the PATROLS '3D In Vitro HepG2 Spheroid model' SOP
- Re-seed $7.5x10^5$ cells in a new flask and top up to a total volume of 15 mL with pre-warmed cell culture medium; Place flask at 37°C, 5% CO_2

- It is recommended to start a new culture after 20 passages

5.7.1.2 HT29-MTX-E12

CCM

- DMEM medium (high glucose) (500 mL)
- 10 % heat inactivated fetal bovine serum (50 mL)
- 5 mL NEAA

Culture / Maintenance

- Cells are seeded at $3x10^6$ cells in 75 cm² flasks and maintained in the above listed E12 culture medium
- Sub-culture cells before they reach 80% confluence¸ generally splitting the cells on Monday and Friday is recommended
- Discard the medium and wash 1x with 5mL pre-warmed PBS
- Discard PBS, add 5 mL fresh pre-warmed PBS and place the flask at 37°C, 5% CO_2 for 5 min
- Discard the PBS and add 3 mL pre-warmed trypsin; place the flask at 37°C, 5% CO_2 for 5 min
- To de-activate the trypsin, add 7 mL pre-warmed E12 cell culture medium to the flask; pipette the cell suspension up and down several times in the flask (to help detach more cells and break up cell clumps)
- Transfer cell suspension to a 15 mL tube and perform a cell count
    - As the cell concentration will be high (>$1.5x10^6$ cells mL^{-1}) it is advised to perform the cell count on a 1:10 diluted cell suspension
    - Ideally cell counts should be performed using an automated cell counter. Alternatively, a manual count can be performed using a haemocytometer following the protocol given in the PATROLS '3D In Vitro HepG2 Spheroid model' SOP
- Re-seed $4x10^5$ cells cm^{-2} in a new flask (i.e. $3x10^6$ cells/flask) an top up to a total volume of 15 mL using pre-warmed E12 cell culture medium; Place flask at 37°C, 5% CO_2
- It is recommended to start a new culture after 20 passages.

5.7.1.3 THP-1

CCM

- RPMI 1640 medium (with L-Glutamine 25 mM HEPES), 500 mL
- 3.3 mL of 0.25 g/mL of D-Glucose stock solution
- 5 mL of 100 mM sodium pyruvate
- 5 mL of 1% Penicillin-Streptomycin (100 U/100 µg/mL)
- 10 % heat inactivated fetal bovine serum (50 mL)
- 2-mercaptoethanol[2] to a final concentration of 0.05 mM (500 µL of 50 mM in 500 mL culture medium

Culture / Maintenance

- Cells are grown in suspension in 25 cm² flasks (standing upright at 37°C, 5% CO_2) at a cell density between 2 and $8x10^5$ cells mL^{-1}
- To count the cells, transfer the cell suspension from the flask to a 15 mL tube; if you have multiple flasks, pool the cell suspensions in one 50 mL tube
- Take 10 µL of the THP-1 cell suspension and dilute 1:1 with Trypan Blue; ideally cell counts should be performed using an automated cell counter. Alternatively a manual count can be performed using a haemocytometer following the protocol given in the PATROLS '3D In Vitro HepG2 Spheroid model' SOP
    - If the concentration is $<8x10^5$ cells mL^{-1}: add 2-4 mL fresh culture medium; do not exceed a total volume of 20 mL/flask
    - If the concentration is $\geq 8x10^5$ cells mL^{-1}: passage cells by keeping $2x10^6$ cells in fresh culture medium (final volume: 10 mL); the flask can be re-used
    - Do not let the cell density exceed $1x10^6$ cells mL^{-1}
- It is recommended to start a new culture after a **maximum of 15 passages**. NB: It is possible that the culture has to be replaced earlier. The PMA responsiveness, morphology and reaction to LPS should be investigated regularly.

[2] Only for cell culture, not for co-culture experiments!

5.7.2 Epithelial co-culture

Experiments should preferably be performed with biological replicates in triplicate. Two different seeding ratios are possible for the epithelial transwell co-cultures, 9:1 and 8:2 Caco-2/E12 cells, respectively. The maintenance protocol is identical for both ratios. The required cell numbers for both ratios are given in Table 5.1.

Cell seeding and maintenance

- Prepare cell suspensions of Caco-2 and one of E12 cells as described in the sub-culture instructions in Section 5.7.1.1 and 5.7.1.2 above
- Perform cell counts as described for Caco-2 and E12 cells separately
- Add 1.5 mL Caco-2 CCM to a transwell-suitable 12-well plate
- unpack the needed amount of transwell inserts and place the inserts on the medium-containing wells of the 12-well plate
- from the concentration of the Caco-2 and E12 cell suspensions calculate the required volume to obtain the following cell numbers for a 9:1 or 8:2 ratio of the two cell types

Tab. 5.1 Required cell numbers for different seeding ratios of epithelial transwell co-cultures

	9:1 ratio (cells/transwell)	8:2 ratio (cells/transwell)
Total	1.62 x10^5	1.62 x10^5
Caco-2	1.46 x10^5	1.29 x10^5
E12	0.16 x10^5	0.324 x10^5

- Top up the medium in the apical compartment to a total of 0.5 mL/transwell
- Place plate at 37°C, 5% CO_2
- Change the medium on both the apical and basolateral compartment every 2-3 days, e.g. Monday, Wednesday, Friday; follow the scheme in Table 5.2 to

gradually transition the cells basolaterally from MEM-based Caco-2 to RPMI-based THP-1 medium (without mercaptoethanol!)

- Maintain the culture for up to 21 days before using the epithelial barriers to establish the triple culture

Table 5.2 Basolateral culture medium transition from MEM-based Caco-2 to RPMI-based THP-1 culture medium

Times of medium change	Caco-2 medium (µL)	THP-1 medium (ME-free, µL)
1st	1500	0
2nd	1000	500
3rd	1000	500
4th	750	750
5th	750	750
6th	500	1000
7th	500	1000
8th	0	1500
9th	0	1500

Monitoring of barrier development

- To routinely follow barrier development, measure TEER as described in **Section 5.7.3** of SOP "Triple culture of the intestine combining Caco-2, HT29-MTX-E12 and THP-1 cells" every 2-3 days; once the basic TEER readings stabilise, TEER measurements can be reduced, e.g. day 7 and 14 post-seeding, and 24h before the establishment of the triple culture with THP-1 cells

Day 2

- Check each flask for their response to PMA before proceeding
    - >90% of the cells should be firmly attached

- >50% of the cells should have developed a distinct macrophage-like phenotype

An example of how well-differentiated THP-1 cells should look like is given in Figure 5.1A. If the differentiation did not work well, cells will resemble the example image of Figure 5.1B.

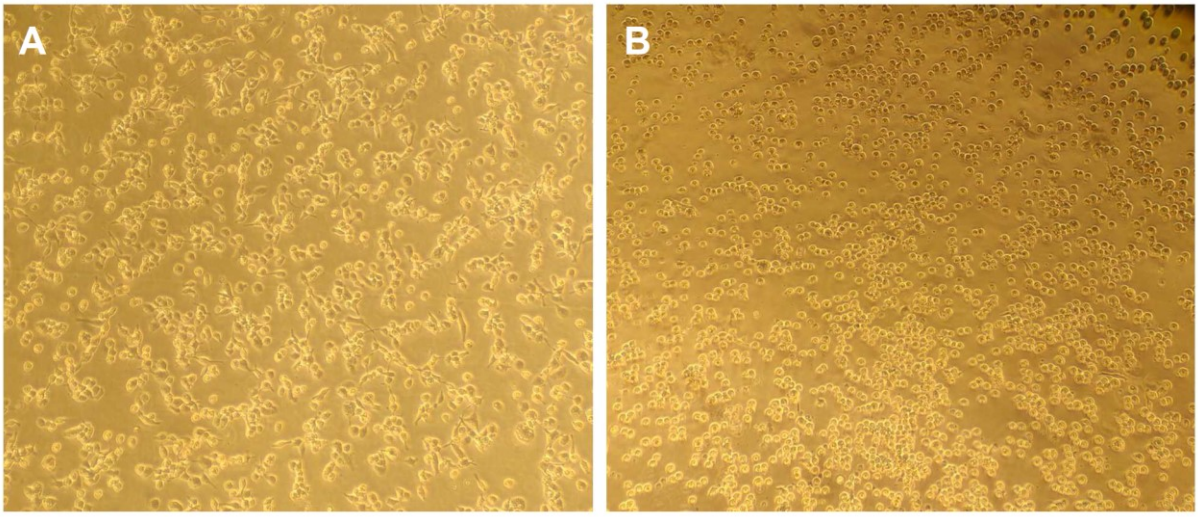

Fig. 5.1 Response of THP-1 cells to 24h PMA-exposure (A) Well-differentiated, (B) cells that did not respond well

- Discard the PMA-containing CCM and wash 2x with 5mL pre-warmed PBS
- Add 1 mL pre-warmed Accutase (1x) per flask
- Incubate for 5 min at 37°C
- Check under the light microscope for cell detachment; if many cells remain attached place back at 37°C for another 5 min
- If the cells are detached (Figure 5.2): add 3 mL fresh pre-warmed CCM to stop the Accutase activity
    - NB: The cells will not retain their amoeboid shape throughout Accutase detachment and subsequent re-attachment.
- Count cells (preferably with Trypan Blue) and re-seed $1.8x10^5$ THP-1 cells in a volume of 1,5 mL THP-1 cell culture medium containing ME onto transwell-suitable 12-well plates
- Place the cells on 12-well plates at 37°C, 5% CO_2 for 1-1.5h for re-attachment

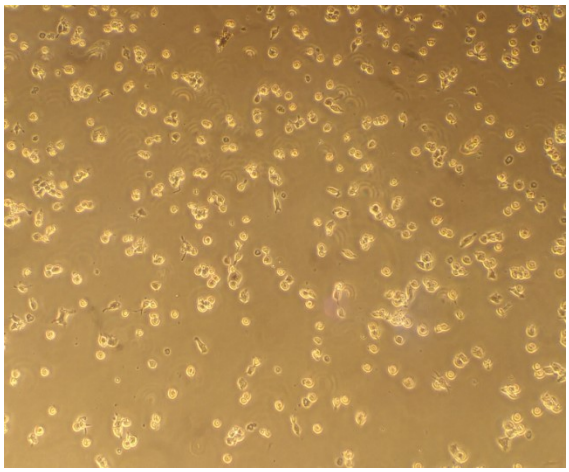

Fig. 5.2 Re-attached PMA-differentiated THP-1 cells

5.7.3 PMA-differentiation of THP-1 cells

Day 1

- Count THP-1 cells as described above (with Trypan Blue in 1:1 dilution)
- Seed 3x10^6 THP-1 cells in 5 mL THP-1 CCM (with ME) in a 25 cm² flask
- Take an aliquot of PMA (100 µM PMA stock solution) and add to the flask to obtain a final concentration of 100 nM PMA/flask
- **NB: PMA IS LIGHT AND TEMPERATURE SENSITIVE! Keep protected from light! Do not keep PMA at room temperature for more than 10 min!**
- Place the flask horizontally at 37°C, 5 % CO_2 for 24h
- NB: Prepare as many flasks as necessary; if possible, do not rely on one flask, even if few cells are needed
- Carefully discard the apical culture medium and replenish with 0.5 mL fresh pre-warmed MEM-based Caco-2 medium
- Carefully place the transwell inserts using sterile forceps in the wells containing re-attached PMA-differentiated THP-1 cells
    - The plate(s) can be used immediately for experimentation, e.g. exposure to substances
    - If plate(s) are not immediately used, place the plate(s) at 37°C, 5% CO_2 until further use / the first time point [e.g. in some circumstances it might

be preferable to let the co-culture establish and equilibrate itself for 24h before use]

- To follow up on the triple culture, measure TEER after 4, 24, and 48h (optionally also at 18 and 42h) of culture with THP-1 cells

5.7.4 Activation of THP-1 cells

- After THP-1 cells have re-attached on the 12-well plates, carefully discard the ME-containing culture medium
- Add 1.5 mL fresh, pre-warmed RPMI-based CCM without ME
- Prepare LPS/IFNγ stock solution
    - Add 400 µL pre-warmed RPMI-based CCM without ME to a 1.5 mL safe-lock tube
    - Take a working aliquot of LPS and IFNγ (10 µg mL^{-1}) from -20°C/-80°C and quickly thaw under the laminar flow hood
    - Add both compounds to the RPMI-containing tube, diluting both compounds 1:10
    - Mix well by inverting the tube several times carefully, do not vortex
- Add 15 µL of the LPS/IFNγ solution to each THP-1 containing well (1:100, 10 ng mL^{-1} activation concentration)
- Place the plate back at 37°C, 5% CO_2 for 4h

5.7.5 Triple culture establishment

- 4-5h before the start of the triple culture, discard the IFNγ-containing medium from the basolateral compartment
- Wash the basolateral compartment including the basolateral transwell filter side twice with 1 mL pre-warmed PBS
- Replenish the basolateral side with 1.5 mL fresh, pre-warmed ME-free RPMI-based CCM
- Place back at 37°C, 5% CO_2 for at least 4h and not more than 6h

- To start the triple culture, take a transwell plate with differentiated Caco-2/E12 cells out of the incubator and measure the TEER of each transwell as described in SOP 'Triple culture of the intestine combining Caco-2, HT29-MTX-E12 and THP-1 cells'
- Arrange the transwell inserts to triplicates based on the TEER readings as exemplified in Figure 5.3, so that the differences between the conditions are as low as possible

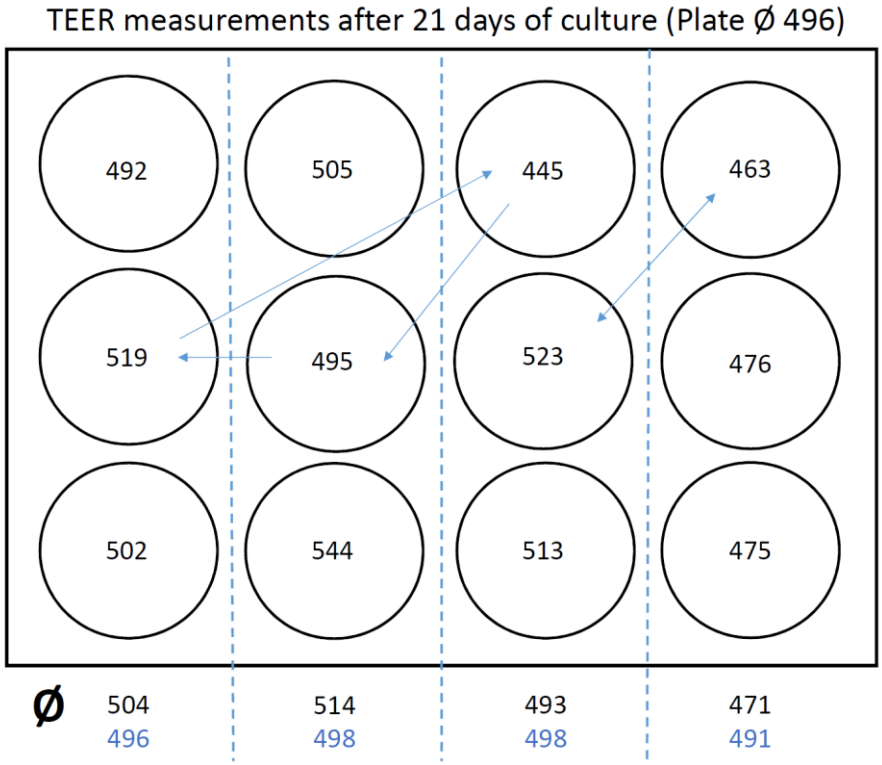

***Figure 5.3 Example**: How to arrange transwell inserts based on TEER measures (Ø black numbers = initial average of three transwells in a column, blue numbers = average of three transwells in a column after re-arrangement)*

- Carefully discard the apical culture medium and replenish with 0.5 mL fresh pre-warmed MEM-based Caco-2 medium
    - **In case of co-exposure experiments with BA:**

        - Weigh a sufficient amount of sodium butyrate to prepare a 100 mM solution
        - Add a sufficient amount of sterile PBS to the weighed sodium butyrate to obtain a 100 mM solution
        - Filter sterilize the solution before use
        - Prior to adding the CCM to the apical compartment, prepare a 1:100 dilution of the butyric acid stock in the culture medium to obtain a working concentration of 1 mM
        - Add 500 µL of the CCM + 1 mM BA to the apical side of the transwell
- Carefully place the transwell inserts using sterile forceps in the wells containing re-attached PMA-differentiated, LPS/IFNγ-activated THP-1 cells
    - The plate(s) can be used immediately for experimentation, e.g. exposure to substances
    - If plate(s) are not immediately used, place the plate(s) at 37°C, 5% CO_2 until further use / the first time point [e.g. in some circumstances it might be preferable to let the co-culture establish and equilibrate itself for 24h before use]
- To follow up on the triple culture, measure TEER after 4, 24, and 48h (optionally also at 18 and 42h) of culture with THP-1 cells

The epithelial co-culture and triple culture set-up is schematically summarized in Figure 5.4.

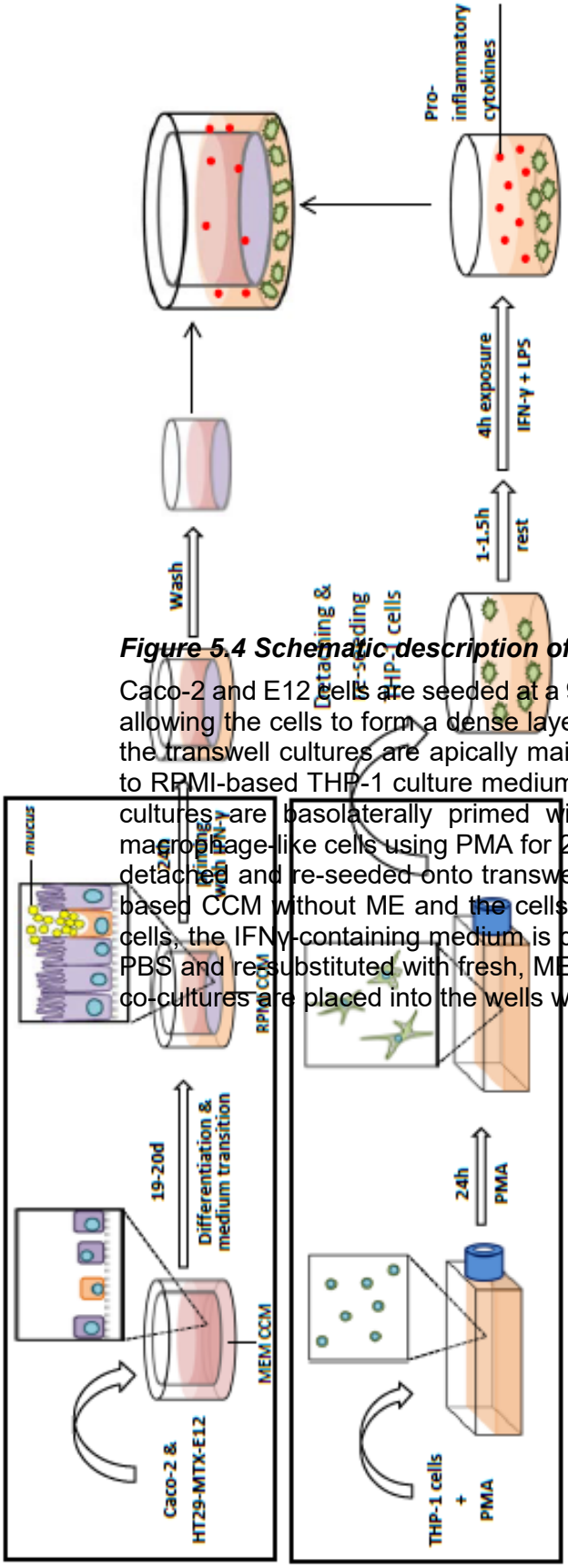

Figure 5.4 Schematic description of the triple culture set-up

Caco-2 and E12 cells are seeded at a 9:1 ratio onto transwell filters. This epithelial co-culture is ma
allowing the cells to form a dense layer and differentiate into enterocyte- and goblet-like cell types
the transwell cultures are apically maintained in MEM-based Caco-2 culture medium, they are ba
to RPMI-based THP-1 culture medium (Table 5.1). At 24h before the planned start of the triple cu
cultures are basolaterally primed with IFNγ (Section 5.7.3). On the same day, THP-1 cell
macrophage-like cells using PMA for 24h (Section 5.7.4). After 24h, the now adherent, amoeboid-
detached and re-seeded onto transwell-suitable well plates. After re-attachment, the their CCM is
based CCM without ME and the cells are activated with LPS and IFNγ for 4h (Section 5.7.5). Af
cells, the IFNγ-containing medium is discarded from the transwell cultures. The cell layers are ba
PBS and re-substituted with fresh, ME-free RPMI-based CCM. After 4h, the transwells containing
co-cultures are placed into the wells with activated THP-1 cells (Section 5.7.6)

5.8 Quality control & acceptance criteria:

The following quality control and acceptance criteria are specified for triple cultures based on 90:10 epithelial cultures. If the 80:20 ratio is chosen, results might differ.

5.8.1 Negative control

- with each inflamed-like triple culture, include 3 wells of a stable triple culture (according to PATROLS SOP 'Triple culture of the intestine combining Caco-2, HT29-MTX-E12 and THP-1 cells') treated with cell culture medium alone as a reference
- basolateral results (e.g. for cytokines, LDH release, …) of inflamed triple cultures should be compared to the levels of stable triple cultures (this should always be done when the cell lines, co- and triple cultures are newly established in a laboratory. Once the protocols are reproducibly established these controls do not have to be run routinely anymore.) Whereas the basolateral cytokine release should be strongly increased in the inflamed vs the stable samples, the LDH activity should not be significantly different.
- the apical results of cytokine and LDH release as well as DNA damage of the inflamed triple-culture should be compared to samples of he stable triple culture. The apical IL-8 release of inflamed co-cultures should be slightly elevated after 48h compared to the stable control. The LDH release should be increased by 30-50% at least. The DNA damage (quantified by alkaline comet assay) is only minimally affected after 48h.

5.8.2 Positive control

- DNA damage in differentiated Caco-2/E12 transwell cultures: 1 mM methyl methanesulfonate for 30 min
- Barrier disruption and cytotoxicity: Diclofenac (2 mM, 24h)
- THP-1 activation with LPS and IFNγ should always induce a significant release of IL6, IL8 and TNFα

5.8.3 Quality control for inflamed triple cultures

In addition to the endpoints recommended in PATROLS SOP 'Triple culture of the intestine combining Caco-2, HT29-MTX-E12 and THP-1 cells' – i.e. TEER, LDH activity, cytokine release, mucus secretion – the following controls are suggested:

1. DNA damage by alkaline comet assay
2. Mucin expression by RT-qPCR

1) Alkaline Comet Assay (based on NanOxiMet protocol 'Fpg-modified single cell electrophoresis')

Materials & Reagents

Reagent	Supplier / Cat. N°	CAS
NaCl	Roth, #0962.1	7647-14-5
EDTA	Sigma, #E9884	60-00-4
Tris base	Roth, #4855.2	77-86-1
NaOH	Merck, #1.064998.0500	1310-73-2
Demineralized H_2O	NA	NA
Agarose	Sigma, #A9539	9012-36-6
Low-melting point (LMP) agaorse	Sigma, #A9414	9012-36-6
Methyl methanesulfonate (MMS)	Sigma, #129925	66-27-3
EtOH	Roth, #9065.3	64-17-5
Glass slides*	e.g. Thermo Scientific Superfrost Plus, #10149870	NA
Cover slips	24x60mm e.g. Thermo Scientific, #10461541	NA
	24x50mm, e.g. Thermo Scientific, #10318963	NA
Electrophoresis chamber	NA	NA
Microwave	NA	NA
Diamond glass cutter	e.g. Sigma, #Z169064	NA
Metal tray	NA	NA

Pencil	NA	NA
Balance	NA	NA
Heating place	NA	NA
Microscope	e.g. Olympus BX60 microscope coupled with U-RFL-T UV burner	
Ice and Ice bucket	NA	NA
2 mL safe-lock tubes	e.g. Eppendorf, #0030120094	NA

**It is suggested to use adhesion microscopy slides to allow for better retention of the agarose gel. If possible, the use of slides with coloured marking area is suggested for easier identification and handling in the electrophoresis chamber*

Preparations

Lysis buffer (pH 10)

- 2.5 M NaCl 146.1 g L^{-1}
- 100 mM EDTA 37.23 g L^{-1}
- 10 mM Tris 1.21 g L^{-1}

Adjust pH to 10 using NaOH

➔ *NB: EDTA only starts to dissolve above pH 8. When EDTA dissolves, the pH decreases again (below pH 8) and the reaction is stopped. Regularly check and if necessary adjust the pH with NaOH until EDTA is completely dissolved.*

➔ Prepare for 1 litre but add only 900 mL water

➔ **add 10% DMSO and 1% Trion X-100 to the buffer shortly before use**

Denature/Electrophoresis buffer (pH 13)

- 0.3 µM NaOH ~15 g L^{-1}
- 1 mM EDTA 0.37 g L^{-1}

If the pH is <13 add more NaOH

Neutralisation buffer (pH 7.5)

- - 0.4 M Tris 48.44 g L^{-1}

  - Adjust pH to 7.5 by HCl

Agarose for slide coating

➔ Prepare 50 mL agarose solution by adding 50 ml PBS buffer to 0.75 g agarose in a 100 mL Erlenmeyer flask

➔ boil it in a microwave until the solution is translucent

Low melting point agarose

- 0.05 g in 10 mL PBS buffer

➔ Prepare solution in a small Erlenmeyer flasks with a wide top. Boil in microwave until the solution is translucent; put a cell culture dish on top to prevent PBS from evaporating

Agarose slide preparation

- Mark the slides with a pencil at the top of the corner (to allow easy distinction of upside and downside) and make a scratch with a diamond cutter at the end of the slide for an optimised agarose adherence (Figure 5.5)
- Dip microscopy glass slides into water to clean from dust and particles
- Let it air-dry over night

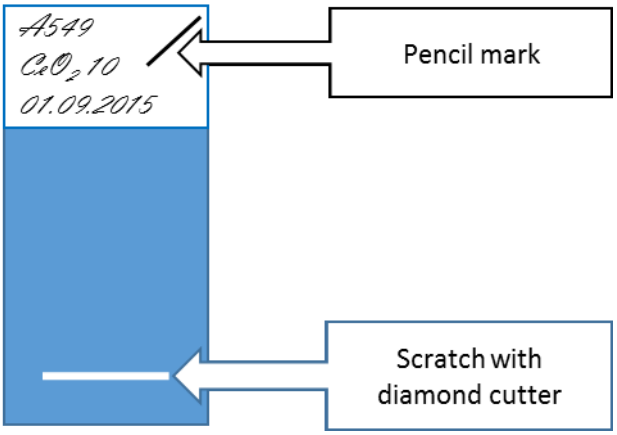

Figure 5.5 Marking of the slide. Label the slide with cell type, particle type and concentration and date (Source: NanOxiMet SOP)

- Heat up the hot plate (switch on 1 hour before gel slide preparation to adequate setting to obtain ~38°C)
- Fill beakers with water and place on the hot plate to later use as a water bath
- Prepare 50 mL agarose solution by adding 50 mL PBS buffer to 0.75 g agarose in a 100 mL Erlenmeyer flask
- boil it in a microwave until the solution is translucent
- Transfer the agarose to a 50 mL tube and place in the water bath beaker on the hot plate

  NB: Agarose has to be kept on the hot plate to avoid gelation

- Dip the slide in the agarose gel. **Wipe off the back side of the slide** with a tissue and put the slide face up on a tissue; let slides air-dry at least 3 hours (better overnight)
- Place the dry agarose-covered slides back into the packaging and mark with your initials and date

Positive control

Each cell line/type might require an individual positive control and/or exposure strategy.

The following chemicals and exposure strategies were tested successfully:

Cell type/specification	Chemical	Concentration	Time
Undifferentiated Caco-2	Etoposide	100 µM	1h
	MMS	1 mM	0.5 h
Undifferentiated E12	Etoposide	100 µM	1h
	MMS	1 mM	0.5 h
Caco-2/E12 transwell cultures	MMS	1 mM	0.5 h

The following substances were tested in differentiated epCC but did not result in useful outcomes: etoposide, H_2O_2, chlorpyrifos

Sample generation from 12-well transwells

- after supernatants were collected, transwell filters are washed twice on both apical and basolateral side with pre-warmed PBS
- prepare a dry surface (e.g. a new 6-well plate of the lid of the 12-well plate by pipetting a drop of 100 µL pre-warmed trypsin for every transwell onto it
- discard the PBS wash from both apical and basolateral side
- carefully place the transwell insert with the basolateral side onto the trypsin drop
- add 100 µL pre-warmed trypsin to the apical side of each filter
- place the filters at 37°C, 5% CO_2 for 10 min
- after 10 min, check for cell detachment under the microscope

NB: the detachment will not be strongly visible. However, the cells will be increasingly spherically shaped.

- To stop the trypsin activity, add 300 µL of cold. 20% FBS-containing MEM-based CCM
- Vigorously pipet the medium/trypsin mix up and down to detach the cells from the transwell filter
- Transfer the cell suspension to a 2 mL safe-lock tube and place on ice immediately

Slide loading with cell samples

- 3h before the sample generation, take sufficient aliquots of LMP agarose (240 µL per sample) and heat up to 95°C for 1h. Once the LMP agarose is liquified, reduce heat to ~37°C to keep LMP agarose liquid
- It is recommended to take a sample from the cell pellet that has formed at the bottom of the tube instead of vigorously resuspending the cell pellet.
- Take 40 µL cell suspension and add to 240 µL low melting point agarose in a 1.5 mL safe-lock tube
- Pipette 120 µL of the cell mixture in the middle of a pre-coated gel slide.
- Cover it with a cover slip → the cell suspension should be spread evenly over the whole slide without bubbles

NB: prepare two slides for each sample

- Place the slide on an ice-cold metal tray until the LMP agarose/cell mixture has solidified (10-15 min should be sufficient)
- While the gel solidifies, prepare the lysis buffer
    - Calculate with ~100 mL lysis buffer per vertical staining cuvette
    - Prepare a 10% DMSO/1% Triton-X100 solution
    - E.g. if staining cuvette are needed: prepare 180 mL lysis buffer and a mixture of 18 mL DMSO and 2 mL Triton-X100. Just before use add the DMSO/Triton mix to 180 mL lysis buffer and mix well
- Carefully remove the cover slip but pulling it off from the short side of the slide
- Take two slides together back to back and place them into a vertical staining cuvette
- Add the prepared complete lysis buffer to the cuvette

NB: Ensure that the whole gel-part of the slide is covered by lysis buffer!

- Wrap the cuvette with aluminium foil keep it in the refrigerator at least overnight and up to a week

!!! The lysis buffer will leave the nuclei unprotected and, therefore, extremely vulnerable to post-experimental damage, which might lead to false-positive

results. Ensure that the slides are not exposed to light from this stage on until the end of the electrophoresis!!!

Electrophoresis

To avoid light damage, use red light as light source. It might be necessary to adjust to the light for several minutes. Be extremely careful until your eyes adjusted to the light conditions as to not hurt/endanger yourself or damage material/instruments or samples.

- Discard the lysis buffer and wash the slides 3 times for 5 minutes in ice-cold water
- Place the slides gel-side facing up in the electrophoresis chamber in the same direction (Figure 5.6)

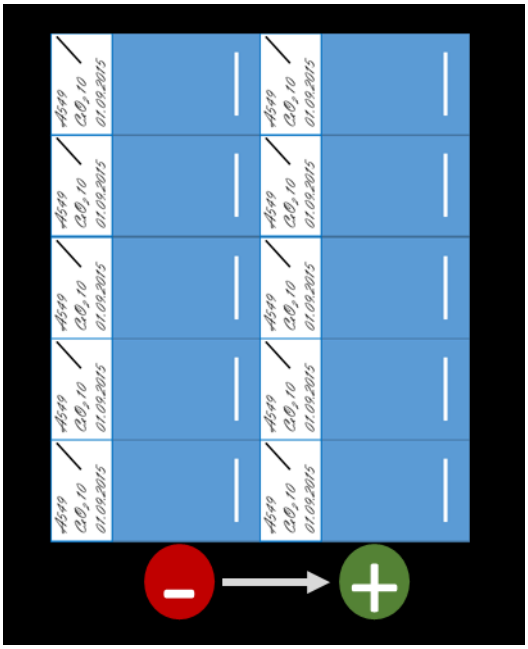

Figure 5.6 Slide orientation in the electrophoresis chamber.

NB: Depending on the size of the electrophoresis chamber it might be necessary to think about your slide arrangement. As the electrophoresis is always slightly different between individual runs, each run should contain a negative and positive control

- Pour Denature/Electrophoresis buffer into the chamber so that the slides are completely covered by a thin film of the buffer; volume is depending on electrophoresis tank and amount of sample slides

- Cover the chamber with the lid and incubate for 20 min to allow the unwinding of the DNA strands to take place
- Before running the electrophoresis check that all slides are in the correct arrangement and direction
- Turn on the power supply and set the voltage to 24-26V (check: V/cm) for IUF tank: adjust current to 280 mA

NB: the voltage and current can be adjusted via the buffer volume. Addition of buffer results in a reduction of voltage and increase in current. Removal of buffer causes an increase in voltage and reduction in current.

- Run the electrophoresis for 10 minutes
- After 10 min switch off the power supply
- Carefully take the slides from the chamber and place, back to back, in a vertical staining cuvette
- Add ice-cold neutralisation buffer to the cuvette and wash for 5 min, repeat twice
- Add 100% EtOH to a vertical staining cuvette (if not used, place a lid on the cuvette to avoid excessive evaporation); carefully take two slides at the time from he neutralisation buffer and swing the slides in 100% ethanol
- Place slides on absorbent paper and allow to air-dry for 0.5-2h

Ethidium bromide staining

Work with a red light source

- Ethidium bromide (EB) is kept at 4°C
- Prepare a 1:100 dilution of ethidium bromide in H_2O (calculate how much you will need by multiplying your number of slides and the required volume per slide (i.e. 40 µL)
- Pipet 40 µL of the ethidium bromide solution onto each slide, place a cover slip on each slide to evenly distribute the ethidium bromide solution
- Place all slides in slide book at keep at 4°C protected from light until analysis

NB: slides are stable for several weeks. However, at some point the agarose becomes brittle and the slides will be increasingly difficult to analyse.

Microscopic analysis/Scoring

NB: This part is only applicable to the instruments and software specified here. Individual adaption might be necessary.

- Turn on the UV lamp (Olympus U-RFC-T) and the microscope (Olymbus BX 60) at least 5 minute before use
- Before start the scoring make sure that the camera is connected
- Turn on the green filter (unmarked) and choose the objective with a 40x magnification
- Turn on the computer
- Open the software **Comet Assay IV** (Desktop)
    - User ID: Supervisor
    - Password: password
  - OK
- Select configuration: **default.cfg**
  - OK
- Select a proper site on the slide
- Move out the lever to transfer the image to the software
- 
- Click on the head of a comet
- The software defines automatically the start of the head (blue line), the middle (green line) of the head and the end of the comet (red line)
- Score 50 nuclei / comets per slide
- It is recommended to express the results as % DNA in tail derived from the averaged results of two slides ± standard deviation

2) Quantification of mucin expression by RT-qPCR

Materials & Reagents

Substance	Supplier, Cat Nr	CAS
DNAse	Sigma, #AMPD1-1KT	NA
Ethanol	Roth, #9065.3	64-17-5
iCycler iQ External Well Factor Solution	Bio-Rad, #170-8794	Mixture
iQ™ SYBR® Green Supermix	Bio-Rad, #170-8884	Mixture
iQ5 PCR detection Systems	Bio-Rad	NA
iScript™ cDNA Synthesis Kit	Bio-Rad, #170-8890	Mixture
Nuclease free water	Qiagen, #129114	NA
PBS	Sigma, #D8537	NA
PCR Thermocycler	--	--
Spectrophotometer	--	--
qPCR film	VWR, #732-3228	NA
Primer Pairs	Eurofins	NA
Roche High Pure RNA Tissue Kit	Sigma, #12033674001	
SuperPlate PCR Plate, 96-well, semi-skirted	VWR, #732-1493	NA
uDROP Plate	Thermo Scientific, #N12391	NA

RNA isolation from detached cells

- Pre-cool centrifuge to 4°C
- Collect the cells in 2 ml Eppendorf Vials and centrifuge for 5 min, 300 g at 4°C
- Discard medium, add 500 µL ice-cold PBS to one safe lock tube and re-suspend cell pellet; use this volume to re-suspend the other replicates of that condition and pool replicates
- Centrifuge at 350 g for 5-10 min (fewer cells = longer centrifugation)
- Discard PBS carefully, re-suspend cells in 500 µL fresh ice-cold PBS
- Centrifuge at 350 g for 5-10 min

- Discard PBS and suspend cell pellet in 200 µL ice-cold PBS
- Add 400 µL Lysis buffer and vortex for 15 s (from now on all steps, including centrifugation, should be performed at room temperature)
- Put Filter Tube into collection Tube, transfer sample into Filter Tube, centrifuge 20 s at 8,000 g and discard the liquid
- For 6 samples add 63 µL DNase I to 567 µL DNase I Incubation Buffer and add 100 µL of the mixture to the filter
- Incubate for 15 min at room temperature
- Add 500 µL Wash Buffer I, centrifuge 20 s at 8,000 g and discard the liquid
- Add 500 µL Wash Buffer II, centrifuge 20 s at 8,000 g and discard liquid
- Add 200 µL Wash Buffer II, centrifuge 2 min at 13,000 g and discard liquid and collection tube
- Insert filter tube into 1.5 mL Eppendorf vial, add 30 µL Elution Buffer and centrifuge 1 min at 8,000 g
- If you do not continue with cDNA synthesis the same day, store RNA at -80°C

Second DNase I digestion (Sigma #AMPD1) and reverse Transcription

The DNase I digestion that is performed with the Roche High Pure RNA Tissue Kit might not be sufficient to remove all residual gDNA from the RNA. You can check this by performing a qRT-PCR of your sample and the according no reverse transcriptase control for 2 or 3 genes. Preferably test highly expressed genes. If there is residual gDNA, the no reverse transcriptase control will give a signal and T_M will either correspond with the melt peak of the sample or show a higher T_M (DNA containing introns). If there is residual gDNA, a second DNase I digestion is recommended.

- Thaw reaction buffer and stop solution.
- Quantify RNA with µDROP plate (see above; no previous heating step)
- For each sample transfer 3x0.5 µg = 1.5 µg RNA into a 0.2 ml Eppendorf vial.
- Top up to 24 µl with nuclease free H_2O.
- Add 3 µL reaction buffer.
- Add 3 µL DNase I.

    - DNase I does not congeal at – 20°C. Do only take it out of the freezer when it is added and maintain it on ice.
- Mix thoroughly by pipetting up and down.
- Incubate for 30 min at room temperature and pipet up and down again.
- Thaw iScript buffer in the meantime.
- After 30 min more at room temperature, add 3 µL stop solution and mix well.
- Use the program '70gradinactiv' at thermal cycler to perform 10 min heat inactivation of DNase I.
- Spin down samples.
- Split each sample into 3x 11 µL.
- For no reverse transcriptase control, add 5 µl nuclease free H_2O and 4 µL iScript buffer to one of three samples (prepare Mastermix before).
- For reverse transcription, add 4 µL nuclease free H_2O, 4 µL iScript buffer and 1 µL reverse transcriptase to two of three samples (prepare Mastermix before).
- Subsequently, follow the Reaction Protocol for cDNA synthesis (see above)

Reaction mix preparation

- Thaw cDNA, 10x primers and iQ SYBR Green Supermix (protected from light) on ice
- Switch on PCR machine 10 minutes before starting the Run (each of the two modules has an individual Power Switch!)
- Prepare on ice enough master master mix for all reactions by adding all required components, except the cDNA template, then protect from light:
-

Components	**Volume per Reaction**
iQ SYBR Green Supermix	12.5 µL
10x Forward primer	2.5 µL
10x Reverse primer	2.5 µL
H_2O	2.5 µL
	---
	20 µL

- Pipet 5 µL cDNA (1:15 diluted) in each well of a 96-Well PCR plate
- Mix the assay master mix thoroughly to ensure homogeneity and pipet 20 µL into each well of a 96-Well PCR plate
- Seal plate with a transparent foil and protect from light

Realtime PCR measurement

Program thermal cycling protocol on the real-time PCR machine:

Setting/Mode	Polymerase activation and DNA Denaturation	Amplification		Melt curve Analysis
		Denaturation	Annealing/extension and plate read at an optimized temperature	
Temperature	95°C	95°C	60°C	55-95°C
Time	3 min	15 sec	45 sec	-

40 cycles

How to set these parameters and design the plate layout will depend on the type of instrument (manufacturer, model, etc.) and is therefore not described in detail.

The following primer pairs and concentrations were tested to be working well for the here described model set-up:

Table 3 Human primer pairs for the detection of IL8, mucins (MUC 1, 2, 5AC, 13, 20) and the reference genes β-actin, GAPDH, RPLP0

Gene	Sequence (5' → 3')	Working conc.	Amplicon
b-actin	fw CCTGGCACCCAGCACAAT rv GCCGATCCACACGGAGTACT	60 nM 60 nM	70 bp
GAPDH	fw CCCCCACCACACTGAATCTC rv GCCCCTCCCCTCTTCAAG	37.5 nM 37.5 nM	65 bp
IL8	fw ACTCCAAACCTTTCCACCC rv CCCTCTTCAAAAACTTCTCCAC	60 nM 60 nM	168 bp
MUC1	fw AGACGTCAGCGTGAGTGATG rv GACAGCCAAGGCAATGAGAT	37.5 nM 37.5 nM	139 bp
MUC2	fw GTCCGTCTCCAACATCACCT rv GCTGGCTGGTTTTCTCCTCT	60 nM 60 nM	287 bp
MUC5AC	fw CAGCACAACCCCTGTTTCAAA rv GCGCACAGAGGATGACAGT	60 nM 37.5 nM	100 bp
MUC13	fw CAGAGACAGCCAGATGCAAA rv CGGAGGCCAGATCTTTACTG	60 nM 37.5 nM	175 bp
MUC20	fw GTGCAGGTGAAAATGGAGGT rv ACGCAGTAAGGAGACCTGGA	60 nM 37.5 nM	152 bp
RPLP0	fw TCCTCGTGGAAGTGACATCG rv CTTGGAGCCCACATTGTCTG	37.5 nM 37.5 nM	174 bp

3) Cytokine release by enzyme-linked immuno-sorbent assay (ELISA)

Materials & Reagents

Substance	Supplier, Cat Nr	CAS
R&D DuoSet antibodies	R&D; TNF-a: DY210; IL-8: DY208; IL-6: DY206	NA
NaHCO3	e.g. Roth, # HN01.1	144-55-8
Demineralised H2O		
Tween-20	e.g. Sigma, #P1379	9005-64-5
PBS (non-sterile)	e.g. Sigma, #D8537	NA
Bovine Serum Albumin (BSA)	e.g. Sigma, #A7906	9048-46-8
H2SO4	e.g. Sigma, #258105	7664-93-9
TMB Peroxidase EIA Substrate Kit	Bio-Rad, #1721067	Mixture
Nunc Maxisorb 96 well plates	Sigma, # M9410	--
Spectrophotometer	--	--

To prepare

Coating buffer:

- Weigh 4.2 g $NaHCO_3$
- Dilute in 500 mL MilliQ H_2O to obtain 0.1 M $NaHCO_3$
- Adjust pH to 8.2
- Aliquots (12 mL) can be stored at -20°C

Washing buffer:

- Add 0.25 mL TWEEN-20 to 500 mL PBS (pH 7.2) (→ 0.05 %)
- Store at room temperature (RT)

Blocking buffer

- Weigh 15 g BSA

- Solve in 500 mL PBS (→ 3% BSA/PBS)
- Filter before use or storage
- Aliquots (50 mL) can be stored at -20°C

Stop solution

- Prepare 1M H_2SO_4 by slowly adding 27.8 mL (of 18 M stock) to 473 mL H_2O [NB: throughout the mixing, an exothermic reaction takes place. Carefully add the acid to H_2O in several steps using low volumes each time]

Day 1

Plate preparation

- Calculate the amount of samples you will have per cytokine (NB: shall samples be analysed in duplicates or single? Don't forget the standard curve, i.e. 2x7 concentrations plus 2x blank)
- Dilute capture antibody (ab) to the indicated concentration in coating buffer
- Add 100 µL of primary antibody solution to each well
- Seal / cover plate and incubate over night at room temperature (RT)

Day 2

- remove liquid and primary ab from wells
- wash 3xwith 200 µL washing buffer
- after the last wash, thoroughly tab the plate on absorbent paper to remove residual buffer

Blocking

- add 200 µL blocking buffer to each well and incubate for at least 1h
- remove the liquid and wash plate 3x with 200 µL washing buffer
- after the last wash, thoroughly tab the late on absorbent paper to remove residual buffer

Standard curve

- prepare a standard dilution range for each cytokine tested *[NB: concentrations of stock and concentration range required is cytokine-dependent and needs to be confirmed in R&D-supplied documents]*
- analyse each concentration in duplicate

Samples

- add 100 µL of your samples or standard to each well [it might be necessary to prepare dilutions of samples in case the cytokine concentration exceeds the upper detection limit] → incubate for 2h at RT
- discard liquid and wash 4 times with 200 µL washing buffer
- after the last wash, thoroughly tab the late on absorbent paper to remove residual buffer

Secondary ab

- prepare secondary ab at required concentration (check R&D sheets as this might differ between cytokines and LOTs) in reagent diluent (1% BSA in PBS)
- add 100 µL of secondary ab to each well and incubate for 2h at RT
- discard the liquid and wash plates 3 times with 200 µL washing buffer
- after the last wash, thoroughly tab the late on absorbent paper to remove residual buffer

HR-peroxidase

- prepare HRP in reagent diluent (1:40) and add 100 µL to each well; incubate for 30 min at RT protected from light
- discard the liquid and wash plates 3 times with 200 µL washing buffer
- after the last wash, thoroughly tab the late on absorbent paper to remove residual buffer

TMB

- mix ELISA kit TMB substrate A and B 9:1 and add 100 µL to each well; observe the colour reaction but incubate for at least 20 min, better 30 min
- stop the reaction by adding 50 µL Stop solution to each well
- measure plate at 450nm

If possible plot standard curve as 4-parameter log fit instead of as linear function. 4PL model equation has a maximum and a minimum built into the model which are more reasonable to describe biological systems. There is no biological system that will increase or decrease forever as the curve goes to infinity (basically what a linear curve fit does).

5.8.4 Acceptance criteria for triple cultures

1) For Caco-2/E12 epithelial co-cultures

The same acceptance criteria as laid out in SOP 'Triple culture of the intestine combining Caco-2, HT29-MTX-E12 and THP-1 cells' apply

2) Inflamed triple culture

- 4h after the start of the triple culture, the TEER (Figure 5.7) should be reduced by at least 10-15%
- The maximum of TEER reduction should be reached after 24h
- After 24h, the TEER of the inflamed triple culture should gradually increase again
- The TEER should not be significantly reduced anymore compared to the stable triple culture after 72h
- In presence of BA in the inflamed triple culture, the minimum TEER is increased from 74 ± 6 % to 79 ± 6 after 18h.

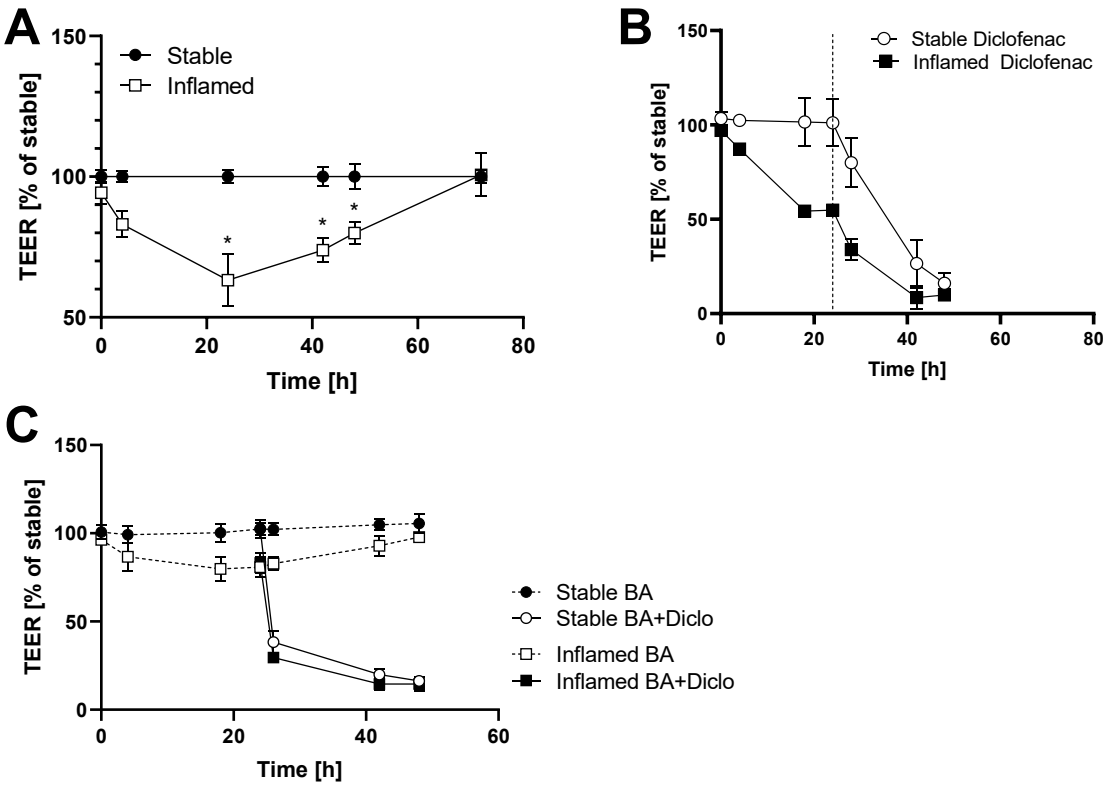

Figure 5.7 Barrier integrity measured as TEER of (A) inflamed triple cultures, (B) stable and inflamed triple cultures treated with Diclofenac and (C) triple cultures treated with butyric acid and co-exposed to Diclofenac compared to the stable triple culture control (average ± SD, N=3; Diclofenac N=2)

Cytokine release (Figure 5.8)

- The apical release of IL8 might be slightly increased after 48h and >96h of inflamed triple culture
- The basolateral levels of TNFα, IL8 and IL6 should be highly increased from 24h on
    - NB: these cytokines follow very different kinetics
    - TNFα should be detectable early on (t_4) at high concentrations and reach its maximum at t_{24}
    - IL6 is minimally detectable at t_4 and but should gradually increase over time
    - IL8 should be highly released at all time points and increase continuously throughout the culture period.

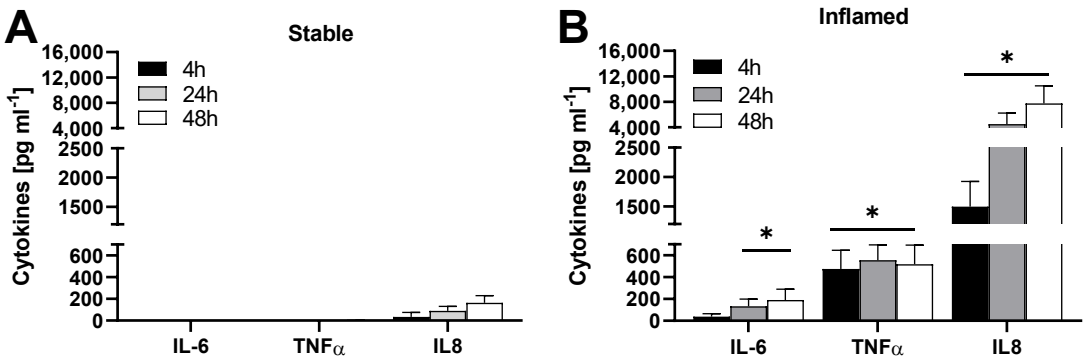

Figure 5.8 Release of TNFα, IL6 and IL8 in (A) stable and (B) inflamed triple cultures after 4h, 24h, and 48h (average ± SD, N=3)

LDH release (Figure 5.9)

- The basolateral cytokine release might be slightly increased. This increase is usually not statistically significant.
- The apical LDH activity should be increased by 30-50 compared to the stable culture control after 48h. At earlier time points, the apical LDH activity might be slightly – but not significantly – increased.
- After treatment with Diclofenac (2 mM) for 24h high LDH activity is detected in AP and BL supernatants of both stable and inflamed triple cultures; the release if overall higher in inflamed conditions
- In presence of 1 mM BA, the LDH release after Diclofenac exposure is increased further in both stable and inflamed triple cultures

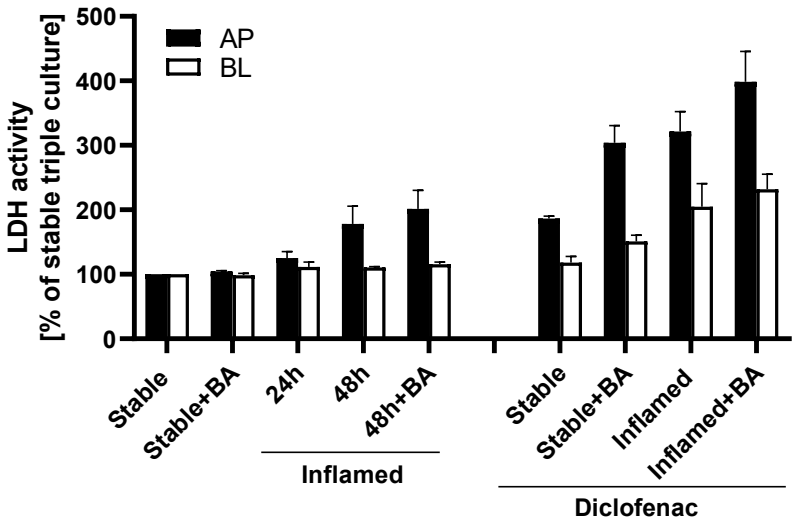

Figure 5.9 LDH activity in stable (S) and inflamed (I) triple cultures after 24h and 48h, with and without BA treatment and / or exposure to Diclofenac (2 mM) (average ± SD, N=3; Diclofenac w/out BA N=2; control = LDH activity in stable triple cultures at the respective time point)

DNA damage (Figure 5.10)

- The baseline DNA damage in stable triple cultures is ~5% after both 48h and 103h
- After 48h inflamed triple culture the quantified DNA damage was not considerably increased compared to the stable culture
- The treatment with 1 mM butyric acid did not cause an effect in either model
- MMS (1 mM, 30 min) should yield a DNA damage of >25%

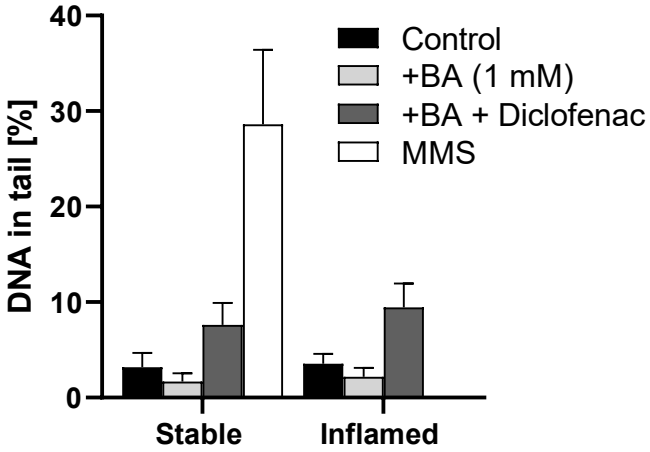

Figure 5.10 DNA damage quantified as % DNA in tail by alkaline comet assay after 48 stable (S) and inflamed (I) triple culture (Control), after 48h incubation with butyric acid (+BA) and co-exposure of butyric acid and Diclofenac (average ± SD, N=3; MMS was used as positive control)

Mucin expression (Figure 5.11)

After 48h inflamed triple culture (A)

- Expression of IL8 should be notably but not extremely elevated
- Expression of MUC1 is highly increased
- Expression of MUC2, MUC5AC and MUC20 is reduced, whereas MUC13 is not changed considerably

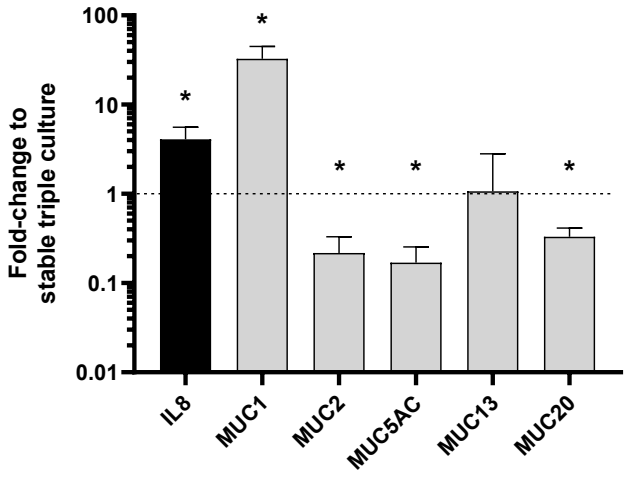

Figure 9 Gene expression of IL8 and the mucins MUC1, MUC2, MUC5AC, MUC13 and MUC20 in 90:10 Caco-2/E12 epithelial cultures after (A) 48h inflamed triple culture (average ± SD of N=3; results were normalised against stable triple cultures as control and β-Actin as reference gene. The depicted fold changes were derived from the mean of the ΔΔCT-values)

6 Data Analysis and Reporting of Data:

6.1 TEER

- Express the TEER values as % of the negative control (i.e. the average of the epithelial co-culture triplicates)
- In case of exposure experiments, e.g. using ENM, expressing the TEER results as % of the untreated triple culture control is recommended

6.2 Cytokine release

- The standard curve should be plotted as 4-parameter logfit unless the supplier/manufacturer of supplies states otherwise
- The cytokine release can be expressed as
    - total values (usually pg mL^{-1} or ng mL^{-1})
    - fold increase compared to the negative control
- basolateral results of stable co-cultures should initially be compared to the cytokine levels of PMA-differentiated THP-1 monocultures

6.3 LDH release

- The LDH release can be expressed as
    - absorbance value
    - total value (usually pg mL^{-1}) if a standard curve is prepared
    - fold increase compared to the negative control

6.4 DNA damage

- the DNA damage should be evaluated on 2 slides per condition of three biological replicates and at least 3 independent experiments.
- The results can be expressed as
    - Absolute percentage of DNA in tail
    - Relative change to the stable culture control

7 Publications:

The basic principles of this method are described in

KÄMPFER, A. A. M., URBAN, P., GIORIA, S., KANASE, N., STONE, V. & KINSNER-OVASKAINEN, A. 2017. Development of an in vitro co-culture model to mimic the human intestine in healthy and diseased state. *Toxicol In Vitro,* 45**,** 31-43.

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