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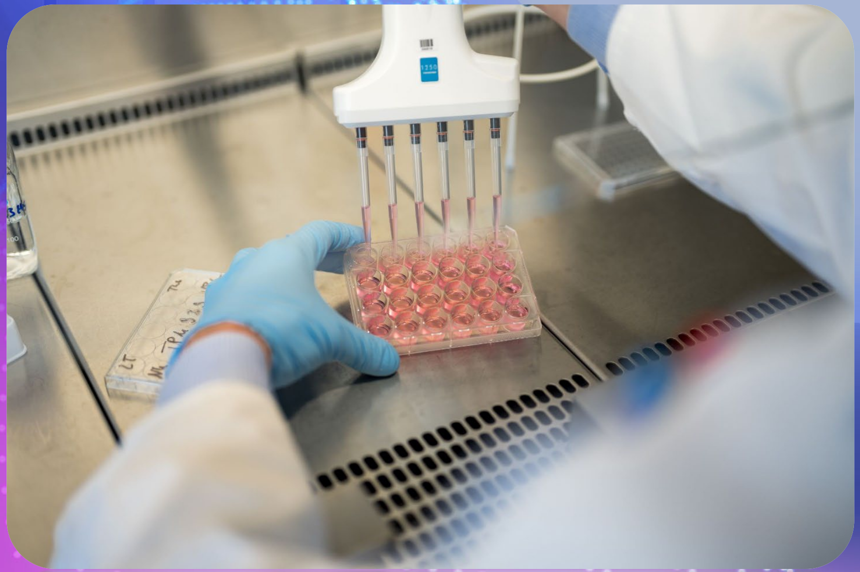
NEXT GENERATION PRODUCTS

Optimisation and validation of the µFlow *in vitro* micronucleus test using TK6 cells



Edgar Trelles Sticken

2024-10-14 CORESTA Edinburgh

Theme of the conference

“ADVANCING TOBACCO HARM REDUCTION THROUGH SCIENTIFIC COLLABORATION”

- CORESTA offers an excellent platform for collaborative research and publishes guidelines to ensure companies adhere to scientifically sound product assessment practices.

- For tobacco product assessment the CORESTA *in vitro* toxicity Test battery recommends a cytotoxicity assay (NRU), and a bacterial mutagenicity assay (Ames assay) paired with a mammalian genotoxicity assay (micronucleus assay or mouse lymphoma assay or chromosome aberration assay [IVT-225-CTR]). Extensions for NGP testing are under preparation.

- Focus of the presentation: implementation and optimisation of the flow cytometric *in vitro* micronucleus test (IVMNT) as screening tool for conventional and NGP testing.

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The *in vitro* Micronucleus Test (IVMNT)

- Is a well established method to identify the genotoxic potential of chemical substances or complex mixtures like smoke from combustible tobacco, and aerosols/extracts of non-combustible next generation products or also neat E-liquids.
- In our collaborative scientific endeavours, the micronucleus assay serves as a critical component and provides valuable data for the support of regulatory submission and the concept of harm reduction.
- CORESTA directed proficiency tests indicate potential for standardisation (e.g., cell lines, evaluation method etc.).

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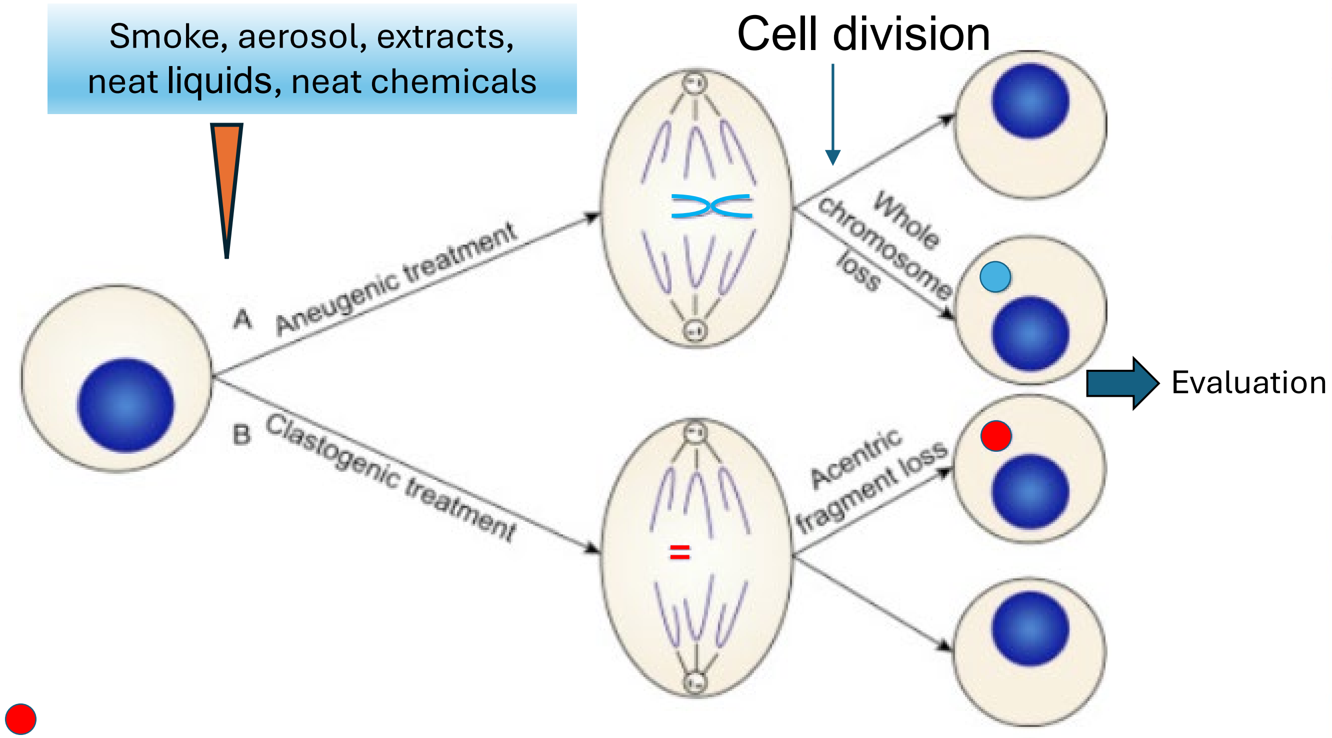
Mechanisms of micronucleus induction

Test substances (smoke, aerosols, extracts, neat liquid, neat chemicals) can act as clastogens or aneugens.

Aneugen: Act on the cytoskeleton thereby interfering with the distribution of chromosomes leading to micronuclei consisting of a **whole chromosome** in one of the daughter cells.

Clastogen: induce chromosome breaks
➔ leads to micronuclei consisting of chromosome fragments in a daughter cell.



→ Increase in MN frequency indicates genotoxic potency

Image from: Genetic Toxicology Testing | ScienceDirect

Evaluation Methods

- Manual microscopy: labour-intensive, time-consuming, variable (interindividual).
- Automated microscopy: advanced systems for micronuclei detection; may need verification for false positives (semi-automated [SAM]).
- High content screening: combines automated microscopy with image analysis for multiparameter measurements.
- Flow cytometry: laser-based cell/nuclei analysis in a fluidic stream; high sensitivity and specificity; can also be paired with biomarkers for mechanistic insights.

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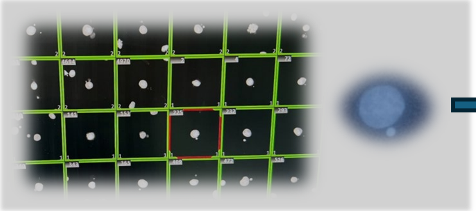
Methods used at Imperial's laboratories

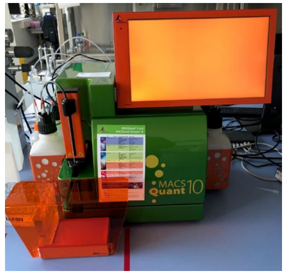
Semi-Automated microscopy (SAM) and Flow cytometry (FC) (both without Cytochalasin B)

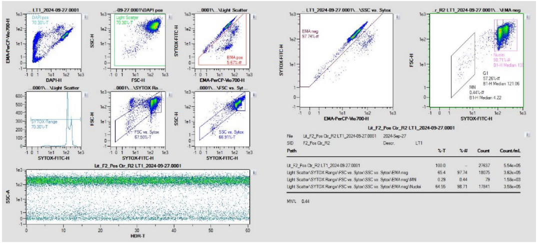
Metafer (SAM)



Cells	Total Particulate Matter	E-liquids, OND extracts, neat chemicals	Fresh whole smoke/aerosol at the ALI
V79 Chinese hamster lung fibroblasts	SAM	SAM Substituted by	SAM
TK6 Human lymphoblasts suspension cell line	FC	FC	NA

MACSQuant X (FC)





Implemented
& partially accredited

NA: Not Applicable FC: full validation not finalised

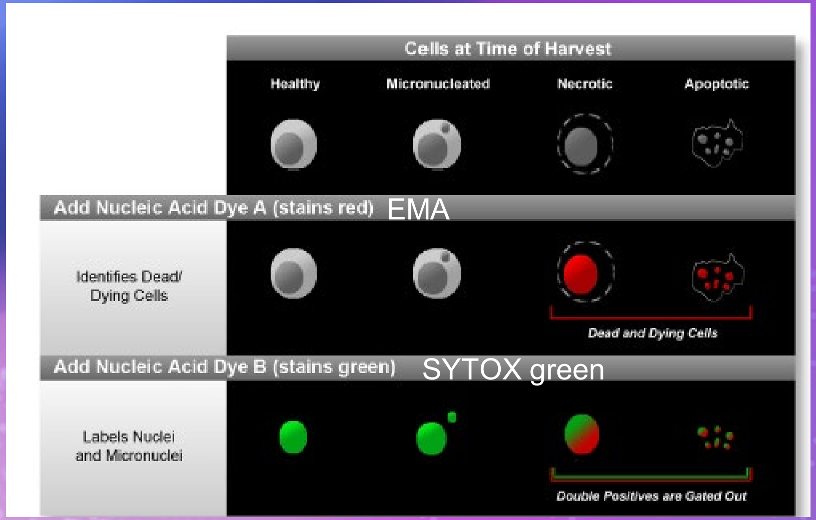
FC: ISO 17025 accredited; ALI: Air liquid Interphase

OECD requirement vs. first results

- Flow cytometric procedure described in the commercially available µFlow kit worked fine *per se.*

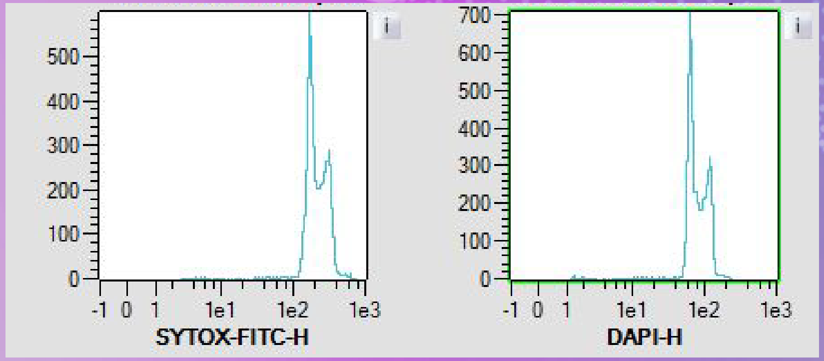
- ***But*** background frequency varied too much
  - Problem: OECD requires that statistically significant increases in MN frequencies from an experiment should also be significantly increased when compared to the lab's historic negative control data base to be deemed positive. With high background variability the comparison might result in an insignificant comparison → **false negative.**
  - Needed to find a way to reduce background variability.

Staining and gating strategy for flow cytometric evaluation

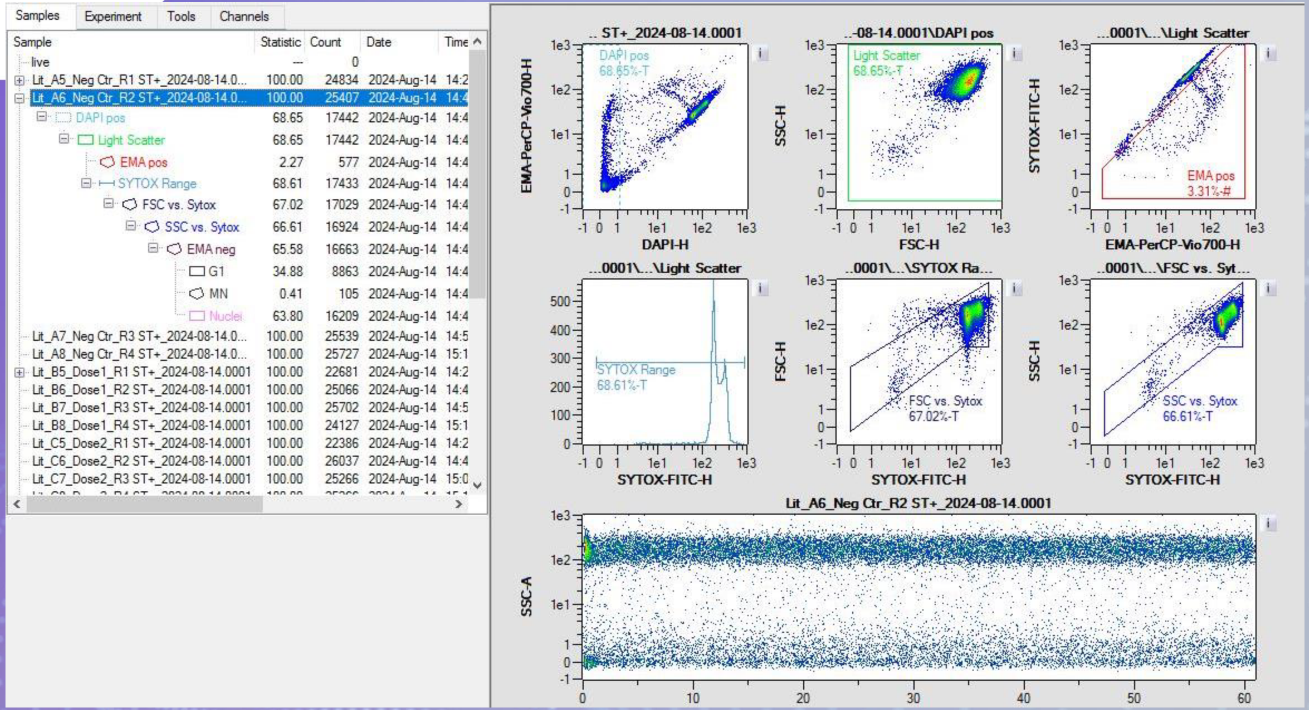


+ DAPI in last step

- Quenching of Sytox by DAPI?



Parallel stains result in stable signals



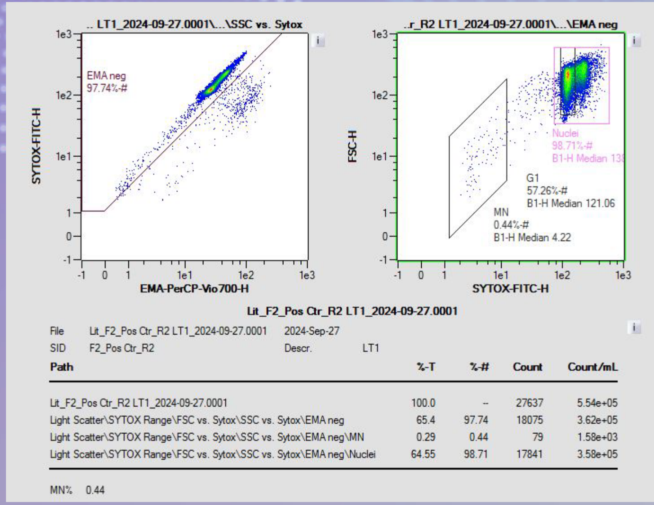
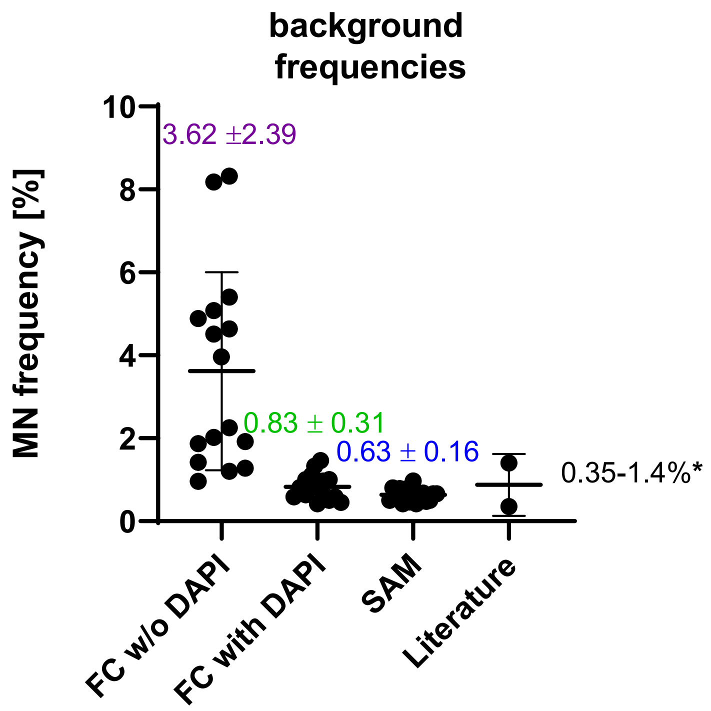


Image from Litron Laboratories homepage: "Instruction-Manual-In-Vitro-MicroFlow-For-rea.aspx(litronlabs.com)"

MN frequency comparison to microscopy method and literature data under ST+S9 schedule



Test with a negative genotoxicity E- Liquid

Test	MN Mean (n=4) by short Term +S9 [%]	
	w/o DAPI correction	DAPI corrected
- Ctrl.	**4.85**	**0.71**
C1	**4.23**	**0.82**
C2	**4.58**	**0.71**
C3	**4.58**	**0.78**
C4	**5.22**	**0.62**
CPA (2µg/ml)	**12.65**	**1.77**

Flow cytometric (FC)-Results obtained with DAPI stain/gate showed good accordance with literature data and also with internal results obtained with semi-automated microscopy (SAM).

*: lit.-data gives a range only (multiple data points not available)

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Results from validation study with positive controls using the modified method

Indirect clastogen cyclophosphamide A (CPA)

CPA ST+S9	day 1	day 2	day 3	day 4
		intraday-Variability		
Mean (n=3) [%MN]	1.23	1.71	3.03	2.17
STD	0.04	0.07	0.20	0.50
Repeatability (CV) [%]	**3.6**	**3.9**	**6.5**	**23.1**
		Interday variability		
Mean (n=12) [%MN]		2.04		
STD		0.72		
Intermediate precision (CV) [%]		**35**		

Direct clastogen mitomycin C (MMC)

MMC ST-S9	day 1	day 2	day 3	day 4
		Intraday-Variability		
Mean (n=3) [%MN]	1.15	1.18	1.74	1.35
STD	0.07	0.10	0.13	0.22
Repeatability (CV) [%]	**6.2**	**8.3**	**7.2**	**16.4**
		Interday variability		
Mean (n=12) [%MN]		1.35		
STD		0.27		
Intermediate precision (CV) [%]		**20**		

Aneugen vinblastine (VBL)

VBL LT	day 1	day 2	day 3	day 4
		Intraday variability		
Mean (n=3) [%MN]	2.31	3.76	4.75	2.41
STD	0.08	1.09	0.26	0.13
Repeatability (CV) [%]	**3.4**	**29.1**	**5.4**	**5.3**
		Interday variability		
Mean (n=12) [%MN]		3.31		
STD		1.16		
Intermediate precision (CV) [%]		**35**		

Intermediate precision (CV) for Background frequencies:

ST+S9 28.9%
ST-S9 16.7%
LT 20.3 %

Validation results matched criteria*→ succesful
Do we find what was seen with Metafer regarding positive liquids?

*Validation Criteria: Intermediate prec.:35%; Intraday var.: 30%

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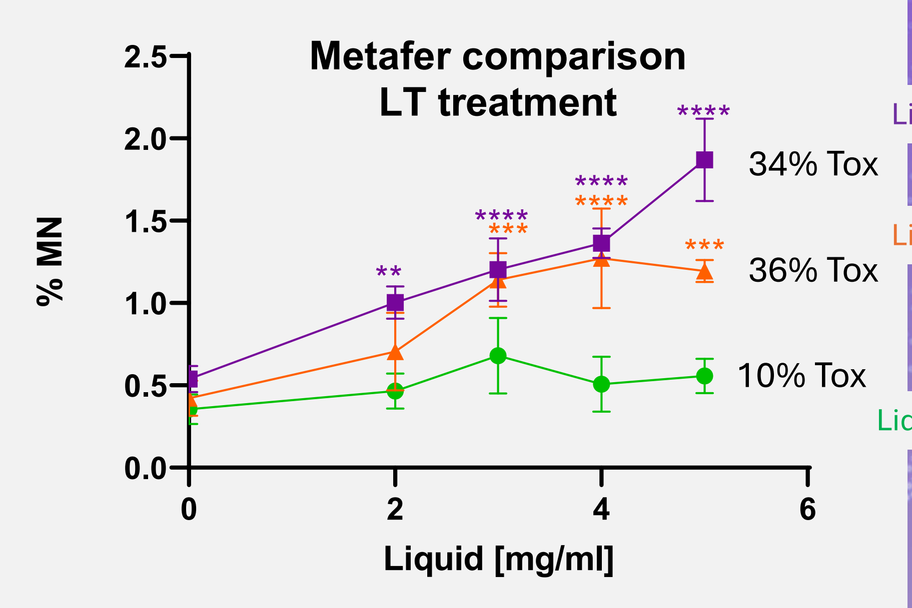
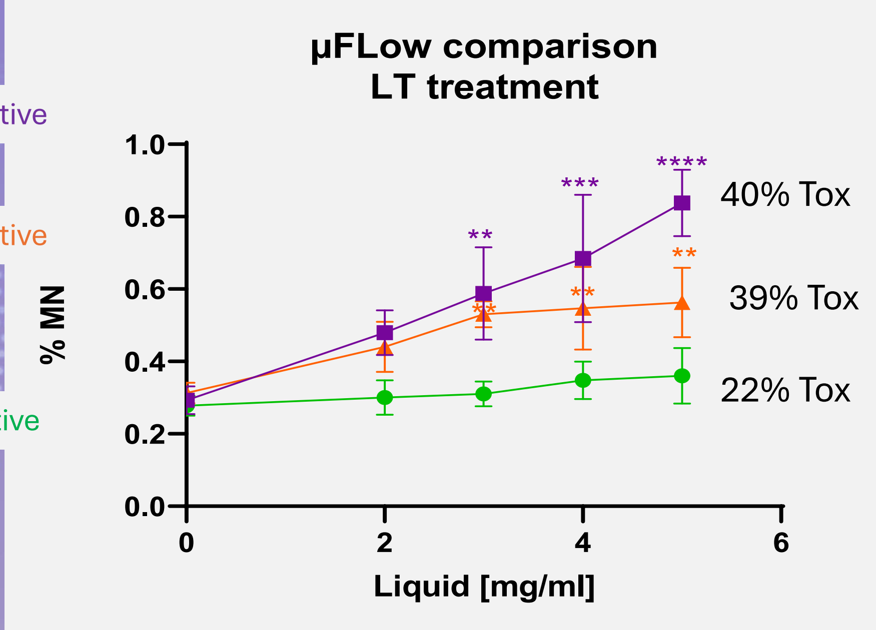
Comparison to microscopic results

Question:
Do we get similar results with the µFLow method when compared to results from semi-automated microscopy?
Stress test: Two E-liquids which were already reported as genotoxic positive under long term treatment conditions and one E-liquid reported as negative in old method



Liquid 1: Genotoxic positive
Liquid 2: Genotoxic positive
Liquid 3: Genotoxic negative



→ **Same findings with significantly increased efficiency (i.e. reduced lab turn-around times/costs)**

(n=4 per dose leve; stats: ANOVA with post hoc Dunnett test P<0.05*, p<0.01**, p<0.001 ***, p<0.0001****)

Summary of Results

- Supplementation of µFlow-kit with DAPI staining works
  - ✓Cost efficient adaptation with additional staining increased specificity to match literature and historical lab result from microscopic version
- Fully automated evaluation and calculation of Tox and MN frequencies established.
- Validation for qualitative assessment complete.

→ what about quantitative assessment?

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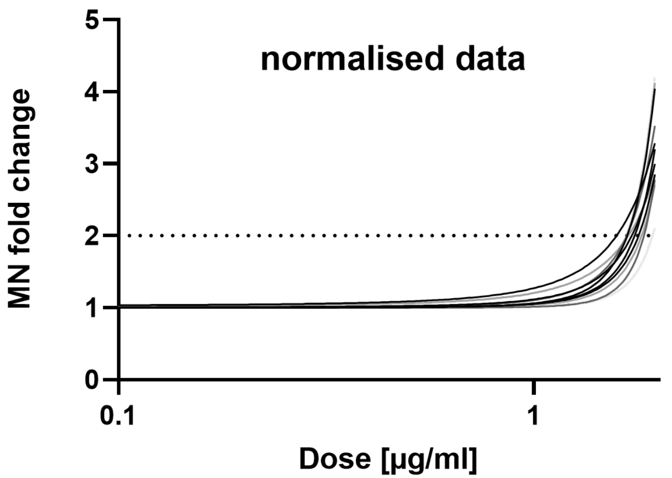
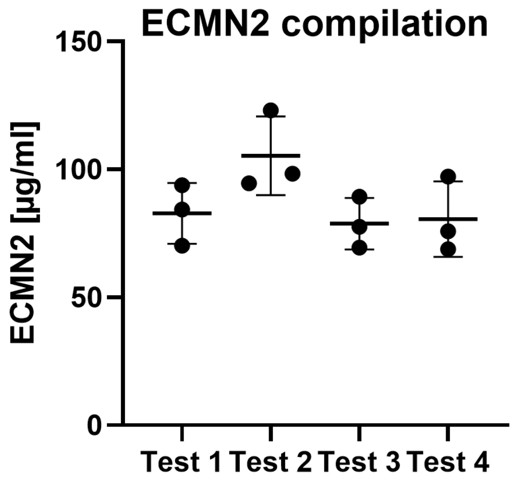
Suggestion for quantitative assessment with 1R6F TPM ST+S9 as example

- Although variability was reduced a quantitative approach for TPM evaluation needs a data normalisation step.
- Calculation of the dose necessary to increase MN frequency 2-fold by non-linear regression analysis (ECMN2)





[µg/ml]	ECMN2 Day 1	ECMN2 Day 2	ECMN2 Day 3	ECMN2 Day 4
	84.36	94.62	77.51	75.81
	70.24	123	89.34	68.77
	93.84	98.37	69.4	97.16
Mean	82.8	105.3	78.8	80.6
STD	9.7	12.6	8.2	12.1
Rep.CV [%]	11.7	12.0	10.4	15.0
Mean	86.9			
STD	15.2			
IP CV [%]	17.5			

Summary:
With the FC method the robustness of the test was increased to also allow for a quantitative assessment / comparison of positive products. Results with TPM indicate that ECMN2 calculation of normalised data by non-linear regression provides a good measure to assess genotoxicity beyond Yes/No.

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Main achievments and next steps

- The modified µFLow method allows a faster assessment of extracts and E-liquids (60% reduced work load when compared to microscopic version). Time to market can be decreased.
- Internal TPM validation work is in progress.
- Expansion of the method to adherent cell line from human origin (e.g. BEAS-2B) to allow the testing of fresh whole smoke and aerosols.
- Implementing modifications to also cover Mode of Action, i.e. combining FC analysis with appropriate markers for aneugenicity / clastogenicity.

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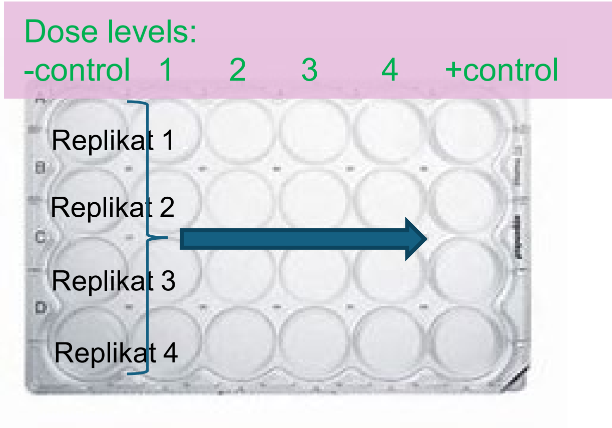
Open for questions now

Thank you

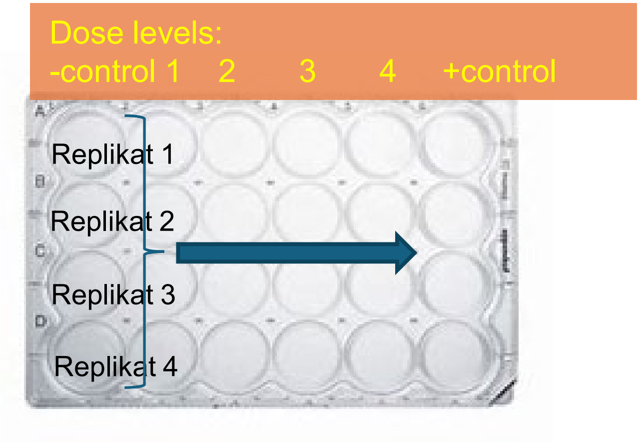
Acknowledgement:
Catrin Lietz and Andreas Bosnak, IMB/ Reemtsma
Arne Knoerck, Miltenyi

Backup Slide 1(Final setup for product testing)

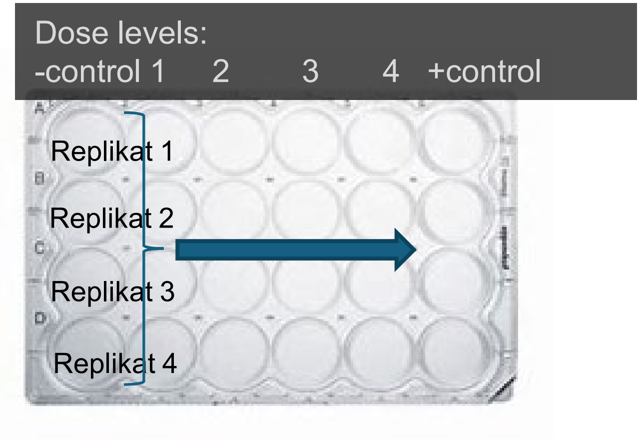
Treatment 1



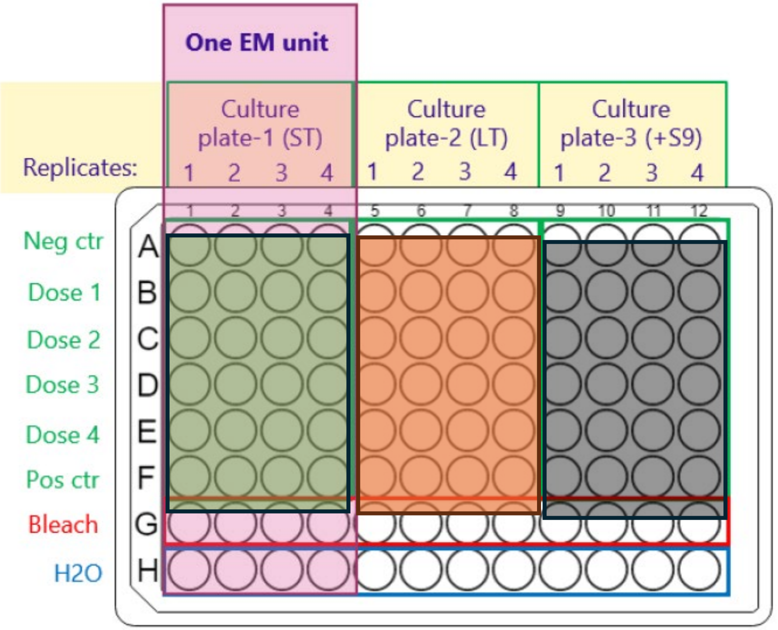
Treatment 2



Treatment 3

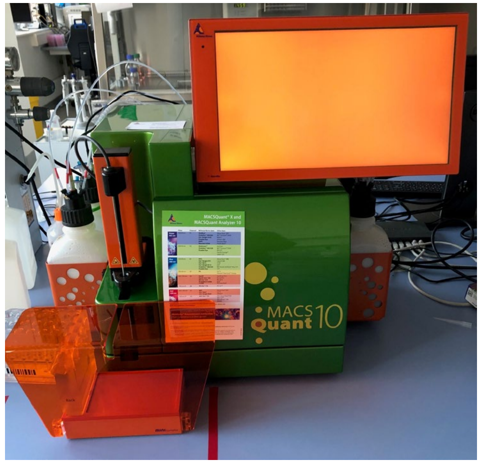


Mirroring 3 x 24 well plates on one 96 well plate turned by **90°**



One plate to process and measure with 100% automated evaluation

analysis



Backup slide 2 (Validation results with adapted meth

indirect clastogen

CPA ST+S9	day 1	day 2	day 3	day 4
date	26.09.2023	17.10.2023	19.10.2023	24.10.2023
Replicate values [%MN]	1.21	1.62	2.99	2.88
	1.18	1.73	2.80	1.92
	1.29	1.78	3.28	1.73
intraday-Variability				
Mean (n=3) [%MN]	1.23	1.71	3.03	2.17
STD	0.04	0.07	0.20	0.50
Repeatability (CV) [%]	3.6	3.9	6.5	23.1
Interday variability				
Mean (n=12) [%MN]	2.04			
STD	0.72			
Reproducibility (CV) [%]	35			

direct clastogen

MMC ST-S9	day 1	day 2	day 3	day 4
date	26.09.2023	17.10.2023	19.10.2023	24.10.2023
Replicate values [%MN]	1.21	1.25	1.65	1.55
	1.05	1.24	1.66	1.46
	1.19	1.04	1.92	1.04
Intraday-Variabilität				
Mean (n=3) [%MN]	1.15	1.18	1.74	1.35
STD	0.07	0.10	0.13	0.22
Repeatability (CV) [%]	6.2	8.3	7.2	16.4
Interday variability				
Mean (n=12) [%MN]	1.35			
STD	0.27			
Reproducibility (CV) [%]	20			

aneugen

VBL LT	Tag 1	Tag 2	Tag 3	Tag 4
date	26.09.2023	17.10.2023	19.10.2023	24.10.2023
Replicate values [%MN]	2.21	4.74	4.81	2.50
	2.35	4.30	5.03	2.50
	2.38	2.23	4.41	2.23
Intraday variability				
Mean (n=3) [%MN]	2.31	3.76	4.75	2.41
STD	0.08	1.09	0.26	0.13
Repeatability (CV) [%]	3.4	29.1	5.4	5.3
Interday variability				
Mean (n=12) [%MN]	3.31			
STD	1.16			
Reproducibility (CV) [%]	35			

Reproducibility /Variability for Background frequencies:

ST+S9 28.9%
ST-S9 16.7%
LT 20.3 %

Validation results are ok.
Do we find what was seen with Metafer regarding positive liquids?

Opportunities recognised and implemented

- Cell culture procedures were adapted:
  - treatment times and
  - recovery times

- **Supplementation of µFlow kit with DAPI* staining**

  Background frequency could be stabilised using an additional step for DNA staining .

* 4',6-diamidino-2-phenylindole, DNA specific stain