CELET course 2024

The CYTOKINESIS BLOCK MICRONUCLEUS (CBMN) ASSAY

14 November 2024

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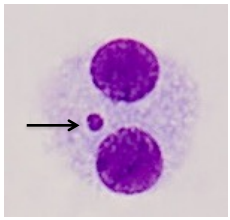
1

The cytokinesis-block micronucleus assay Contents

- What are micronculei and how are they formed
  - The cytokinesis-block micronucleus (CBMN) assay
- The CBMN assay for radiation dose assessment
  - micronucleus dose response
  - application of the CBMN assay for radiation dose assessment in humans
    - ✓ development of an automated CBMN assay for high-throughput analysis (biological dosimetry, biomonitoring)
    - ✓ development of a CBMN centromere assay for the detection of low doses
- The CBMN assay for assessing individual radiosensitivity and cancer predisposition
- Practical issues: NDI, Scoring criteria, protocols

2

What are micronuclei (MN) and how are they formed



Micronuclei are:

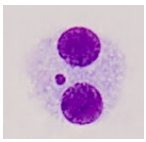
- small extranuclear bodies found in interphase cells after cell division

Micronuclei represent:

- acentric chromosome fragments (MN-ac) or whole chromosomes (MN-wc) that are not incorporated in the daughter nuclei during cell division

3 3

What are micronuclei (MN) and how are they formed



MN-ac (acentric chromosome fragments)

- exposure to clastogenic agents (eg ionizing radiation)
- result of non- or misrepaired DNA double strand breaks (DSB)

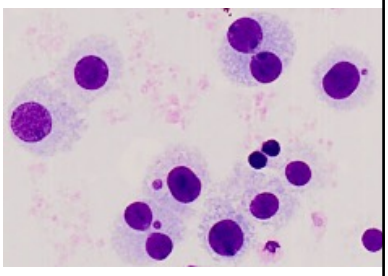
MN-wc (whole chromosomes)

- exposure to aneugenic agents
- a small number appear spontaneously
  - ➢ result of whole chromosomes that are unable to interact with the spindle

MN are by this not radiation specific!

4 4

The cytokinesis-block micronucleus (CBMN) assay

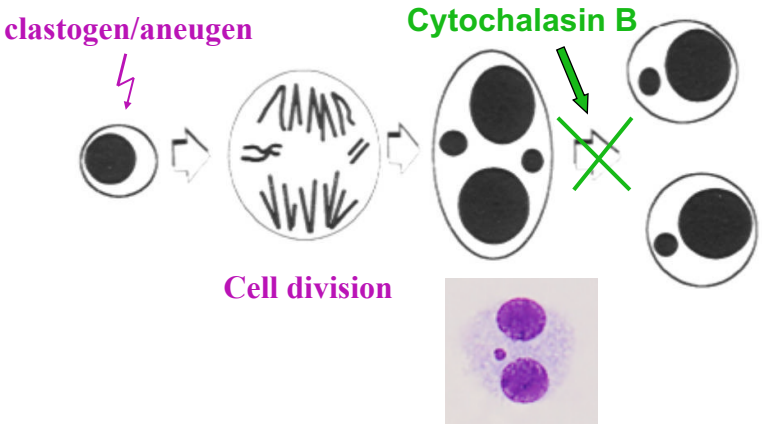


- The CBMN assay has been developed by Fenech and Morley in 1985
- In the CBMN assay Cytochalasin B , a cytoplasmic division inhibitor, is added to the cell culture to identify cells that underwent one division. These cells are identified as binucleate (BN) cells
- The CBMN assay is often applied to peripheral blood lymphocytes (PBL), but any cell that can divide can be used
- The CBMN assay is often used as a general toxicology test, mutagen (radiation) sensitivity test and as a biomarker for cancer predisposition

5 5

The Cytokinesis-block micronucleus assay (CBMN assay)

Principle of the assay



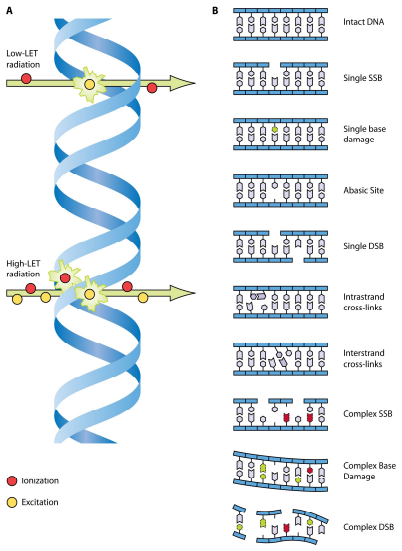
6

The CBMN assay for radiation dose assessment

- Ionizing radiation is a strong clastogenic agent and thus a potent inducer of DSB and by consequence also of MN
- In the CBMN assay for radiation dose assessment, MN are typically scored in 500 -1000 binucleate cells
- Radiation-induced MN contain mainly acentric chromosomal fragments (MN-ac) resulting from non-repaired or misrepaired DNA dsb.

7

Radiation-induced DNA damage



DNA damage: simple and complex DSB

↓

misrepair (NHEJ, HR) or non-repair

↓

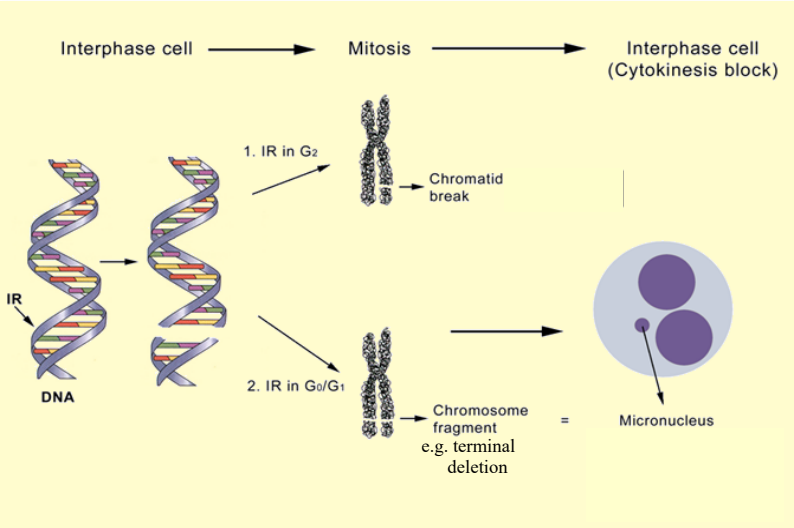
chromosomal aberrations, micronuclei

JAEA R&D Review 2007 and Georgakilas 2008

8

Formation of chromosomal aberrations and micronuclei

1 lesion aberrations



9

2 lesion aberrations

Examples of 2-lesion *Chromosome-type* aberrations

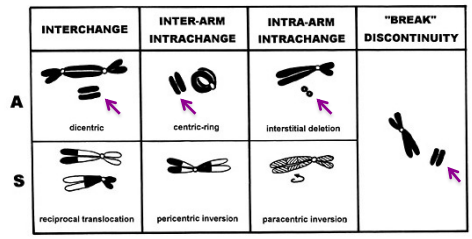


Figure 1 IR in G0/G1

Examples of 2-lesion *Chromatid-type* aberrations

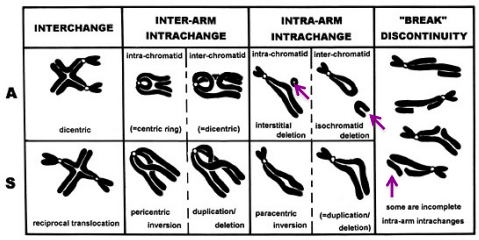
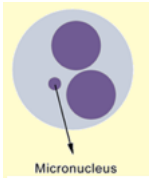


Figure 2 IR in late S/G2

(J. Savage)

Formation of chromosomal aberrations and micronuclei



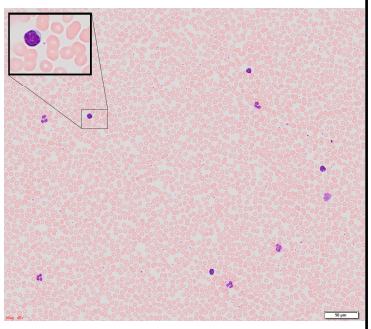
10

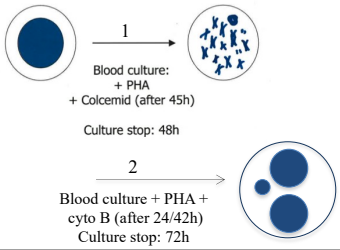
The CBMN assay for radiation dose assessment in humans

peripheral blood lymphocytes (PBL)

- advantages of using PBL:

- Easily obtained in large quantities by venipuncture
- The majority of peripheral blood lymphocytes reside at the G_0 phase of the cell cycle; constantly redistributed between blood and other tissues (= millions of microscopic dosemeters)
- Phytohaemagglutinin (PHA) converts resting lymphocytes into dividing cells allowing visualization DNA lesions in (1) metaphase chromosomes (eg. chromosome aberration assays) or (2) interphase cells after division (CBMN assay)
- The assay can be performed on fresh whole blood cultures, frozen whole blood cultures and isolated lymphocyte cultures (fresh and frozen)





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The cytokinesis-block micronucleus assay for cryopreserved whole blood

Elien Beyls, Ans Baeyens & Anne Vral

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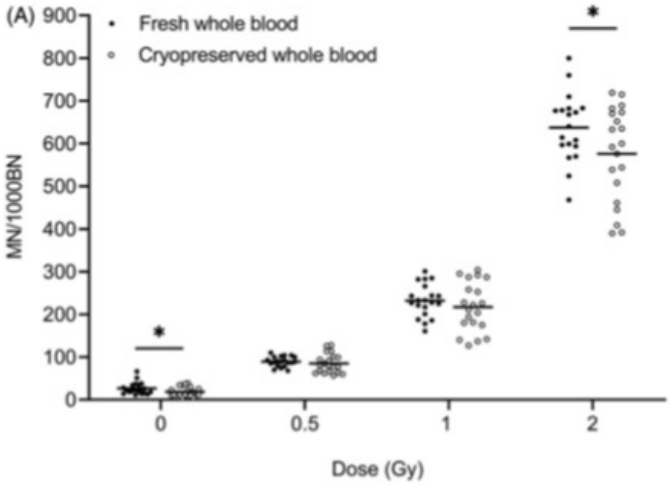
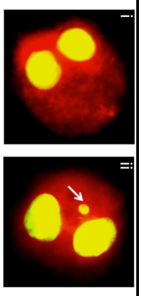
To link to this article: https://doi.org/10.1080/09553002.2021.1941378

Published online: 01 Jul 2021.

12

Comparison of MN results obtained in fresh and frozen blood samples





Acridine orange staining

- similar response in fresh and frozen blood (n=20)
- no effect of cryopreservation time (2wk – 1 year) on MN yields

13

Article

The Cytokinesis-Block Micronucleus Assay on Human Isolated Fresh and Cryopreserved Peripheral Blood Mononuclear Cells

Simon Sioen, Karlien Cloet, Anne Vral and Ans Baeyens *

J. Pers. Med. **2020**, *10*, 125; doi:10.3390/jpm10030125 www.mdpi.com/journal/jpm

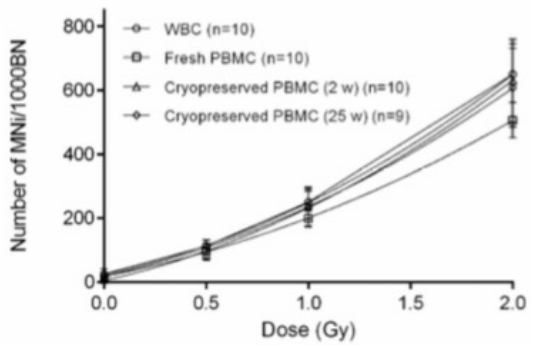
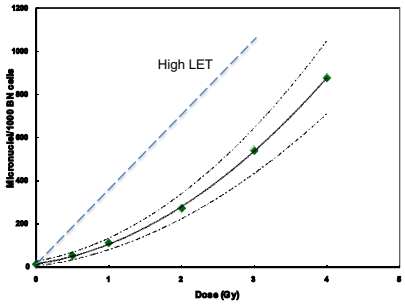


Figure 2. Comparison of the number of micronuclei (MNi) per 1000 binucleated (BN) cells for whole blood cultures (WBC), fresh, 2 weeks, and 25 weeks cryopreserved peripheral blood mononuclear cells (PBMCS) after 0, 0.5, 1 and 2 Gy exposure. The error bars represent SD of the mean.

14

MN dose response curve

- Radiation-induced MN are strongly correlated with radiation dose and quality
- the in vitro MN dose-response calibration curves follow the same shape as described for the standard dicentric assay
- for low LET radiation, a linear-quadratic dose response is observed:
  $y = c + \alpha D + \beta D^2$
- for high LET radiation a linear dependence 'y = c + αD' is observed, with high LET radiation being more effective in generating MN at the same dose levels.
- for doses > 5 Gy the response levels off.
  This phenomenon is also observed for other cytogenetic endpoints and is interpreted as selection against heavily damaged cells that cannot enter mitosis.



15

The MN dose response curve

- Recently, a number of specialized curve-fitting computer programs for cytogenetic endpoints such as dicentrics, translocations and micronuclei have been developed:
  - chromosomal aberration calculation software, CABAS (Deperas et al., Rad. Prot. Dos., 124, 115-123, 2007)
  - Dose Estimate (Ainsbury and Lloyd, Health Physics,98, 290-295, 2010)
  - Biodose Tools (RENEB network, in progress) https://aldomann.shinyapps.io/biodose-tools-beta/_w_b7e02338/_w_d125428d/_w_24b759e5/_w_4cf61364/

16 16

Application of the CBMN assay for radiation dose assessment in humans

- accidental exposures (biodosimetry)
- occupational exposures (biomonitoring)
- clinical exposures (radiotherapy, radiation diagnostics)

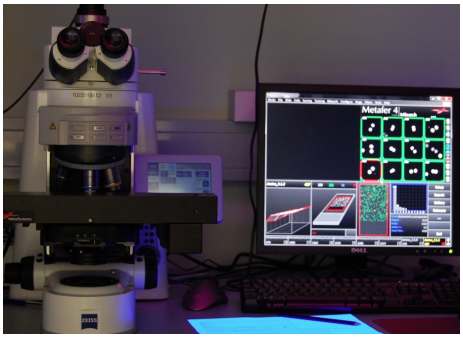
Advantages of the CBMN assay:

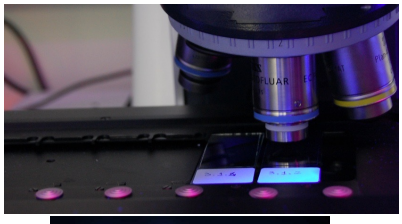
- the assay is easy to perform
- the scoring is easy and quick in comparison to other cytogenetic endpoints
- possibility of automated scoring => high-throughput analysis (triage biodosimetry, biomonitoring)

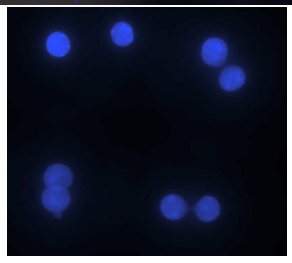
17

The CBMN assay for automated scoring

- the MN software module (MNScore) integrated in the metaphase finder system MSearch of Metasystems



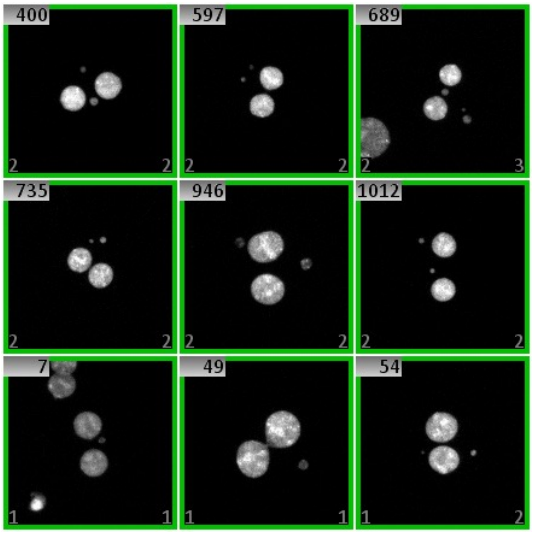




DAPI staining

18

The CBMN assay for automated scoring



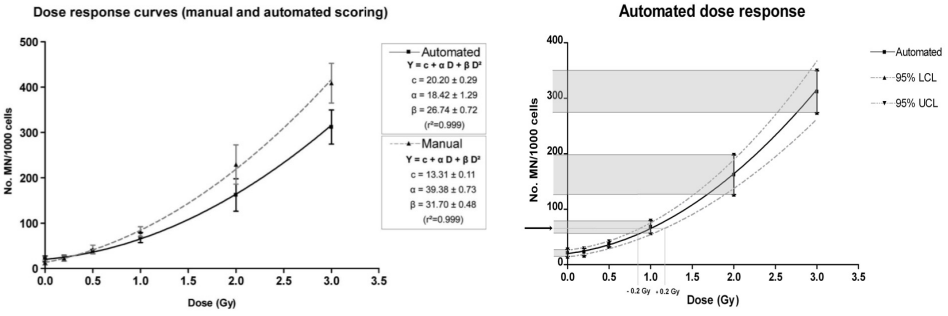
MN module for automated MN scoring of Metasystems

- on 1 slide automated scoring of MN in about 1000-2000 BN cells can be performed in less than 8 minutes

Galleries of BN cells with MN captured by MNScore of Metasystems

19

The CBMN assay for automated scoring



Willems et al., IJRB 86(1),2-11, 2010

Galleries of BN cells with MN captured by MNScore of Metasystems

20

RENEB
Realising the European Network of Biodosimetry

Running the European Network of Biological Dosimetry and Retrospective Physical Dosimetry

Inter-laboratory comparisons

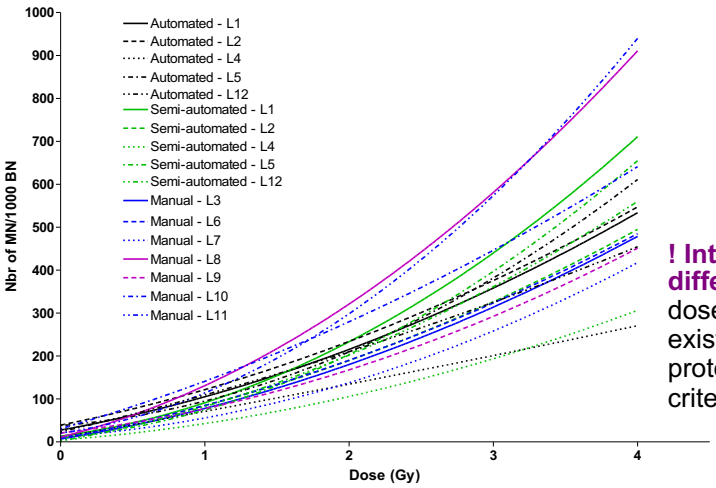
CBMN assay for triage biodosimetry in case of large –scale radiation

21

MN dose response curves

RENEB
Realising the European Network of Biodosimetry

ILC 2017



! Interlaboratory differences in MN dose response curves exist (different protocols, scoring criteria, etc).

Depuydt J, Baeyens A, Barnard S, Beinke C, Benedek A, Beukes P, et al. RENEB intercomparison exercises analyzing micronuclei (Cytokinesis-block Micronucleus Assay). INTERNATIONAL JOURNAL OF RADIATION BIOLOGY. 2017;93(1):36–47.

ILC 2017

Table 4(b). Individual dose estimates reported by the laboratories participating in the second intercomparison exercise (laboratories: L1–L16), stratified by scoring procedure. Mean dose estimates ± SEM per scoring procedure are also presented.

		Estimated doses True doses (Gy)	
Scoring method	Laboratory	0.85	2.7
Automated	L1	0.87	2.72
	L8	0.27	2.82
	L4	0.46	2.39
	L5	0.48	2.73
	L12	1.15	3.42
	L2	0.29	2.09
	Mean ± SEM (Gy)	0.58 ± 0.14	2.69 ± 0.18
Semi-automated	L1	1.00	2.55
	L8	0.51	2.86
	L4	0.95	3.03
	L5	0.85	2.79
	L12	1.15	2.79
	L2	0.66	2.55
	L13	0.66	2.05
	L15	1.17	3.65
	Mean ± SEM (Gy)	0.87 ± 0.09	2.78 ± 0.16
	Mean ± SEM (Gy) (without L13, L15)	0.85 ± 0.1	2.76 ± 0.08
Manual	L3	1.10	2.69
	L5	0.83	3.09
	L6	0.92	2.40
	L7	0.38	1.59
	L9	1.13	3.94
	L10	0.99	3.29
	L11	0.76	2.37
	L13	0.70	2.35
	L15	1.34	3.53
	L16	2.03	4.10
	Mean ± SEM (Gy)	1.02 ± 0.14	2.94 ± 0.25
	Mean ± SEM (Gy) (without L13, L15, L16)	0.87 ± 0.10	2.77 ± 0.29
	Mean TOTAL ± SEM (Gy)	0.86 ± 0.08	2.82 ± 0.12
	Mean TOTAL ± SEM (Gy) (without L 13, L 15, L 16)	0.78 ± 0.07	2.74 ± 0.12

Uncertainty interval for triage dose estimation.
0.50 Gy for 0.85 Gy.
0.54 Gy for 2.7 Gy (20% of the true dose).
Grey: values lying outside the uncertainty interval

23

ILC 2021

Vral et al. , RadRes, 199, 2023

TABLE 4

estimate Values Reported by each Laboratory for the 3 Blind Blood Samples According to Scoring Method

ıethod	Code	Delivery time (h)	Reporting time (h)	Estimated doses (Gy) with 95% CIs		
Reference doses				0 Gy	1.2 Gy	3.5 Gy
Manual (n = 11)	L3	45	174	0 (0–0.10)	**2.05 (1.78–2.32)**	4.07 (3.65–4.48)
	L5	53	143	0 (0–0.12)	1.58 (1.33–1.83)	3.13 (2.9–3.36)
	L6	21	238	0 (NA) [a]	**2.11 (1.85–2.38)**	5.34 (5.04–5.64)
	L7	24	191	0 (0–0.35)	1.64 (1.32–1.97)	4.78 (4.5–5.05)
	L8	41	289	0 (NA) [a]	1.57 (1.15–2.00)	4.22 (3.78–4.65)
	L9	45	147	0 (0–0.68)	1.50 (1.17–1.82)	5.13 (4.78–5.49)
	L10	21	356	0.00 (0–0.26)	**2.18 (1.93–2.46)**	4.07 (3.69–4.45)
	L11	69	178	0 (–0.46–0.35)	1.65 (1.16–2.14)	4.95 (4.31–5.58)
	L12	24	78	0 (0–0.27)	1.41 (1.22–1.60)	3.48 (3.28–3.69)
	L13	21	892	0 (0–013)	**2.16 (1.88–2.45)**	5.16 (4.83–5.49)
	L14		155 [b]	0.03 (0–0.21)	1.12 (0.80–1.44)	3.06 (2.65–3.47)
No. (%) of estimates inside correct clinically relevant groups				*11 (100%)*	*7 (64%)*	*11 (100%)*
No. (%) of estimates inside ± 0.5 Gy (no. 1, no. 2) or ± 1 Gy (no. 3) intervals				*11 (100%)*	*7 (64%)*	*6 (55%)*
No. (%) of estimates for which the 95% CI includes the reference dose				*11 (100%)*	*4 (36%)*	*1 (9%)*
Semi-automated (n = 6)	L1	3	76	0.21 (0–0.50)	1.60 (1.21–1.99)	3.70 (3.09–4.31)
	L2	3	93	0.02 (0–0.13)	1.68 (1.44–1.98)	3.84 (3.48–4.25)
	L4	22	314	0.03 (0–0.11)	1.47 (1.03–1.91)	4.03 (3.22–4.85)
	L6	21	238	0.00 (0.00–0.18)	1.70 (1.37–2.02)	4.27 (3.72–4.82)
	L8	41	289	0 (NA) [a]	1.33 (0.98–1.68)	3.53 (3.13–3.93)
	L12	24	78	0.02 (0–0.12)	1.00 (0.80–1.20)	3.12 (2.89–3.35)
No. (%) of estimates inside correct clinically relevant groups				*6 (100%)*	*6 (100%)*	*6 (100%)*
No. (%) of estimates inside ± 0.5 Gy (no. 1, no. 2) or ± 1 Gy (no. 3) intervals				*6 (100%)*	*6 (100%)*	*6 (100%)*
No. (%) of estimates for which the 95% CI includes the reference dose				*6 (100%)*	*3 (50%)*	*4 (67%)*
Automated (n = 5)	L1	3	76	0.36 (0–0.75)	1.79 (1.20–2.39)	4.24 (3.19–5.29)
	L2	3	93	**6.90 (6.41–7.45)**	**6.72 (6.24–7.26)**	5.69 (5.25–6.19)
	L6	21	238	**1.56 (1.08–2.05)**	1.62 (1.18–2.07)	3.73 (3.05–4.40)
	L8	41	289	0 (0–0.07)	1.01 (0.65–1.38)	3.40 (3.00–3.80)
	L12	24	78	0.03 (0–0.14)	**0.95 (0.74–1.17)**	3.11 (2.84–3.37)
No. (%) of estimates inside correct clinically relevant groups				*3 (60%)*	*3 (60%)*	*5 (100%)*
No. (%) of estimates inside ± 0.5 Gy (no. 1, no. 2) or ± 1 Gy (no. 3) intervals				*3 (60%)*	*3 (60%)*	*4 (80%)*
No. (%) of estimates for which the 95% CI includes the reference dose				*3 (60%)*	*3 (60%)*	*3 (60%)*

Notes. Delivery times and reporting times are also presented. Bolded text indicates dose estimates laying outside the clinically relevant group.
[a] In case of very low MN yields, accurate CIs of the estimated dose could not be determined by the Biodose Tools software.
[b] L14 received no blood samples but images from L8. Results were reported 155h after receiving the images.

24

Application of the CBMN assay for radiation dose assessment in humans

”low doses”

Drawback of the CBMN assay for applications in the low dose range:

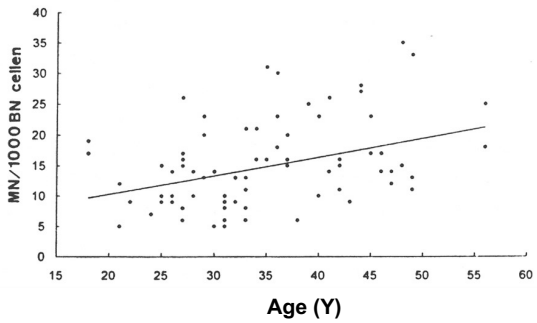
– High and variable background frequency
(2-36 MN/1000 BN lymphocytes (lab UGent)

➔ restricts low dose assessment to doses
> 0,2- 0.3 Gy X-or gamma-rays rays

Factors influencing the MN background levels:

- age, gender, diet, exposure to environmental mutagens

- age: systematic increase with age
- gender: systematically higher in females vs. males
  - ➔ males: 0.24 - 0.44 MN/1000BN/year
  - ➔ females: 0.52 - 0.54 MN/1000BN/year



The CBMN assay for the detection of low doses

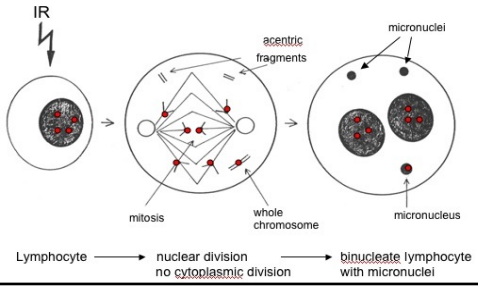
What do background/spontaneous MN contain?

Analysis of MN content was performed using a FISH pan-centromeric probe

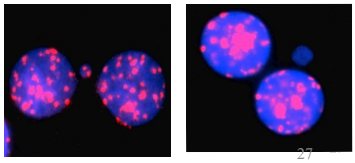
➔ the majority of spontaneous MN contain a centromere (> 70%) ➔ whole chromosome

➔ the age increase is due to an increase in centromere-positive MN

* often the X-chromosome is involved ➔ explains the observed gender difference



MNCM+ MNCM-



27

The CBMN-centromere assay for low dose detection

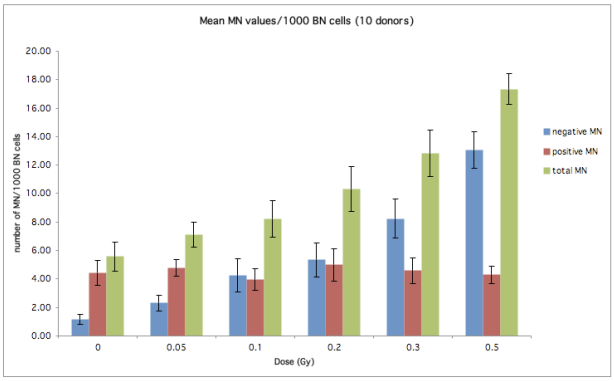


Figure: average of the total number of MN (MNtotal), MNCM+ and MNCM- for 10 donors as a function of dose. Semi-automated scoring results.
(modified from Baeyens et al., IJRB, 2011)

- most radiation-induced MN are CM-
- the number of MNCM+ only shows a very small increase with dose
- scoring of MNCM- lowers the detection limit to 0.05 - 0.1 Gy
- can be combined with automated scoring (will decrease scoring time)

28 28

Application of the CBMN assay for assessing individual radiosensitivity and cancer predisposition

mutations in DNA dsb repair genes and cell cycle checkpoint genes →
↑ non-repair or misrepair of DSB → ↑ chromosomal aberrations

➔ chromosomal instability
➔ enhanced chromosomal radiosensitivity

⇨ both are linked with an increased cancer risk

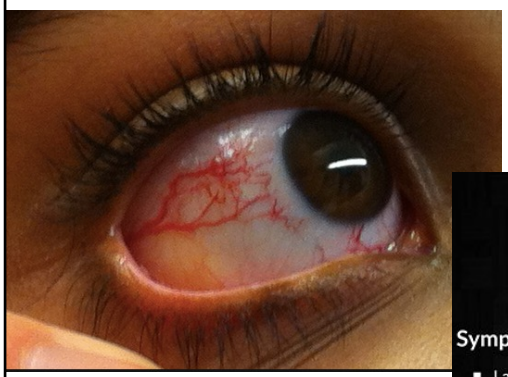
➔ cytogenetic assays are good biomarkers for cancer risk

⇨ the suitability of the CBMN assay as biomarker assay for radiation sensitivity and cancer risk has been demonstrated in several studies, e.g. :

- Ataxia Telangiectasia patients and XCIND syndrome
- a significant percentage of breast cancer patients (BRCA1/2 mutations)

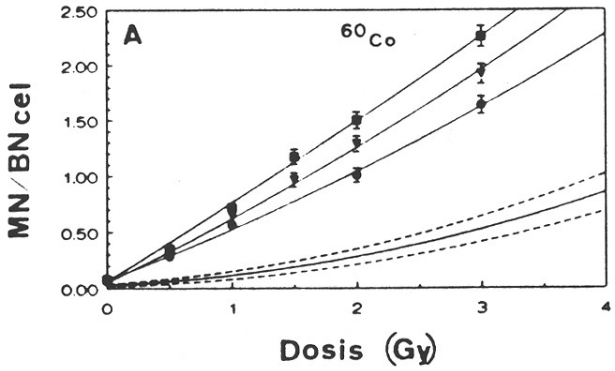
29

Ataxia Telangiectasia



- autosomal recessive neuro-degenerative disorder
- oculo-cutaneous telangiectasia
- cerebellar ataxia

AT patients



3 AT patients

mean of 6 control individuals

- XCIND syndrome: genetic diseases characterised by **X**-ray sensitivity, **C**ancer susecpitbility, **I**mmunodeficiency, **N**eurological abnormalities and **D**SB repair disfunction (includes AT patients (ATM), patients with NBS, SCID, PID, FA, …)
- chromosomal radiosensitivity testing : a valuable adjunctive tool in the diagnosis and therapeutic handling of these patients
- since 2019 we are an accredited lab for radiosensitivity testing in patients with a suspected DNA repair disorder

31

Genetic disorders comprising the XCIND syndrome

Table 1 A chronology of the radiosensitivity disorders

Diagnosis	Described	Primary pathogenesis	Radiosensitivity described
Ataxia-telangiectasia	1926 [1]	Hierarchical ATM protein kinase	[2,3]
Fanconi anemia	1927 [4]	Replication fork/cell cycle checkpoint	[5]
X-linked agammaglob	1952 [6]	BTK gene function	[7]
SCID ADA	1979 [8]	Toxicity of deoxyadenosine	[3]
NBS deficiency	1981 [9]	DNA repair/component of MRN complex	[9]
Mre11 deficiency	1999 [10]	DNA repair/component of MRN complex	[10]
Ligase IV deficiency	1999 [11]	DNA end-joining repair	[12]
SCID-Artemis	2001 [13]	DNA end-joining repair	[13]
SCID-XLF/Cernunnos	2006 [14,15]	DNA end-joining repair	[14,15]
RNF168 deficiency	2009 [16]	Chromatin ubiquitin ligation cascade	[17••,18••]
RAD50 deficiency	2009 [19••]	DNA end-joining repair	[19••]
DNA-PKcs deficiency	2009 [20••]	DNA end-joining repair	[20••]

32

The CBMN assay: practical issues

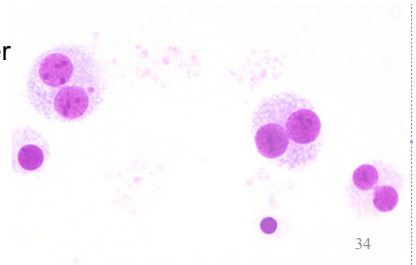
- Scoring criteria for the CBMN assay
- Nuclear division index (NDI)
- Protocols

33 33

Scoring criteria for the CBMN assay

Criteria for manual scoring of BN cells

- the cytokinesis-blocked cells should have the following characteristics:
  (a) The cells should be binucleated (BN)
  (b) The two nuclei in a BN cell should be situated within the same cytoplasm
  (c) The two nuclei in a BN cell should be approximately equal in size, staining pattern and intensity
  (d) The two main nuclei in a BN cell may touch but ideally should not overlap each other
  (e) BN cells should not overlap each other

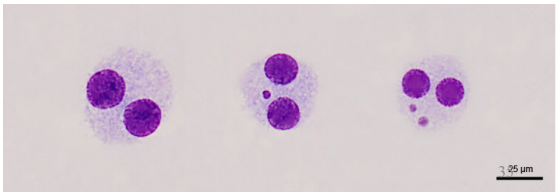


34

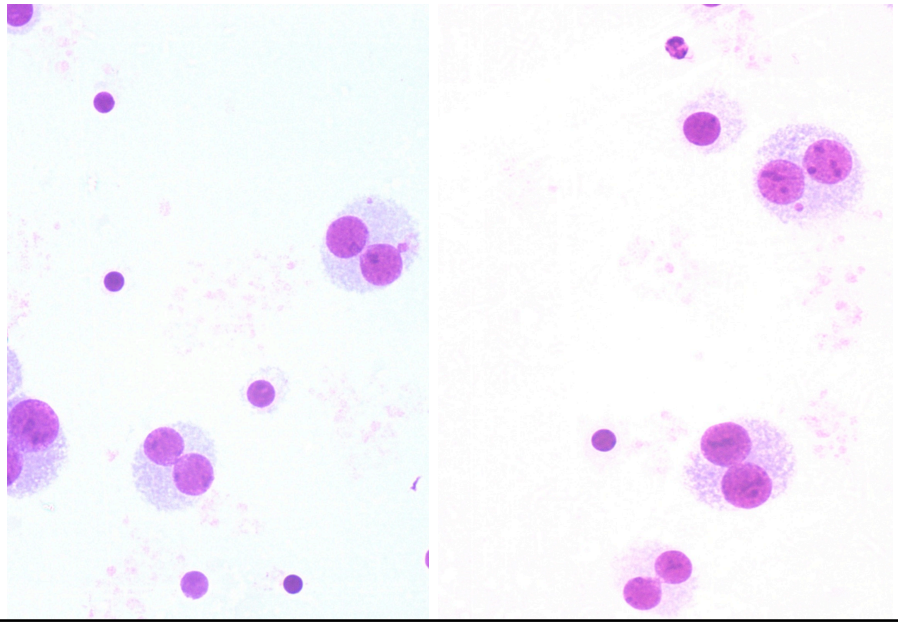
Scoring criteria for the CBMN assay

Criteria for manual scoring of MN

- MN are morphologically identical to but smaller than the main nuclei. They also have the following characteristics:
  - (a) the diameter of MN in human lymphocytes should be smaller then 1/3rd of the mean diameter of the main nuclei
  - (b) MN are non-refractile and can therefore be readily distinguished from artefacts such as staining particles
  - (c) MN are not linked or connected to the main nuclei
  - (d) MN may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable
  - (e) MN usually have the same staining intensity as the main nuclei but occasionally staining may be more or less intense



Criteria for manual scoring of MN



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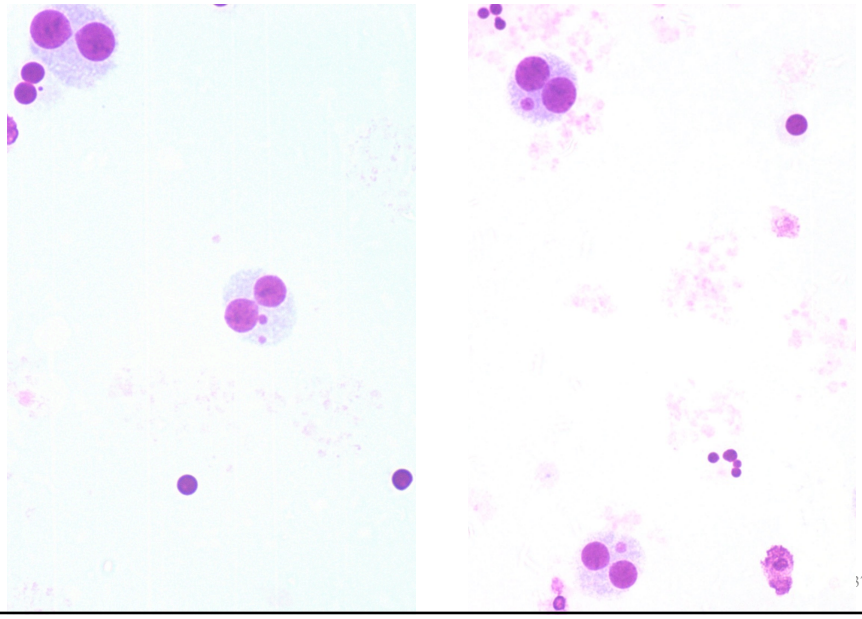
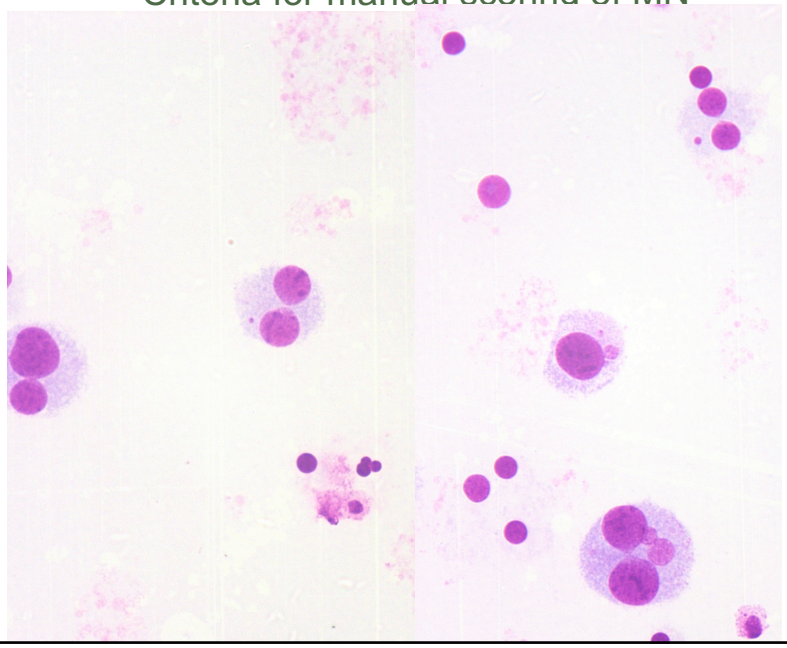


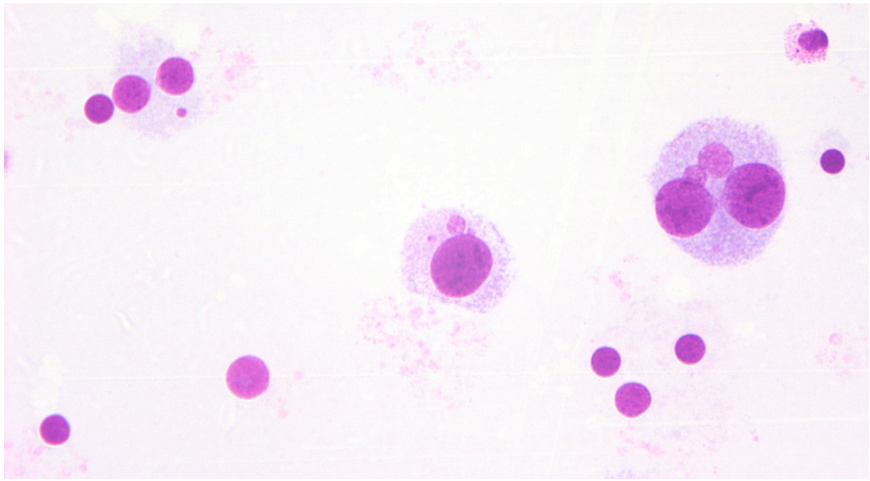
37

Criteria for manual scoring of MN



38

Criteria for manual scoring of MN



39

Nuclear division index (NDI)

- In the CBMN assay, the relative frequencies of cells with 1, 2, 3, etc nuclei (NDI) can be used to define cell cycle progression and how this is affected by radiation exposure
- Calculation of the NDI

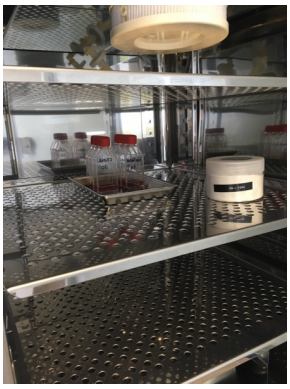
  $NDI = M_1 + 2M_2 + 3M_3 + 4M_4 / N$

  - 500 cells are scored
  - M_1 to M_4 represent the number of cells with one to four nuclei and N is the total number of viable cells scored
- More details about the calculation of the NDI and its uncertainty are described in the IAEA Manual

40 40

Protocols

1. CB-Micronucleus assay for peripheral blood lymphocytes



Blood collection:

Heparinized blood is used and kept at room temperature until use.

In vitro irradiation:

Irradiation of blood sample (or blood culture) at 37° C or room temperature. Controls are sham irradiated.

Blood culture:

for each sample blood cultures are set up immediately after irradiation. Each culture flask contains:

- 0,5 ml of the irradiated (sham irradiated) blood
- 4,5 ml complete RPMI medium (RPMI 1640+ 10%FCS + L-Glutamine and penicillin-streptomycin) + 100 µl of Phytohaemagglutinin (PHA-M Gibco)

- place flasks in a 5% CO2 incubator for 70 hours
- add 20 µl of cytochalasin B after 24h (42h) culture (6 µg/ml) (Sigma)

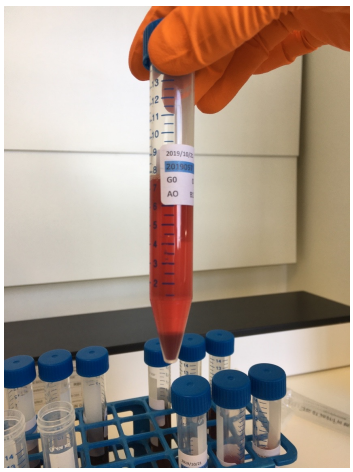
41 41

Protocols

1. CB-Micronucleus assay for peripheral blood lymphocytes

Hypotonic KCl treatment:

- after 70h of culture, transfer whole blood culture to pre-labelled tubes
- Centrifuge tubes at 1000 rpm for 8 min (RT)
- Discard supernatant but leave around 0,5 cm of fluid above the pellet. Resuspend pellet and add 7ml of cold hypotonic (0.075M) KCl under continuous vortexing.
- Centrifuge tubes at 1000 rpm for 8 min



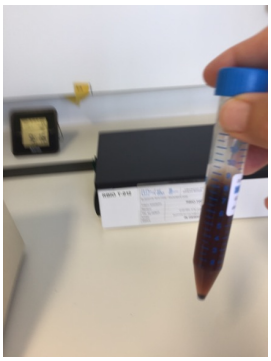
42 42

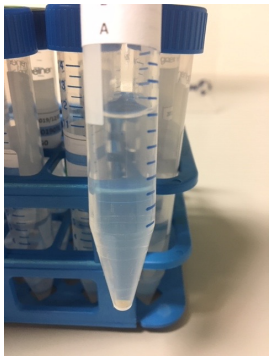
Protocols

1. CB-Micronucleus assay for peripheral blood lymphocytes

Fixation:

- Add 7 ml of freshly prepared cold fixative (4:1:5 methanol : acetic-acid : ringer (0.9% NaCl) solution) while vortexing. Put tubes in refrigerator overnight.
- Repeat fixation step 3 x more with cold 4:1 methanol:acetic acid (until fixative is clear). Store tubes in refrigerator until slide preparation.





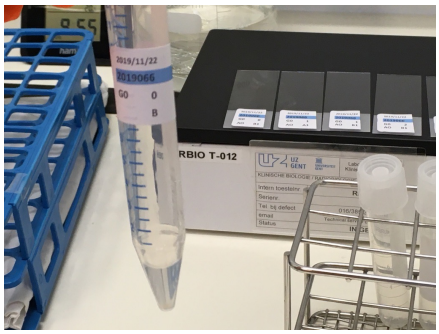
43 43

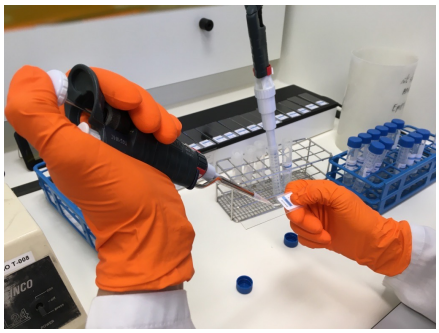
Protocols

1. CB-Micronucleus assay for peripheral blood lymphocytes

Slide preparation:

- discard supernatant and resuspend cells in a small amount of fresh fixative (dependent on the pellet size).
- Per sample 2 slides are prepared. For this a drop of cell suspension (about 40 µl) is dropped on dry clean slides.





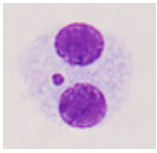
44

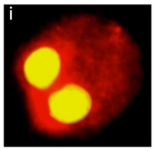
Protocols

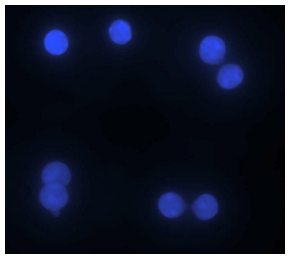
1. CB-Micronucleus assay for peripheral blood lymphocytes

Slide preparation:

- When the slides are dry they are stained with Giemsa or Acridine orange. For the automated MN assay a drop of DAPI-Vectashield is put on the slides and then covered with a coverslip.
- Per sample MN are scored in 1000 binucleate cells (500 binucleate cells per slide).







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Protocols

1. Standard Cytokinesis-Block Micronucleus protocol

- MN practical
- Details of the CBMN assay are also described in the IAEA EPR-Biodosimetry 2011 - Cytogenetic Dosimetry: Applications in preparedness for and response to radiation emergencies, Annex IV.

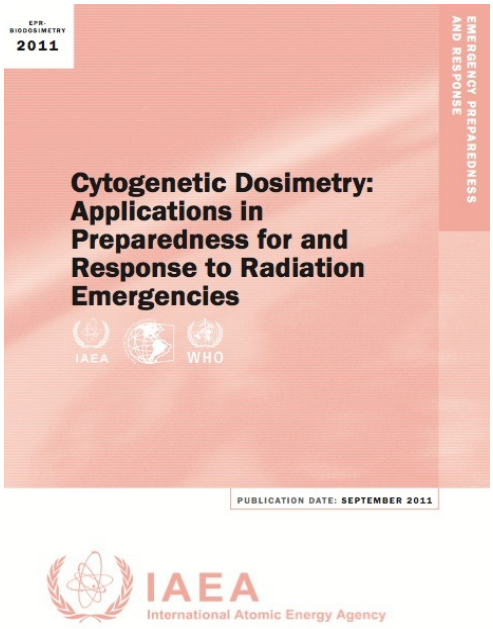
2. Micronucleus-centromere staining protocol

- slides are prepared according to the standard CBMN protocol
- A FISH protocol for detecting centromeres in MN, using a commercial pan-centromeric FISH probe, is described in the IAEA EPR-Biodosimetry 2011.
- a protocol using a home-made pan-centromeric probe by PCR amplification has been published in IJRB (2011) by Baeyens et al.

3. Protocol for the CBMN-cytome assay to detect NPB

- a detailed protocol of the CBMN Cyt assay has been published in Nature Protocols (2007, 2(5), 1084-104) by M. Fenech

46 46



IAEA
International Atomic Energy Agency

https://www-pub.iaea.org/MTCD/publications/PDF/EPR-Biodosimetry%202011_web.pdf

47

Questions?

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48