Micronucleus Detection Using ImageStream® System

Sample Preparation and Data Acquisition

This procedure describes the 3 day core method for preparing cells to measure micronucleus (MN) formation after treatment with cytotoxic or genotoxic compounds. This procedure can be adapted for use with other cell lines and chemicals. Recommendations for setting up and acquiring data on the ImageStream® system are also provided.

Materials

Cytochalasin B: Millipore Sigma C6762-5MG
DMSO: Millipore Sigma D8418-50ML
KCl: Millipore Sigma P9541-500G
10% Ultra-Pure Formaldehyde: Fisher NC9200219
Fetal Bovine Serum: Fisher SH3008803
DPBS: Fisher SH30028.03

1. Stock solutions of #chemical
   a) Initial reconstitution of the chemical in dH2O or DMSO
   b) Further dilution if necessary. Make aliquots
   c) Create stock to dilute prior to treatment. Make aliquots
2. Cytochalasin B is soluble in DMSO:
   a. Aliquots of 750 uL, 200 ug/mL are stored at -20C
3. 75 mM KCl. To make, add 2.79 g to 500 mL of high purity water and sterile filter. Store at 4°C.
4. 4% Formalin. Make 40 mL (16 mL of 10% Formalin + 24 mL of 1X DPBS) in chemical hood. Store at 4°C.
5. 2% FBS in 1X DPBS (wash buffer). Store at 4°C.
6. Muse or other counter for cell counting.

NOTE: For suspension cell lines, the cell seeding day steps should be skipped. Steps where 0.25% Trypsin-EDTA is added should not be performed.

Day 1 – Cell seeding

1. 24 h before the start of treatment, aspirate media and rinse bottom of T75 flask with 2.5 mL DPBS
2. Incubate flask with 2.5 mL 0.25% Trypsin-EDTA at 37°C for detaching (do not wait longer than 5 min)
3. Add 7.5 mL cell media and transfer to 15 mL Falcon tube
4. Seed cells into T25 flasks at a density of _____ cell/mL; final volume is 10 mL media; final cell number is _____ cells per flask. *For Cyt-B, seed at a density of at least double of what a "non-cyt B" experiment requires. Once Cyt-B is added, cell division will be blocked.
5. **For non-Cyt-B:** Remove 100 µL from each flask and perform a pre-treatment cell count and record below

Flasks	Live cell count
1	
2	
3	
4	
5	

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Day 2 – Cell culture and chemical exposure

6. Thaw sufficient aliquots created in 1(c) above to room temperature
7. *Depends on chemical concentration*. Create 2 mL volumes of the following concentrations in 15 mL polypropylene tubes by adding the appropriate volumes of the ___ µg/mL stock solution to sterile water according to the table below. Final dilution 1:10 in step 10.

Tube	Concentration (µg/mL)	_ µg/mL #chemical volume (µL)	Sterile water volume (µL)	Total volume (mL)
1 (control)	0	0	2000	2
2				2
3				2
4				2
5				2

8. **For suspension cell lines:** Seed cells into T25 flasks at a density of _____ cell/mL; final volume is 10 mL (9 mL media + 1 mL chemical); final cell number is _____ cells per flask. *For Cyt-B, seed at a density of at least double of what a "non-cyt B" experiment requires. Once Cyt-B is added, cell division will be blocked.
9. Add 1 mL (10% v/v) of #chemical or sterile water to each flask plus 9 mL of fresh media for a final concentration indicated in the table below:

Flasks	#chemical dose (µg/mL) – initial concentration	#chemical dose (µg/mL) – final concentration in flask
1	Sterile water (control)	Sterile water (control)
2		
3		
4		
5		

10. Incubate for appropriate amount of time (3 or 24 hr). Time of incubation start: _____
11. **For suspension cell lines:** At the end of treatment, spin at 200 x g (~1200 rpm in large bucket centrifuge) for 5 min.
12. Aspirate supernatant and resuspend pellet in 10 mL of fresh media for recover
13. **For short dose:** at the end of 3 hr treatment, aspirate media and replace with 10 mL of fresh media for recovery.
14. **For addition of Cyt-B long dose:** After 3 hr incubation, add 150 µL of Cyt-B (stock concentration = 200 µg/mL) to each flask for a final concentration of 3 µg/mL.
15. **For addition of Cyt-B short dose:** After 3 hr treatment, aspirate media and replace with 10 mL of fresh media for recovery. Add 150 µL of Cyt-B (stock concentration = 200 µg/mL) to each flask for a final concentration of 3 µg/mL.

Day 3 – Cell harvest and staining

16. After 21 h recovery (short dose) or 24 treatment (long dose) (_____), aspirate media and rinse bottom of T25 flask with 1 mL DPBS
17. Incubate flask with 1 mL 0.25% Trypsin-EDTA at 37°C for detaching (do not wait longer than 5 min)
18. Add 9 mL cell media and transfer to 15 mL Falcon tubes
19. **For suspension cell lines:** After 21 h recovery (_____), transfer all samples to 15 mL polypropylene tubes.
20. **For non-Cyt-B:** Remove 100 µL from all flasks and perform a post-treatment cell count and record below (10 mL total volume)

Flasks	Live cell count
1	
2	
3	
4	
5	

21. Spin at 200 x g for 5 min
22. Aspirate supernatant and resuspend pellet
23. Slowly add 5 mL of cold (4°C) 75 mM KCl and incubate at 4°C for 7 min
24. Add 2 mL of 4% Formalin and incubate for 10 min at 4°C
25. Spin at 200 x g for 5 min
26. Aspirate supernatant, resuspend in 100 µL of 4% Formalin and incubate for 20 min at 4°C
27. Add 5 mL of cold (4°C) wash buffer (2% FBS in 1X DPBS)
28. Spin at 200 x g for 5 min
29. Aspirate supernatant and resuspend in 150 µL of cold (4°C) wash buffer (2% FBS in 1X DPBS)
30. Transfer all samples to 1.5 mL Eppendorf tubes
31. If not running cells immediately on ISX, store at 4°C

Prepare samples for running on ISX:

32. Perform a total cell count on each sample (*the volume is now 150 µL) and record the values in the table below (1:100 or 1:10 dilution may be necessary). Display cell counts per 1 million cells (__ x 10^6 cells).

Samples	Dose (µg/mL)	Post processing counts (x10^6 cells/mL)	Volume of Hoechst to add (µL) – (Counts x 0.15 x 5 µL)
1	0		
2			
3			
4			
5			

33. While waiting on counts, add 15 µL of 500 µg/mL RNase for a final concentration of 50 µg/mL (10 µL RNase per 100 µL sample)

34. **Cell staining**
    a. Add the appropriate volume of 100 µg/mL Hoechst 33342 to the samples according to the table above
    b. Incubate at 37°C for 15 min
    c. Add cold (4°C) wash buffer to obtain a final volume of 1 mL
    d. Microcentrifuge at 300 x g for 5 min (1,200 rpm in small bucket centrifuge) and pipette off as much supernatant as possible
    e. Resuspend in 50 µL wash buffer (be very careful with bubbles)
    f. *c-e may not be necessary if there is a large amount of cells. *If very low amount of cells, do not wash, just concentrate the sample.
    g. Run on ISX using the following settings:

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ISX MKII INSPIRE™ settings (40x)

1. Load the template for 40x #cell line with the following parameters:

Parameter	Value
405 laser power	15 mW*
Area BF	100-900
Aspect Ratio BF	1-0.70
Grad RMS BF	55-75
Grad RMS Hoechst	13-21
Hoechst Intensity	2e5-2e6

*The mean peak on the Hoechst Raw Max Pixel histogram should be between 1500 and 2000

Note: Parameters may need to be modified depending on the cell type, treatment, instrument, and overall image quality.

2. Collect 10,000 events for non-CytB and 30,000 events for CytB per sample from the Hoechst+ population (R3)
3. After collection, run 10% bleach through the instrument for 5 min.
4. Discard waste into the correct waste container.
5. For data analysis use the IDEAS® and Amnis® AI templates, or create your own analysis.

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Reference INSPIRE™ instrument settings image: