IN VITRO MUTAGENIC AND DNA AND CHROMOSOMAL DAMAGE ACTIVITY BY SURFACTANT DISPERSION OR SOLVENT EXTRACT OF A REFERENCE DIESEL EXHAUST PARTICULATE MATERIAL

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Genotoxicity

- Genotoxic compounds, e.g., some polycyclic organic molecules, have the potential to cause genetic alterations (mutations) in somatic or germ cells.

- Such alterations in proto-oncogenes or tumor suppresor genes can be a mechanism for the development of cancer in the target organ, or for genetic alterations in germ cells leading to reproductive disorders.

Genotoxicity Screening

- Some industries routinely use a set of in vitro cellular genotoxicity assays to screen materials of human exposure, e.g., food or drugs, for genotoxic activities.
- "First Tier" screening for genotoxicity frequently includes cellular assays for
  - bacterial cell gene mutation
  - mammalian cell chromosomal damage
  - mammalian cell DNA damage

Diesel Exhaust Genotoxicants

- Genotoxic compounds frequently are found in diesel exhaust particulate materials (DPM);
- … in organic solvent extracts, e.g., acetone extracts, of filter-collected DPM
- … with bioassay of the solvent-extracted material for cellular mutagenicity or DNA or clastogenic damage.
- DPM genotoxicant content changes with engineering factors, e.g., engine design, speed and loading

Diesel Exhaust Genotoxicant Content is Affected by Engineering Factors:

Bacterial mutagenic activity of DPM solvent extract versus fuel, torque, load :
McMillian MH, et al., Society of Automotive Engineers Technical Paper 2002-01-1699.

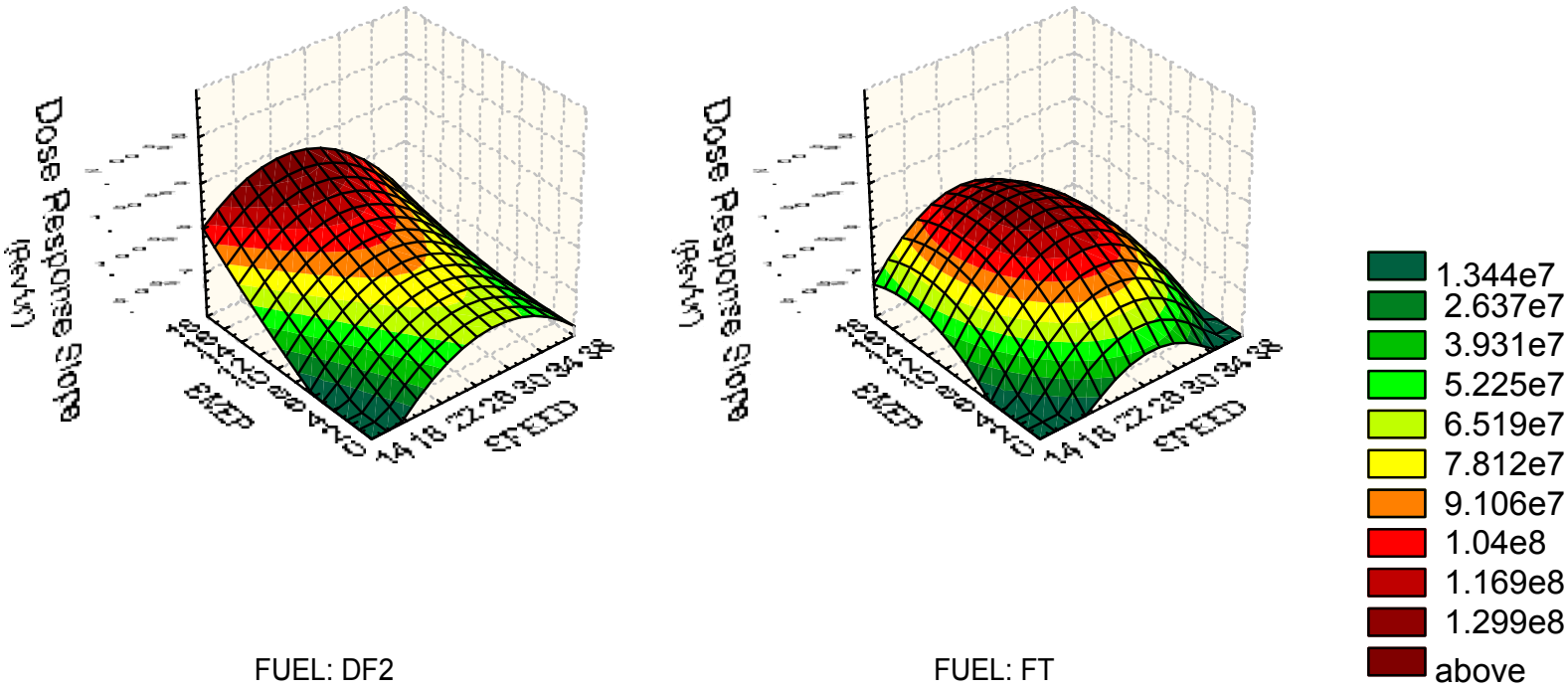
Dose Response vs Engine Speed and Load

FUEL: DF2 z=-3.212e8+2.692e7*x+9.162e6*y-4.783e5*x*x-2.544e5*x*y+1.186e5*y*y

FUEL: FT z=-4.321e8+3.487e7*x+1.937e7*y-6.486e5*x*x-1.844e5*x*y-5.983e5*y

X-axis: Speed

Y-axis: BMEP



FUEL: DF2

FUEL: FT

Question:
Can DPM Particle-Bound Genotoxicants be Bio-available and Active in the Lung?

- Genotoxic activity typically is measured on dissolved material extracted from DPM by organic solvents, e.g., acetone or dichloromethane

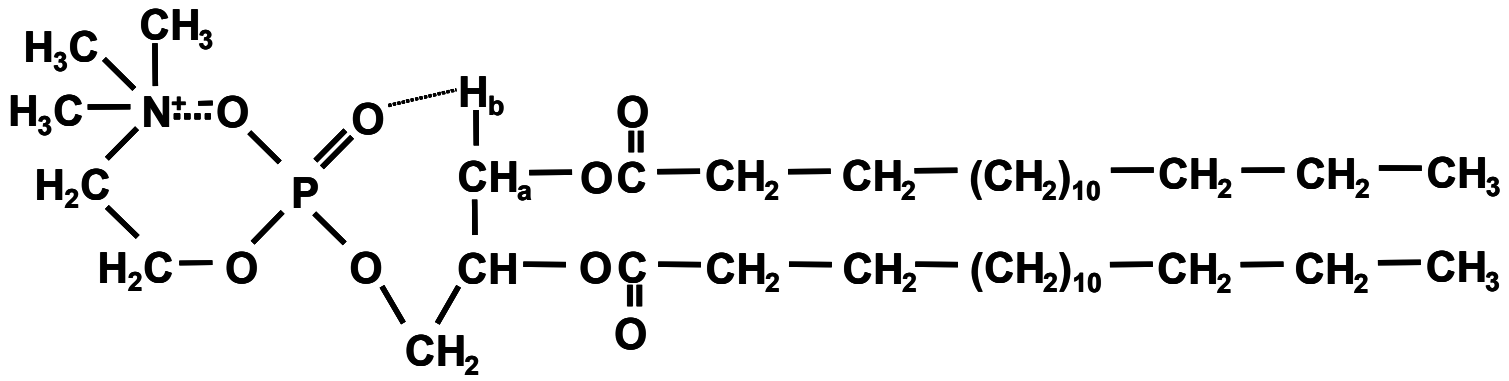
- That does not model the physiological bio-availability of those genotoxicants in the lung

- The deep lung airways are water-wet and coated with surfactants.

- The major component of those surfactants, phospholipids, do <u>not</u> efficiently extract genotoxicants from DPM

DPPC Surfactant Structure:

Palmitate residues associate with DPM hydrocarbon;
zwitterionic phosphatidyl choline head oriented outward
→ molecular conformation provides a
"wettable" DPM surface



Lung Surfactant Does Not Extract DPM Genotoxicants …But the "Solubilized" Particles are Active

extraction of DPM by phospholipid surfactants

→ little or no *in vitro* genotoxicity

but dispersion of DPM into phospholipid surfactants → genotoxic activity

(the non-dissolved, non-extracted whole particles express activity)

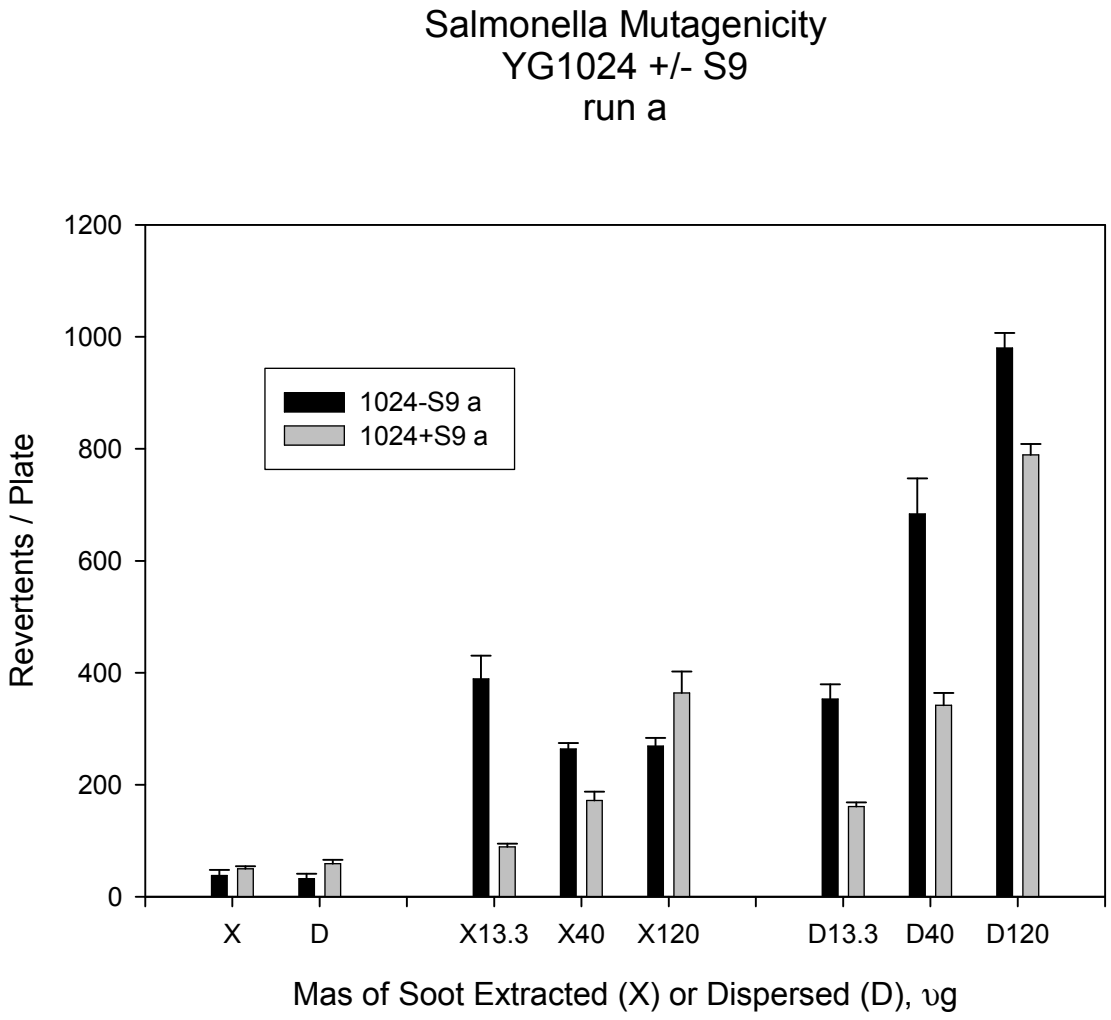
US National Institute of Standards (NIST)
Standard Reference Material 2975
Assayed for In Vitro Genotoxic Activities as a:

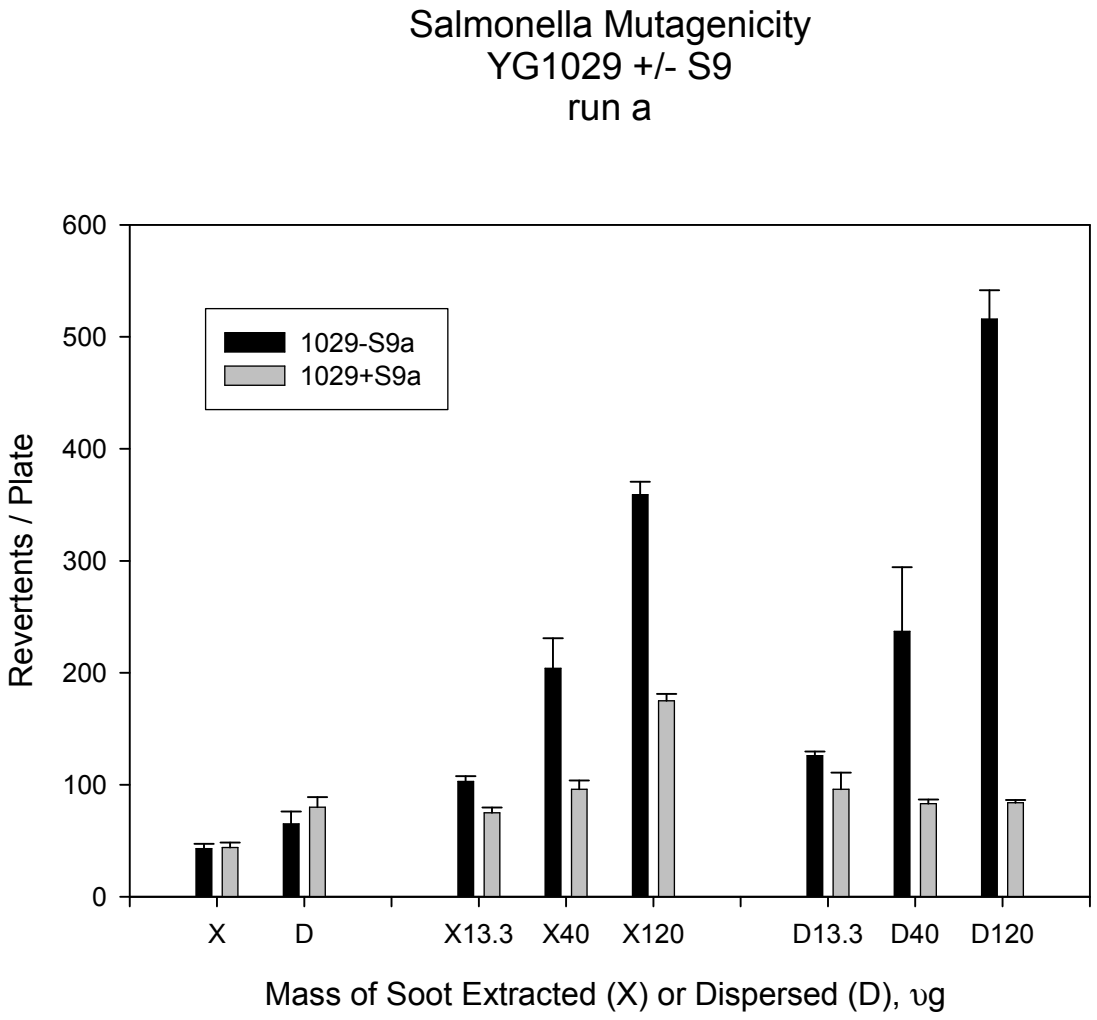
- Solvent extract:
  extract soot with acetone;
  exchange extracted materials into DMSO
  (4% extractables by mass)

- Surfactant dispersion:
  mix soot into aqueous dispersion of a phospholipid component of lung surfactant.

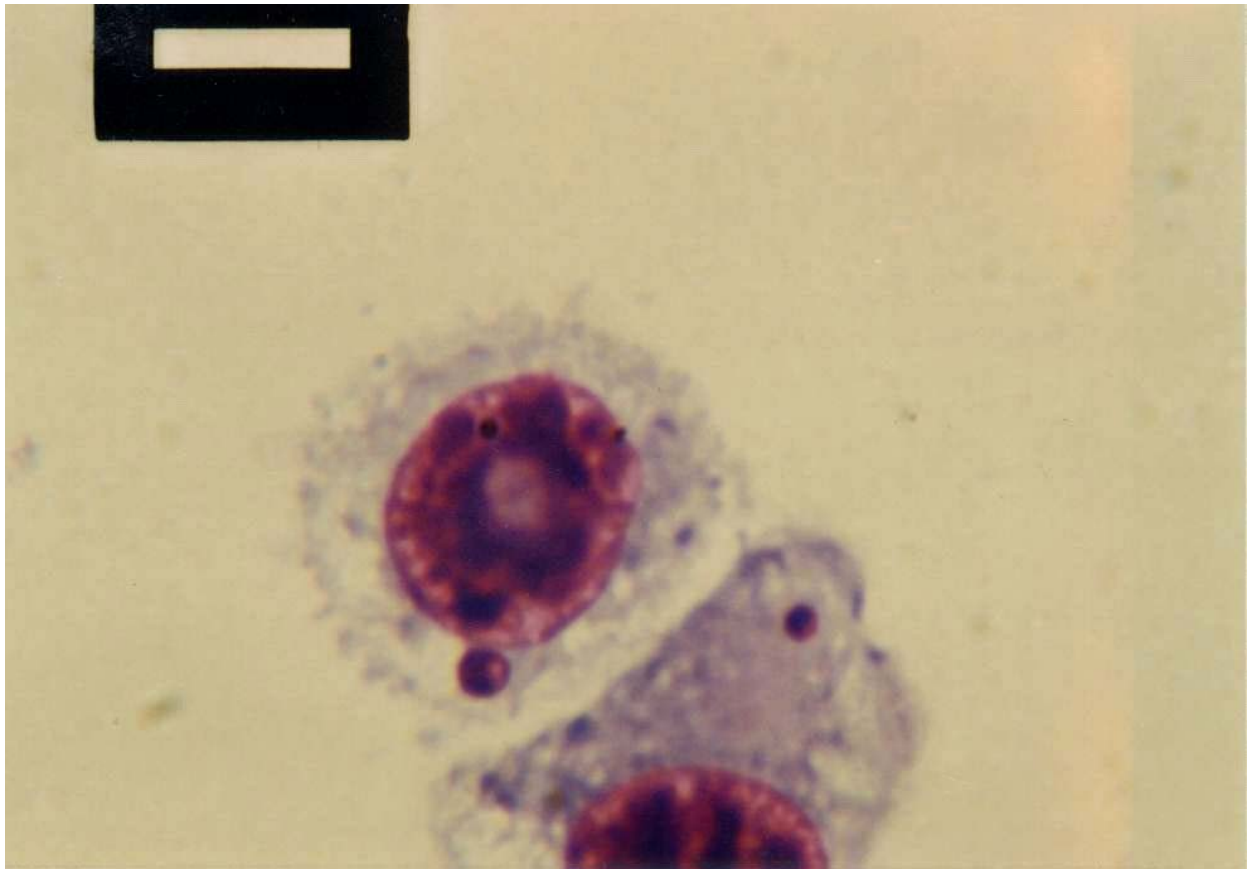
In Vitro Genotoxicity Assays

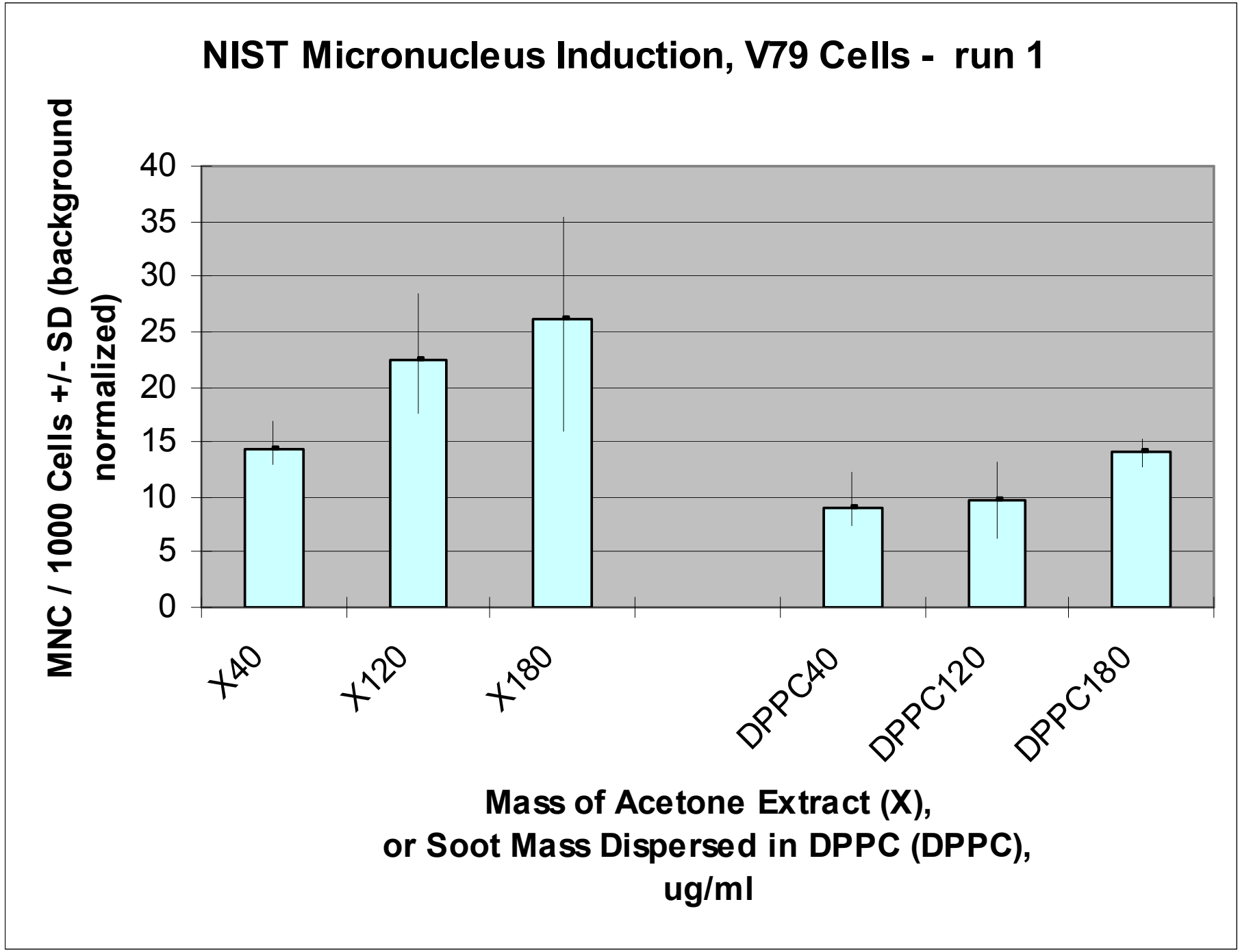
- Mutagenic activity, as measured by the "Ames test" for a reverse gene mutation in Salmonella
- DNA damage in a mammalian cell line as measured by single cell gel electrophoresis "comet" assay
- Chromosomal or clastogenic damage as measured by micronucleus induction

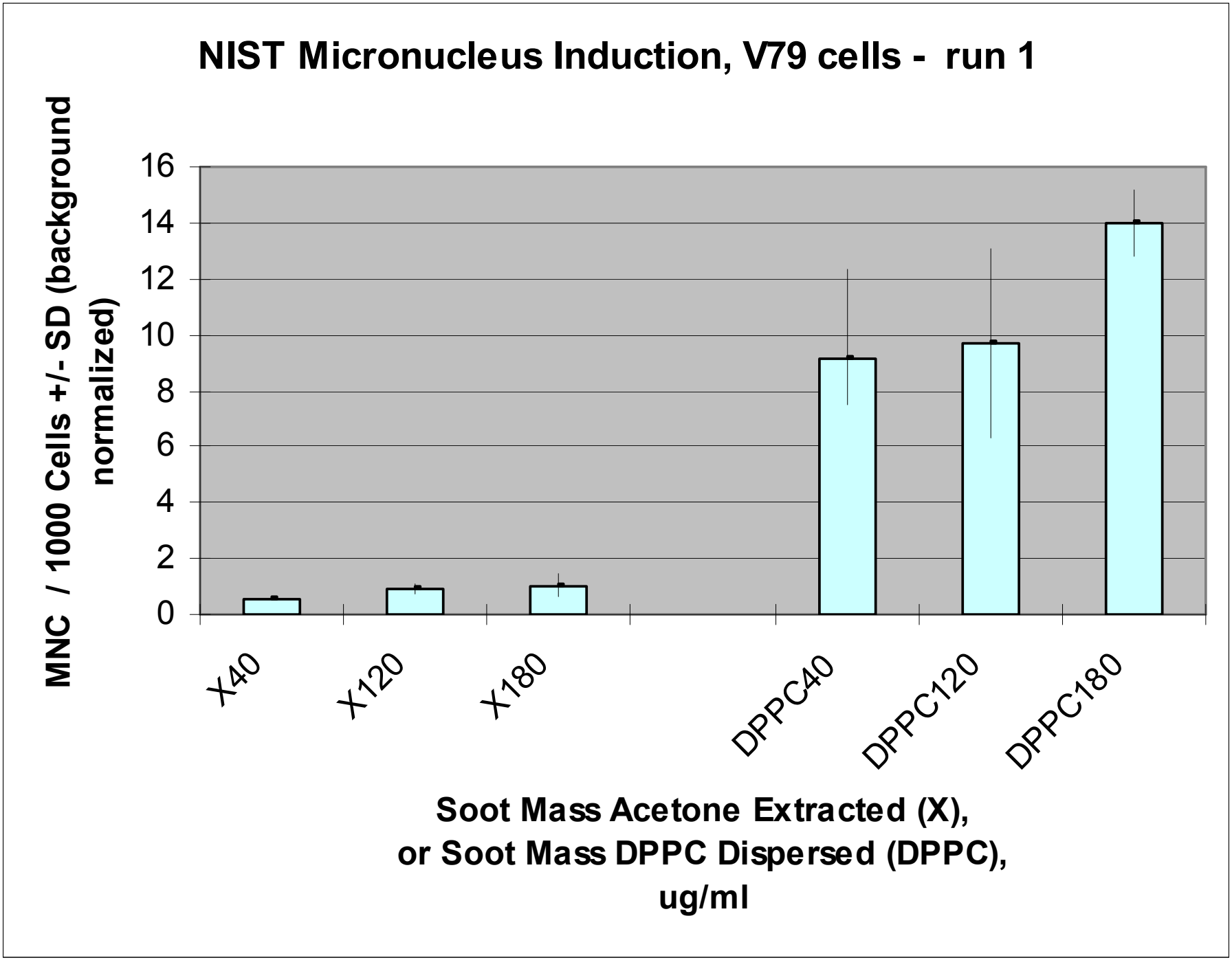

Salmonella Mutagenicity
YG1024 +/- S9
run a
1200
1000
800
600
400
200
0
Revertents / Plate
1024-S9 a
1024+S9 a
X
D
X13.3
X40
X120
D13.3
D40
D120
Mas of Soot Extracted (X) or Dispersed (D), υg


Salmonella Mutagenicity
YG1029 +/- S9
run a
600
500
400
300
200
100
0
Revertents / Plate
1029-S9a
1029+S9a
X
D
X13.3
X40
X120
D13.3
D40
D120
Mass of Soot Extracted (X) or Dispersed (D), υg

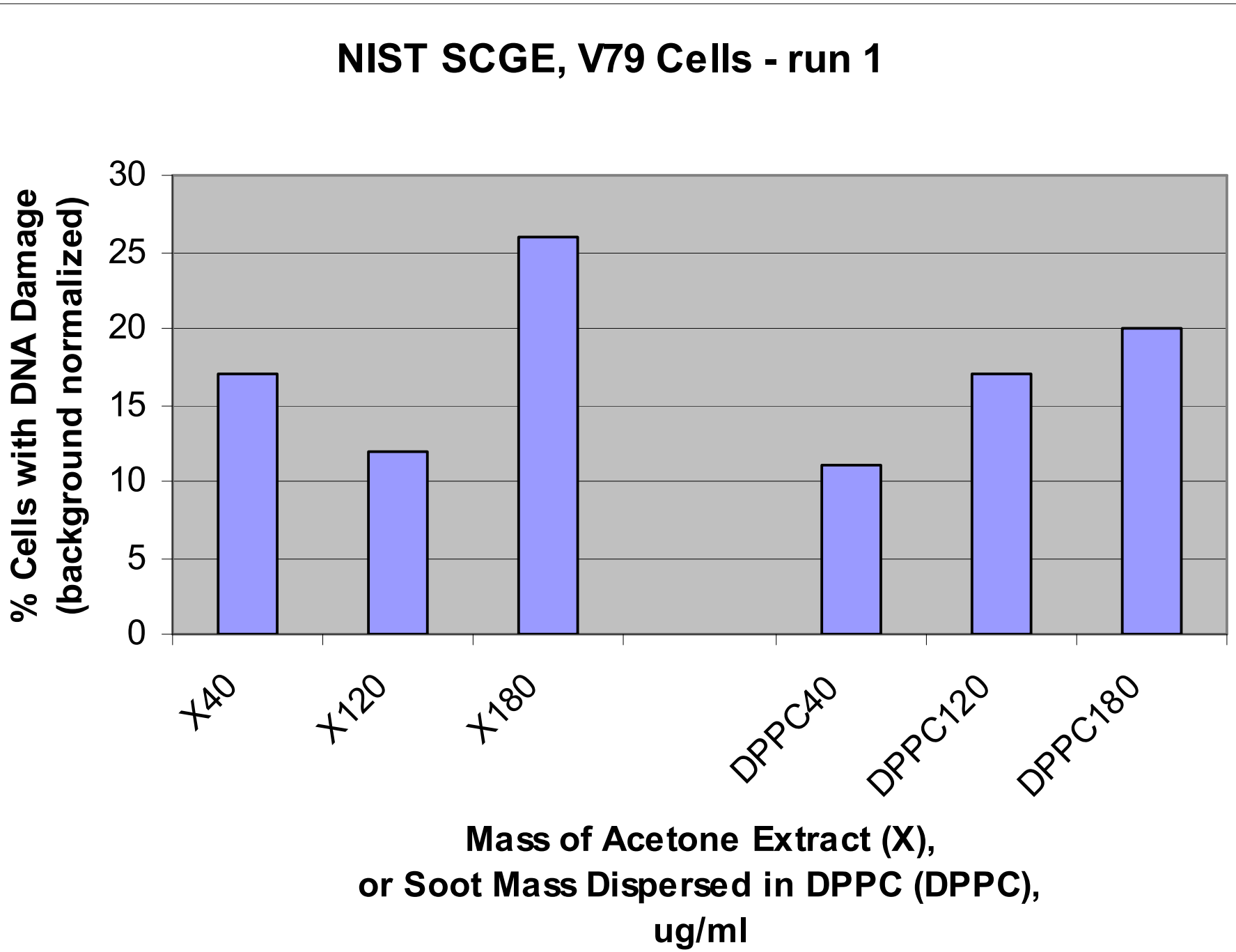
Micronuclei in V79 cells challenged with surfactant-dispersed diesel exhaust particles

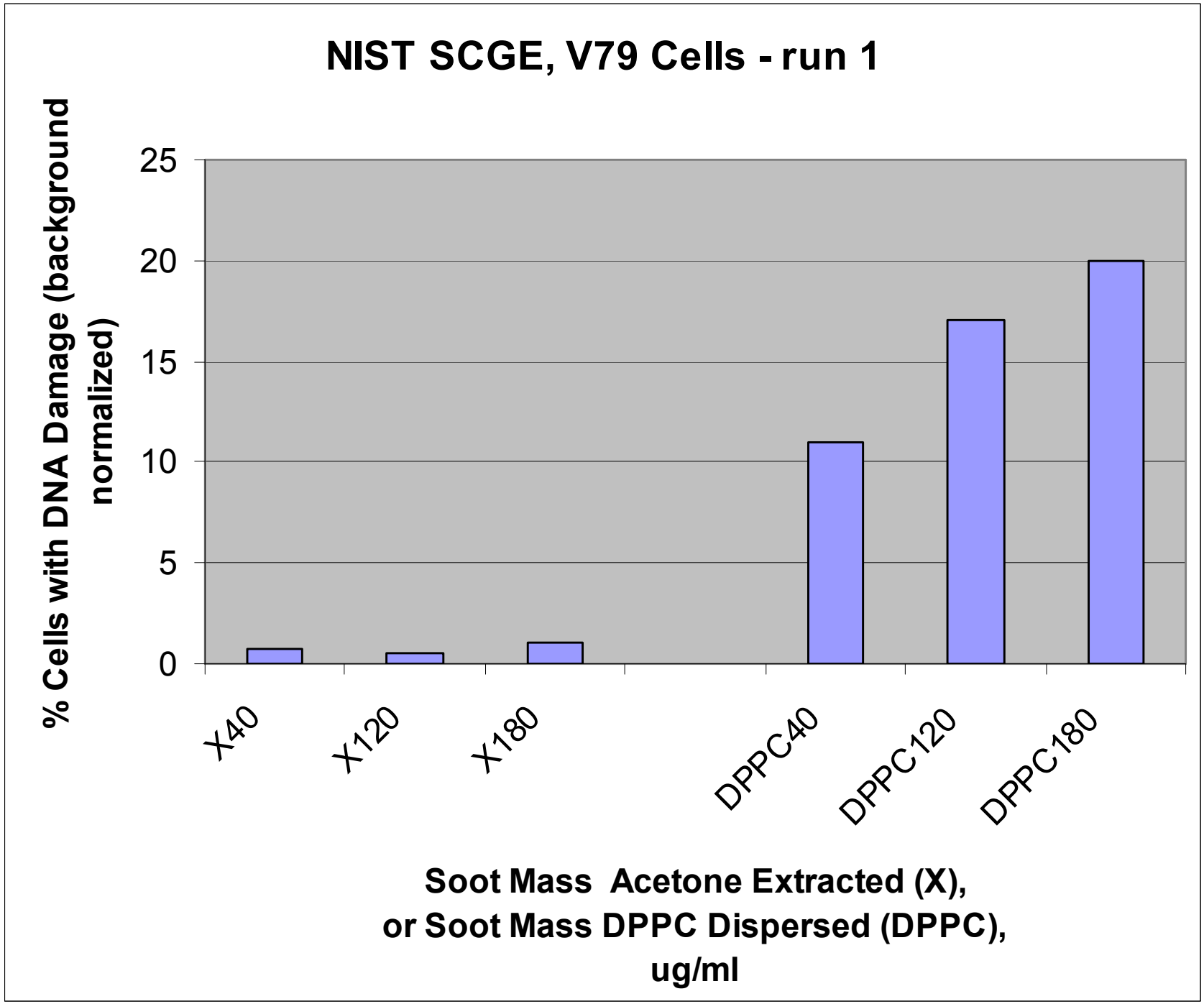



NIST Micronucleus Induction, V79 Cells - run 1
MNC / 1000 Cells +/- SD (background normalized)
40
35
30
25
20
15
10
5
0
X40
X120
X180
DPPC40
DPPC120
DPPC180
Mass of Acetone Extract (X),
or Soot Mass Dispersed in DPPC (DPPC),
ug/ml


NIST Micronucleus Induction, V79 cells - run 1
MNC / 1000 Cells +/- SD (background normalized)
16
14
12
10
8
6
4
2
0
X40
X120
X180
DPPC40
DPPC120
DPPC180
Soot Mass Acetone Extracted (X),
or Soot Mass DPPC Dispersed (DPPC),
ug/ml

Single Cell Electrophoresis ("Comet") Assay showing Damaged Cellular DNA in V79 mammalian cells challenged with surfactant-dispersed diesel exhaust particles


NIST SCGE, V79 Cells - run 1
% Cells with DNA Damage
(background normalized)
30
25
20
15
10
5
0
X40
X120
X180
DPPC40
DPPC120
DPPC180
Mass of Acetone Extract (X),
or Soot Mass Dispersed in DPPC (DPPC),
ug/ml


NIST SCGE, V79 Cells - run 1
% Cells with DNA Damage (background normalized)
25
20
15
10
5
0
X40
X120
X180
DPPC40
DPPC120
DPPC180
Soot Mass Acetone Extracted (X),
or Soot Mass DPPC Dispersed (DPPC),
ug/ml

Discussion:
Reasons for Testing DPM as Soot Dispersed in Surfactant

To retain soot particulate properties,
ultrafine particle size, and structural features
while modeling their conditioning upon deposition in the deep lung.

Those may affect
biological availability or expression of activity
of particle-borne genotoxicants.

Summary of Results

- Salmonella mutagenicity + for both solvent extract and surfactant dispersion of NIST DPM
- Mammalian cell DNA damage "Comet Assay) and clastogenic/chromosomal damage (micronucleus assay) both + for both preparations.
- Genotoxic activities in mammalian cells were stronger for surfactant-dispersed whole soot particles than for the organics extracted from an equal mass of soot.
- This appearance of an ultrafine particulate phase synergistic effect on expression of genotoxic activity is being further analyzed.

Discussion/ Conclusions

- Genotoxic activity can be expressed in vitro by DPM dispersed in a primary lung surfactant.

- → models bio-availability
  of DPM genotoxicants in the lung

- → preserves ultrafine particulate structural effects in bioassays

- →→ particulate structure can significantly affect genotoxic activity,
  as seen here in mammalian cells

Discussion:
Elements of a Strategy

→ DPM collection/ bio-assay
as a dispersion in lung surfactant

→→ Identify critical DPM
composition and structural properties
determining genotoxicity

→→→ Identify engineering factors
controlling DPM properties/genotoxicity

→→→→ Inform engineering development;
inform in vivo testing

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The Analysis of Genotoxic Activities of Exhaust Emissions from Mobile Internal Combustion Engines

Novel Collection and Toxicological Analysis Techniques for IC Engine Exhaust Particulate Matter

Disclaimer: The findings and conclusions in this report have not been formerly disseminated by the National Institute for Occupational Safety and Health and should not be construed to represent any agency determination or policy.

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"Mutagenicity of Diesel Exhaust Particles and Oil Shale Particles Dispersed In Lecithin Surfactant".

Wallace WE, Keane MJ, Hill CA, Xu J, Ong TM, "Mutagenicity of Diesel Exhaust Particles and Oil Shale Particles Dispersed In Lecithin Surfactant". Journal of Toxicology and Environmental Health, 21 163-171 (1987). -- ref. in : IARC Monograph 46, 1989

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Wallace WE, Keane M, Xing S, Harrison J, Gautam M, Ong T, in Environmental Hygiene II, pp.7-10; Eds.NH Seemayer and W Hadnagy, Springer Verlag, Berlin, ISBN 0-387-52735-4 (1990).

"Genotoxicity of Diesel Exhaust articles Dispersed in Simulated Pulmonary Surfactant". Keane MJ, Xing SG, Harrison J, Ong TM, Wallace WE Mutation Research 260 233-238 (1991).

"Micronucleus Induction and Phagocytosis in Mammalian Cells Treated with Diesel Emission Particles". Gu, ZW, Zhong BZ, Nath B, Whong WZ, Wallace W, Ong T. Mutation Research.279: 55-60 (1992).

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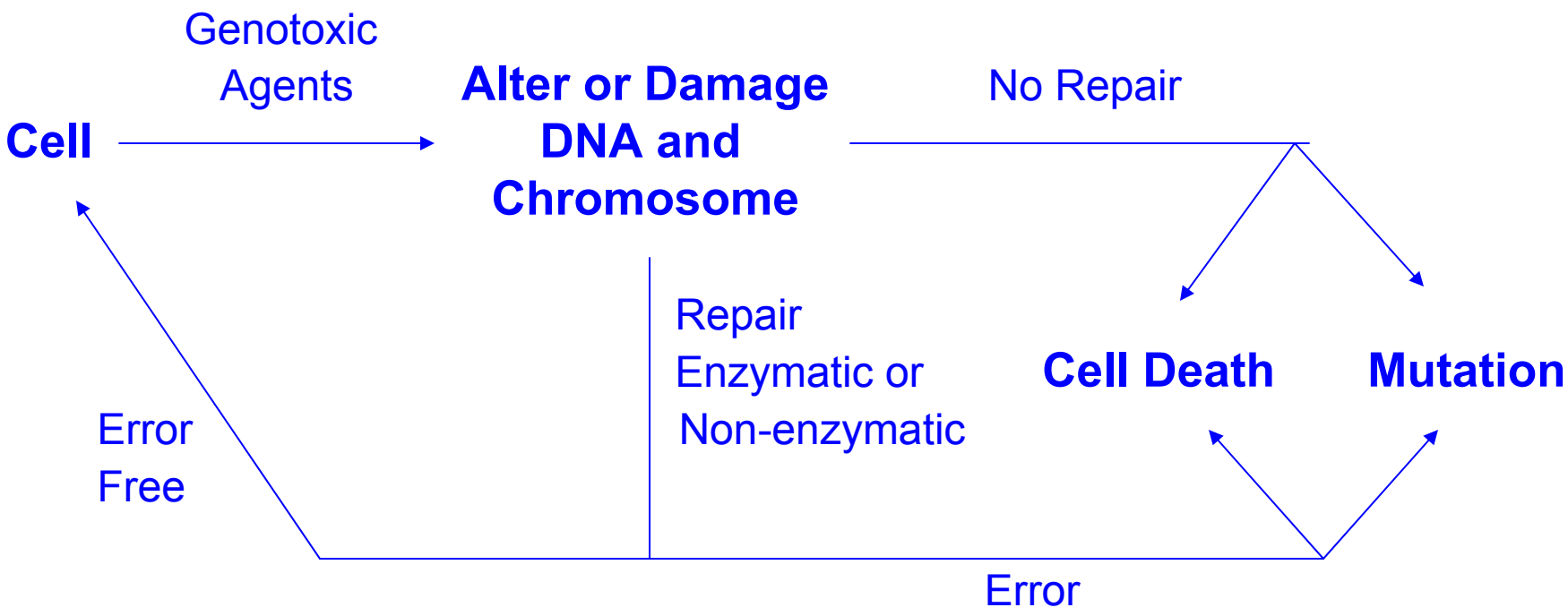
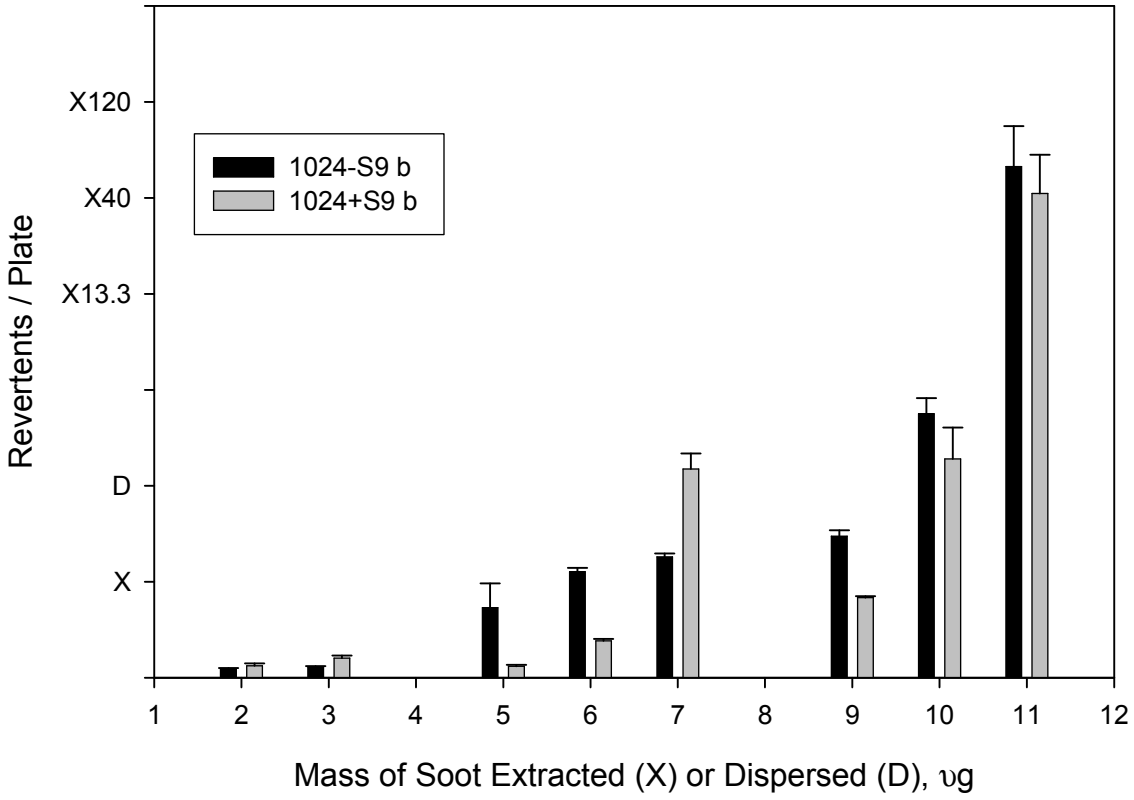
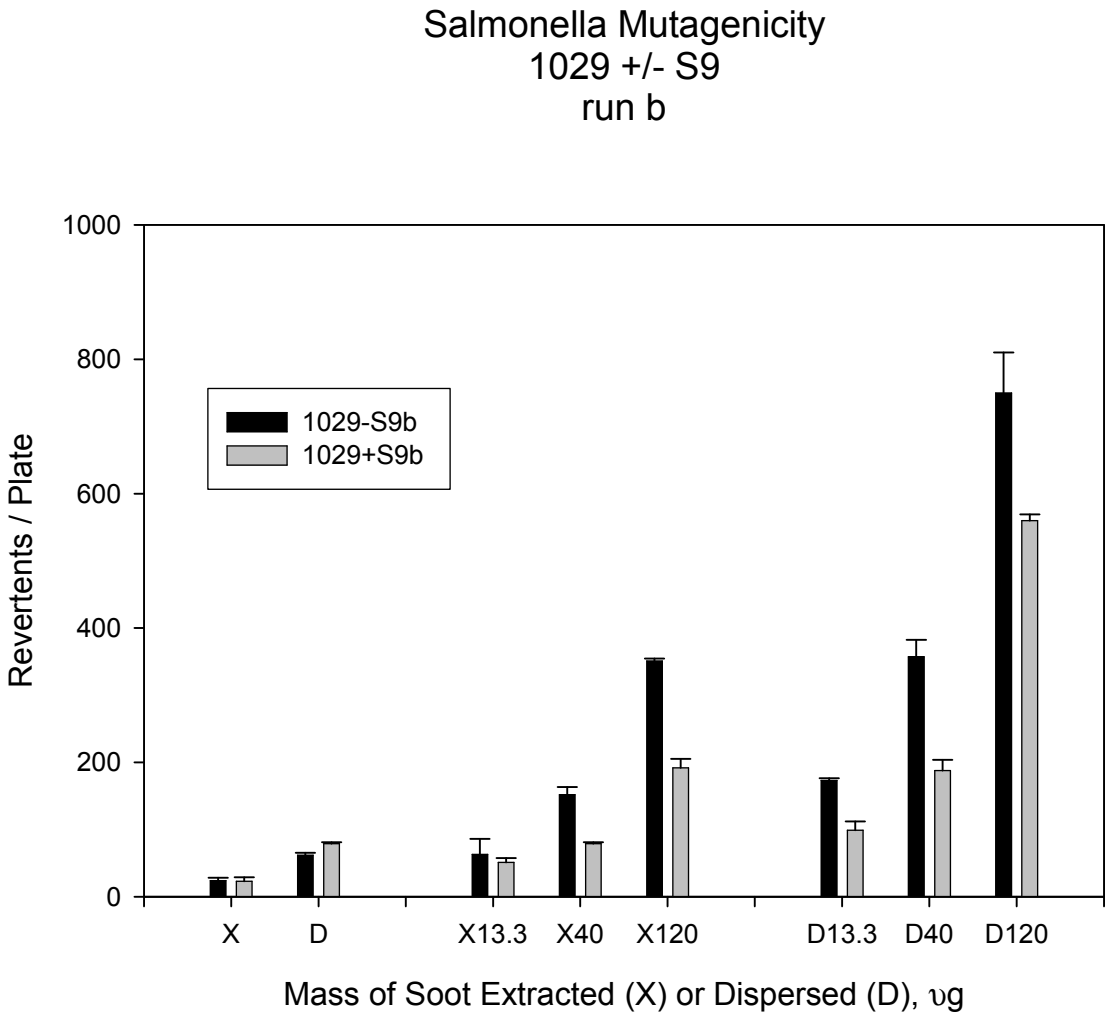

Genotoxic
Agents
Cell
Alter or Damage
DNA and
Chromosome
No Repair
Repair
Enzymatic or
Non-enzymatic
Cell Death
Mutation
Error
Free
Error

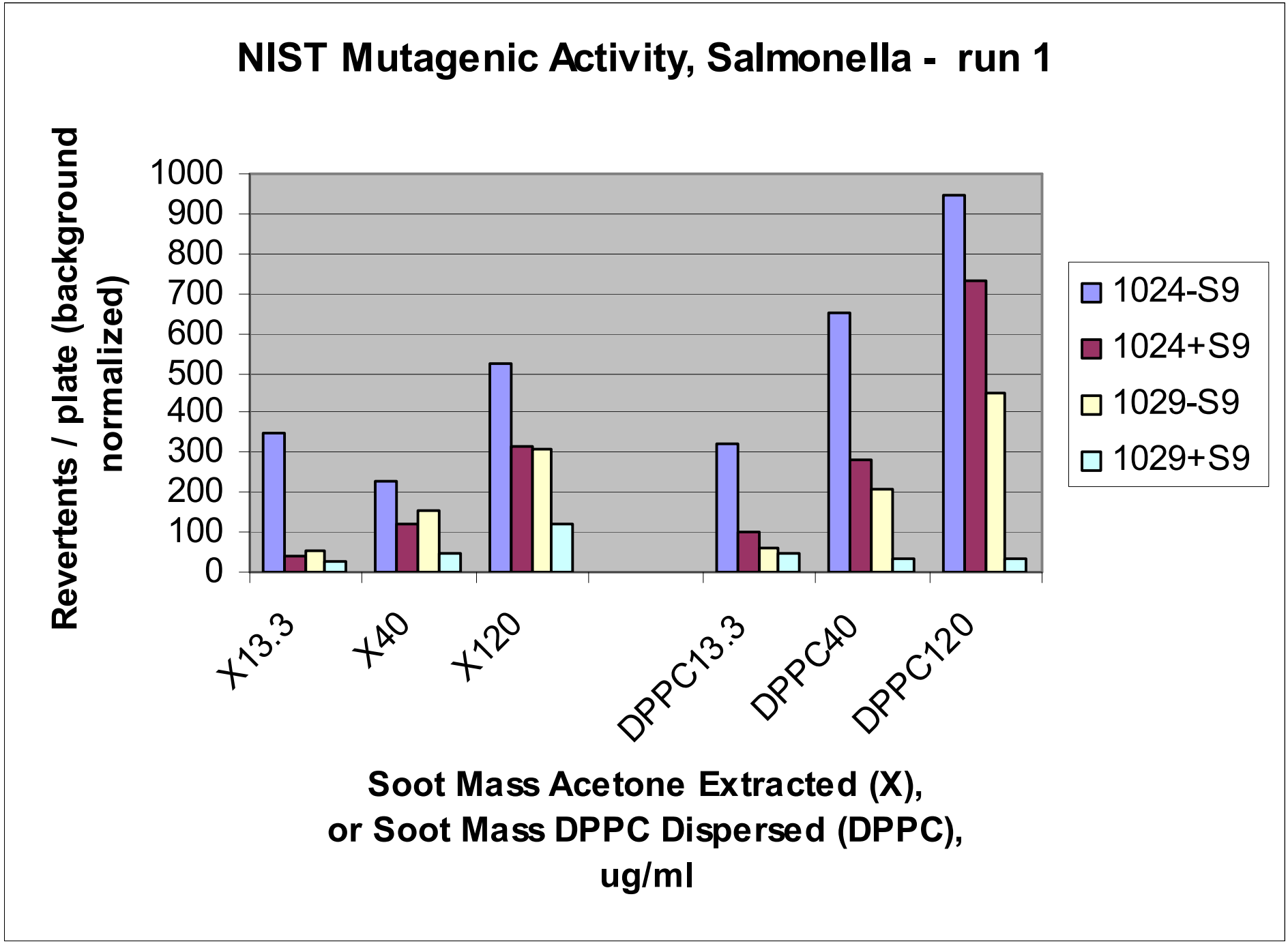
Surfactant Needed for Soot Dispersion

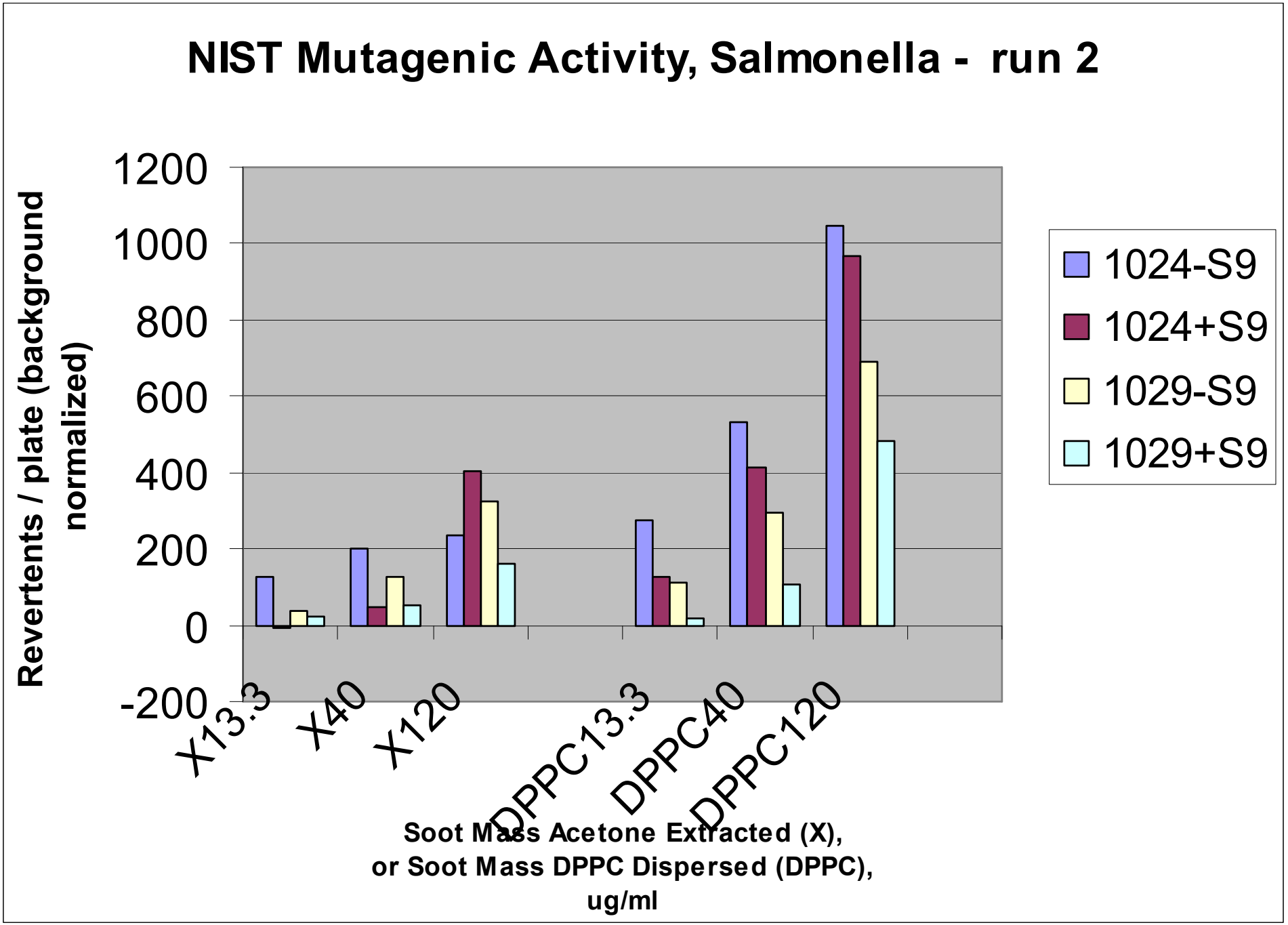
- Diesel soot is ultrafine (nanoparticulate) in size
- → S = specific surface area of soot = on the order of
  100 – 300 m^2/ gram
- DPPC molecules oriented with their hydrophobic lipid tails to the soot particle surface; close packing → about 100A^2 soot particle surface area occupied by a DPPC molecule
- → About S x 10^{-3} g DPPC / g soot for monolayer coverage
- → About 3 x S x 10^{-3} g DPPC / g soot for monolayer + outer bilayer for efficient dispersion
- → About 1 gram DPPC / gram soot for S = 100 m^2/gram

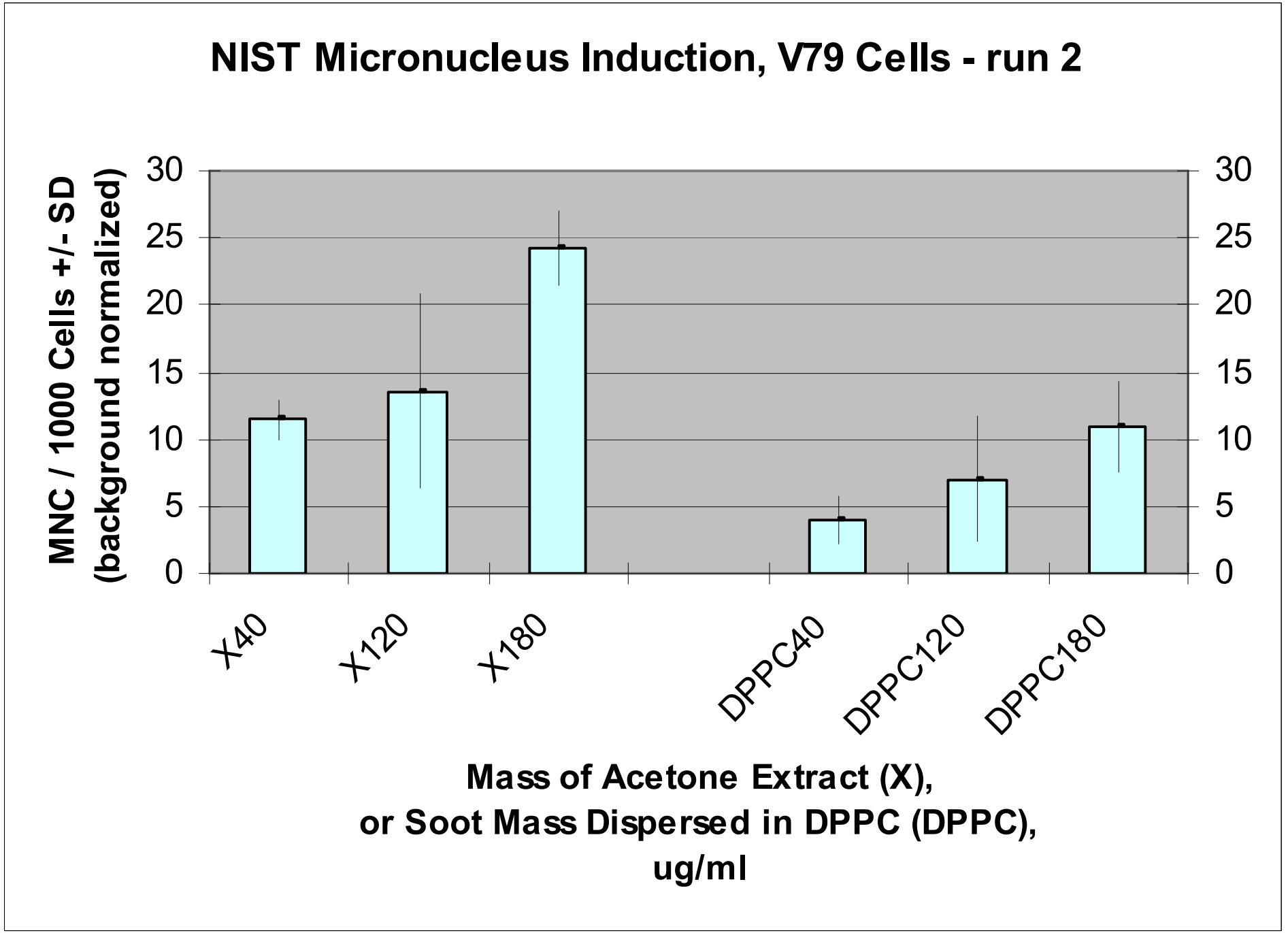
Salmonella Mutagenicity
YG1024 +/- S9
run b

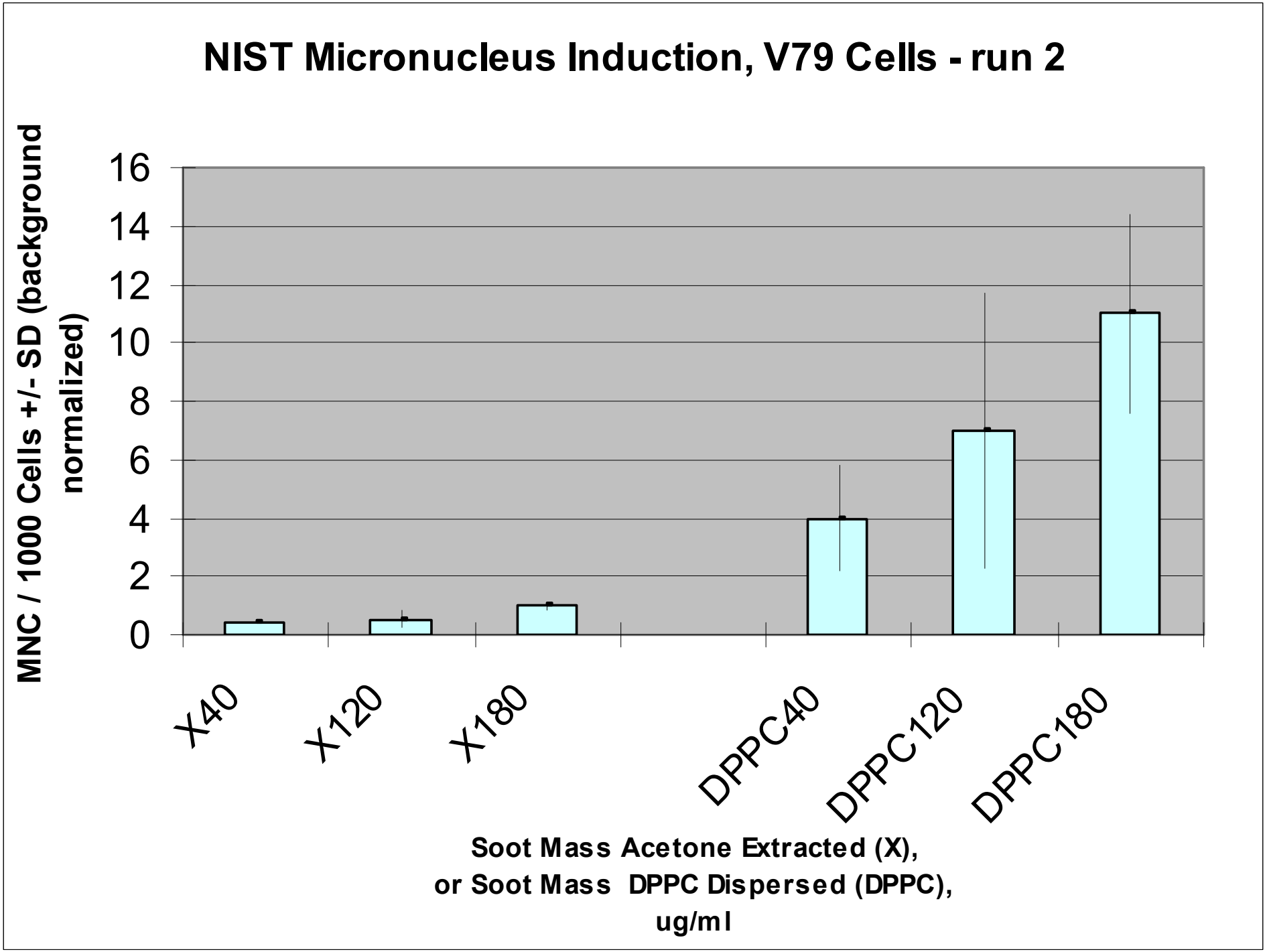


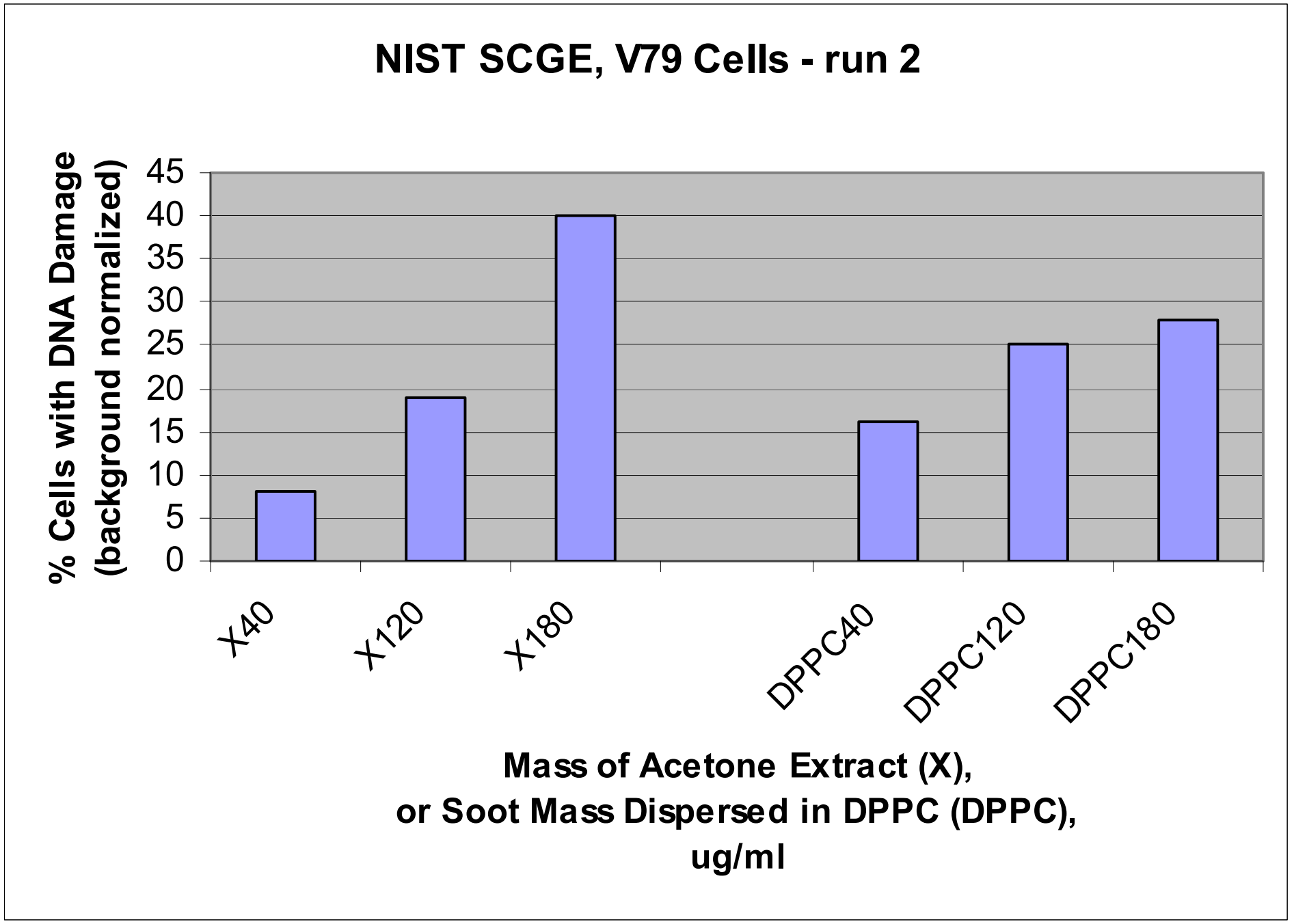

Salmonella Mutagenicity
1029 +/- S9
run b
1000
800
600
400
200
0
Revertents / Plate
1029-S9b
1029+S9b
X
D
X13.3
X40
X120
D13.3
D40
D120
Mass of Soot Extracted (X) or Dispersed (D), υg

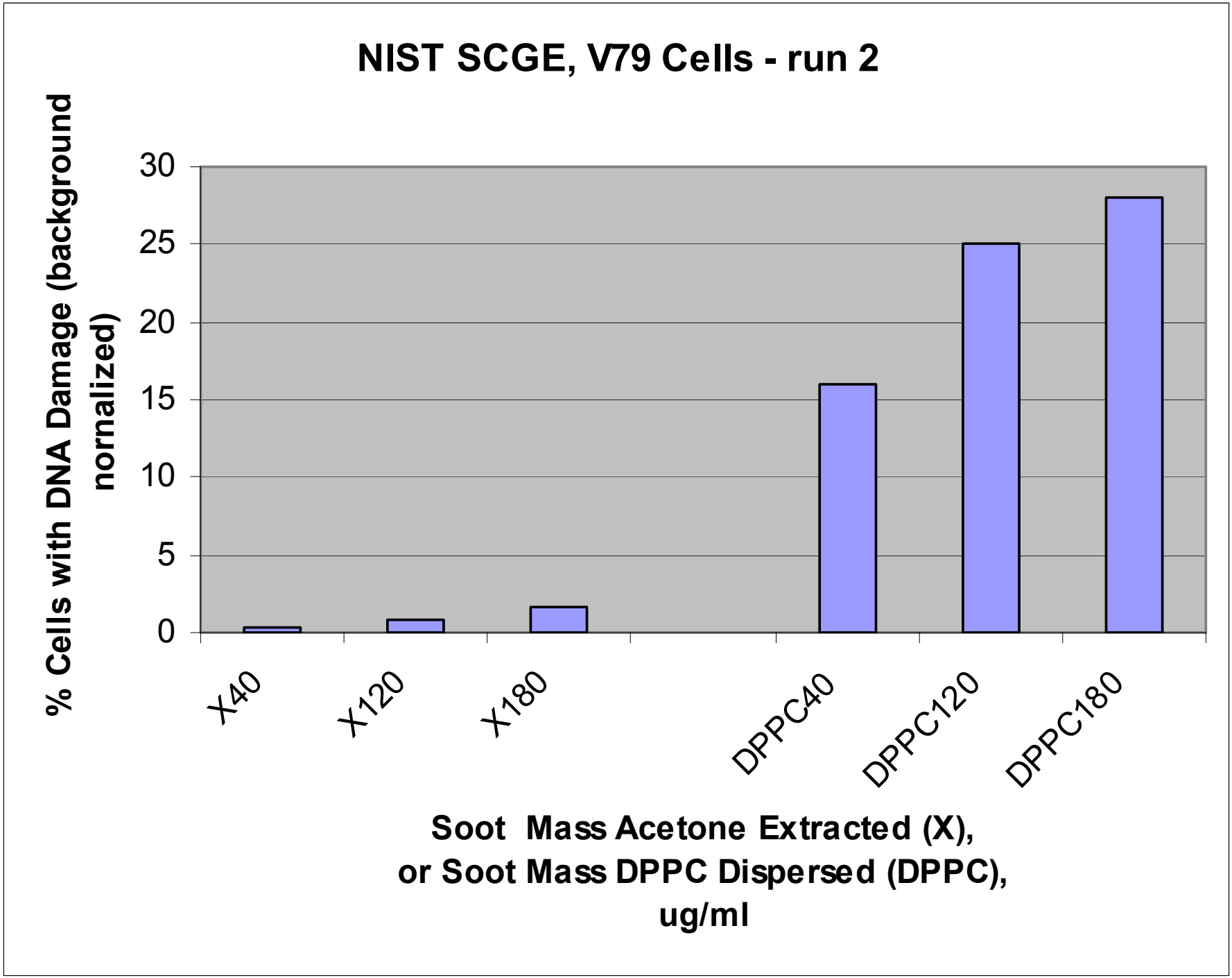

NIST Mutagenic Activity, Salmonella - run 1
Revertents / plate (background normalized)
1000
900
800
700
600
500
400
300
200
100
0
X13.3
X40
X120
DPPC13.3
DPPC40
DPPC120
1024-S9
1024+S9
1029-S9
1029+S9
Soot Mass Acetone Extracted (X),
or Soot Mass DPPC Dispersed (DPPC),
ug/ml


NIST Mutagenic Activity, Salmonella - run 2
Revertents / plate (background normalized)
1200
1000
800
600
400
200
0
-200
X13.3
X40
X120
DPPC13.3
DPPC40
DPPC120
Soot Mass Acetone Extracted (X),
or Soot Mass DPPC Dispersed (DPPC),
ug/ml
1024-S9
1024+S9
1029-S9
1029+S9


NIST Micronucleus Induction, V79 Cells - run 2
MNC / 1000 Cells +/- SD
(background normalized)
30
25
20
15
10
5
0
X40
X120
X180
DPPC40
DPPC120
DPPC180
Mass of Acetone Extract (X),
or Soot Mass Dispersed in DPPC (DPPC),
ug/ml


NIST Micronucleus Induction, V79 Cells - run 2
MNC / 1000 Cells +/- SD (background normalized)
16
14
12
10
8
6
4
2
0
X40
X120
X180
DPPC40
DPPC120
DPPC180
Soot Mass Acetone Extracted (X),
or Soot Mass DPPC Dispersed (DPPC),
ug/ml


NIST SCGE, V79 Cells - run 2
% Cells with DNA Damage
(background normalized)
45
40
35
30
25
20
15
10
5
0
X40
X120
X180
DPPC40
DPPC120
DPPC180
Mass of Acetone Extract (X),
or Soot Mass Dispersed in DPPC (DPPC),
ug/ml


NIST SCGE, V79 Cells - run 2
% Cells with DNA Damage (background nornalized)
30
25
20
15
10
5
0
X40
X120
X180
DPPC40
DPPC120
DPPC180
Soot Mass Acetone Extracted (X),
or Soot Mass DPPC Dispersed (DPPC),
ug/ml

Observations from Surfactant-mediated Genotoxicity Testing of Other DPM:

- Tests with supernatant of centrifuged vs filtered (<0.2 micrometer) DPM dispersion in surfactant → significant activity associated with ultrafine particles
- Tests with particulate residue of organic solvent extracted DPM = no activity → particles without genotoxicants were inactive
- Tests with other phospholipid surfactants (DPPE, DPPA) → comparable bacterial mutagenic activity in DPPC
- Mammalian cell 6-TG mutation not induced by solvent- or surfactant-preparation of DPM.
- Surfactant adsorption suppresses some particulate cytotoxic activities (non-genetic toxicity) → possibly an aid to the expression of genotoxic activity

Salmonella Mutagenicity Assay:

Histidine reversion gene mutation assay in Salmonella typhimurium YG1024 or YG1029 +/- S9 microsomal enzyme activation. Micro-suspension test.

Sample = filter-collected NIST Diesel Exhaust Standard Material 2975.

Surfactant = dipalmitoyl phosphatidylcholine (DPPC) ultrasonically dispersed in physiological sterile saline (PSS); [DPPC] = 2.5 mg DPPC / ml PSS.

Surfactant Dispersion of DPM:

mix DPM into DPPC/PSS dispersion:
[DPM] = 1 mg DPM/ 2.5 mg DPPC / 1 ml PSS.
Dilute with PSS to provide samples of 13.3, 40, 120 microgram DPM in 10 micro-liter PSS.

Solvent preparations of DPM:

dissolve DPM in acetone, evaporate, dissolve in dimethylsulfoxide (DMSO).
[DPM] = 2 mg DPM extract / ml DMSO. Dilute with PSS to provide samples of
13.3, 40, 120 microgram extract in 10 microliter PSS.

Test protocol: Micro-suspension test:

mix samples (0.0133, 0.040, 0.120 mg DPM or solvent-extract of DPM in 0.01 ml PSS,
add .065 ml PSS or S9 preparation, add .025 ml of YG1024 or YG1029 @ 1-2 x 10^8 cells/ml;
Preincubate mixture 30 minutes at 37C;
Then mix with 2.5 ml top agar containing .05 mM biotin + histidine;
Grow cells 48 hr (YG1024) or 72 hr (YG1029)
Count colonies.

Mammalian Cell Micronucleus Assay:

Micronucleus induction in V79 cells.

Sample = filter-collected NIST Diesel Exhaust Standard Material 2975.

Cells = V79: $2x10^6$ cells / ml medium grown 24h prior to challenge.

Positive control = MNNG

Surfactant = dipalmitoyl phosphatidylcholine (DPPC) ultrasonically dispersed in physiological sterile saline (PSS); [DPPC] = 2.5 mg DPPC / ml PSS.

Surfactant Dispersion of DPM:

mix DPM into DPPC/PSS dispersion:
[DPM] = 1 mg DPM/ 2.5 mg DPPC / 1 ml PSS.
Dilute with PSS to samples of 40, 120,180 microgram DPM / ml PSS.

Solvent preparations of DPM:

extract DPM with acetone, evaporate extract, dissolve extract residue in dimethylsulfoxide (DMSO). [DPM] = 2 mg DPM extract / ml DMSO. Dilute with PSS to provide samples of 200, 600, 900 microgram extract in 5 ml medium.

Assay:

24 h incubation of V79 cells in 5 ml medium with 40, 120, 180 microgram/ ml of (X) solvent extract of DPM or (D) DPPC-dispersed DPM.
After 24 hr challenge then rinse; replace 5 ml medium; incubate 24 hr. Harvest / fix / stain cells / prepare slides.
Score 6 x 1000 cells (n=6) for each concentration for each of 2 runs.

Mammalian Cell DNA Damage Assay:

Single-Cell Gel Electrophoresis Assay of Single- or Double-Strand DNA Damage in V79 cells.

Sample = filter-collected NIST Diesel Exhaust Standard Material 2975.

Cells = V79: 2x106 cells / ml medium grown 24h prior to challenge.

Positive control = MNNG

Surfactant = dipalmitoyl phosphatidylcholine (DPPC) ultrasonically dispersed in physiological sterile saline (PSS); [DPPC] = 2.5 mg DPPC / ml PSS.

Surfactant Dispersion of DPM:

mix DPM into DPPC/PSS dispersion:
[DPM] = 1 mg DPM/ 2.5 mg DPPC / 1 ml PSS.
Dilute with PSS to samples of 40, 120,180 microgram DPM / ml PSS.

Solvent preparations of DPM:

extract DPM with acetone, evaporate extract, dissolve extract residue in dimethylsulfoxide (DMSO). [DPM] = 2 mg DPM extract / ml DMSO. Dilute with PSS to provide samples of 200, 600, 900 microgram extract in 5 ml medium.

Assay:

24 h incubation of V79 cells in 5 ml medium with 40, 120, 180 microgram/ml of (X) solvent extract of DPM or (D) DPPC-dispersed DPM.
After 24 hr challenge then perform single-cell gel electrophoresis; read 100 cells for each treatment for each of two runs.