

Appendix A (Continued)

Lab number	Assay number	CytB ^a	Schedule: treatment + recovery (h)	Solvent	Concentration ($\mu\text{g}/\text{ml}$) ^b	Micromononucleated cells ^c		Micromonucleated cells ^c		Survival (%)	Number of cultures analysed	Others ^d	Acc. ^e	Pos. ^f
						Number per 1000 cells	Induction factor	Number per 1000 cells	Induction factor					
		+	3+20	Medium	0	16.5	1.0	18.5	1.0	100	2		Y	NEG
					500	14.0	0.8	18.5	1.0	100	2			
					1000	nd	nd	11.5	0.6	94	2			
					3500	9.5	0.6	19.5	1.1	93	2			
					4000	10.5	0.6	25.5	1.4	88	2			
					4500	10.0	0.6	22.5	1.2	85	2			
					5000	11.5	0.7	22.5	1.2	83	2			
					MMC 0.2	23.0	1.4	120.0**	6.5	80	2	P		
		+	24+20	Medium	0	5.0	1.0	21.0	1.0	100	2		Y	POS
					500	9.0	1.8	40.0**	1.9	117	2			
					4000	8.0	1.6	48.0**	2.3	97	2			
					4500	9.5	1.9	54.5**	2.6	102	2			
					5000	8.0	1.6	34.5*	1.6	88	2			
					MMC 0.1	26.5**	5.3	224.5**	10.7	82	2	P		
Lab 1	Assay 2	-	3+21	Medium	0	21.0	1.0	na	na	100	2		Y	NEG
					100	14.5	0.7	na	na	95	2			
					200	16.0	0.8	na	na	86	2			
					700	17.5	0.8	na	na	88	2			
					800	13.5	0.6	na	na	84	2			
					900	22.0	1.0	na	na	77	2			
					1000	9.0	0.4	na	na	80	2			
					MMC 0.2	155.5**	7.4	na	na	71	2	P		
		-	3+45	Medium	0	26.5	1.0	na	na	100	2		Y	NEG
					100	16.5	0.6	na	na	153	2			
					200	18.5	0.7	na	na	125	2			
					700	17.0	0.6	na	na	7	2	T		
					800	19.5	0.7	na	na	123	2			
					900	17.5	0.7	na	na	123	2			
					1000	15.5	0.6	na	na	98	2			
					MMC 0.2	132.5**	5.0	na	na	97	2	P		
		-	24+0	Medium	0	17.0	1.0	na	na	100	2		Y	NEG
					100	19.0	1.1	na	na	91	2			
					200	18.5	1.1	na	na	97	2			
					700	14.0	0.8	na	na	97	2			
					800	14.5	0.9	na	na	104	2			
					900	16.0	0.9	na	na	101	2			
					1000	15.5	0.9	na	na	101	2			
					MMC 0.1	122.5**	7.2	na	na	39	2	P		

Appendix A (Continued)

Lab number	Assay number	CytB*	Schedule: treatment + recovery (h)	Solvent	Concentration ($\mu\text{g/ml}$) ^b	Micronucleated mononucleated cells ^c		Micronucleated binucleated cells ^c		Survival (%)	Number of cultures analysed	Others ^d	Acc. ^e	Pos. ^f
						Number per 1000 cells	Induction factor	Number per 1000 cells	Induction factor					
		-	24+0	Medium	0	9.0	1.0	na	na	100	1		Y	NEG
					78	14.0	1.6	na	na	95	1			
					156	16.0	1.8	na	na	113	1			
					312	6.0	0.7	na	na	105	1			
					625	8.0	0.9	na	na	116	1			
					1250	7.0	0.8	na	na	117	1			
					5000	16.0	1.8	na	na	111	1			
					MMC 0.1	51.0**	5.7	na	na	74	1			
		-	24+24	Medium	0	21.0	1.0	na	na	100	1		Y	NEG
					78	20.0	1.0	na	na	92	1			
					156	11.0	0.5	na	na	109	1			
					312	16.0	0.8	na	na	nt	1			
					625	17.0	0.8	na	na	103	1			
					1250	14.0	0.7	na	na	94	1			
					5000	8.0	0.4	na	na	76	1			
					MMC 0.1	192.0**	9.1	na	na	nt	1			
		+	3+20	Medium	0	3.0	1.0	9.0	1.0	100	1		Y	NEG
					78	4.0	1.3	9.0	1.0	96	1			
					156	6.0	2.0	19.0	2.1	96	1			
					312	6.0	2.0	7.0	0.8	105	1			
					625	4.0	1.3	11.0	1.2	104	1			
					1250	3.0	1.0	7.0	0.8	102	1			
					5000	3.0	1.0	6.0	0.7	105	1			
					MMC 0.2	26.0**	8.7	49.0**	5.4	95	1			
		+	24+20	Medium	0	7.0	1.0	16.0	1.0	100	1		Y	NEG
					78	6.0	0.9	17.0	1.1	100	1			
					156	5.0	0.7	9.0	0.6	105	1			
					312	7.0	1.0	29.0	1.8	112	1			
					625	9.0	1.3	18.0	1.1	103	1			
					1250	8.0	1.1	24.0	1.5	104	1			
					5000	4.0	0.6	10.0	0.6	101	1			
					MMC 0.1	156.0**	22.3	299.0**	18.7	107	1			
Lab 2	Assay 2	-	3+21	Medium	0	9.5	1.0	na	na	100	2		Y	NEG
					1250	12.5	1.3	na	na	98	2			
					2500	8.0	0.8	na	na	108	2			
					5000	11.5	1.2	na	na	100	2			
					MMC 0.2	52.5**	5.5	na	na	94	2			

–	3+45	Medium	0	11.5	1.0	na	na	100	2	Y	NEG
			1250	11.0	1.0	na	na	121	2		
			2500	8.0	0.7	na	na	119	2		
			5000	10.0	0.9	na	na	122	2		
			MMC 0.2	48.5**	4.2	na	na	126	2		
–	24+0	Medium	0	15.5	1.0	na	na	100	2	Y	NEG
			1250	11.0	0.7	na	na	111	2		
			2500	9.0	0.6	na	na	96	2		
			5000	10.5	0.7	na	na	87	2		
			MMC 0.1	100.0**	6.5	na	na	86	2		
–	24+24	Medium	0	17.0	1.0	na	na	100	2	Y	NEG
			1250	15.5	0.9	na	na	101	2		
			2500	8.5	0.5	na	na	93	2		
			5000	12.5	0.7	na	na	92	2		
			MMC 0.1	198.5**	11.7	na	na	72	2		
+	3+20	Medium	0	4.5	1.0	13.0	1.0	100	2	Y	NEG
			1250	1.5	0.3	9.0	0.7	97	2		
			2500	5.5	1.2	17.0	1.3	97	2		
			5000	2.5	0.6	14.0	1.1	99	2		
			MMC 0.2	5.5	1.2	55.5**	4.3	93	2		
+	24+20	Medium	0	3.0	1.0	10.5	1.0	100	2	Y	NEG
			1250	2.5	0.8	18.5*	1.8	95	2		
			2500	3.0	1.0	12.5	1.2	102	2		
			5000	3.0	1.0	15.0	1.4	100	2		
			MMC 0.1	119.0**	39.7	276.0**	26.3	105	2		
Bleomycin											
Lab 1											
–	3+45	Medium	0	23.5	1.0	na	na	100	2	Y	POS
			0.88	185.0**	7.9	na	na	91	2		
			62.5	187.5**	8.0	na	na	49	2		
			125	174.0**	7.4	na	na	43	2		
			250	144.0**	6.1	na	na	14	2		T
			MMC 0.2	160.0**	6.8	na	na	79	2		
–	24+0	Medium	0	25.0	1.0	na	na	100	2	Y	POS
			3.5	143.0**	5.7	na	na	77	2		
			15	123.0**	4.9	na	na	62	2		
			62.5	101.0**	4.0	na	na	46	2		
			250	44.0**	1.8	na	na	30	2		T
			500	29.5	1.2	na	na	21	2		T
			MMC 0.1	119.0**	4.8	na	na	72	2		
+	3+20	Medium	0	9.5	1.0	20.0	1.0	100	2	Y	POS
			0.17	18.0*	1.9	150.5**	7.5	70	2		
			0.39	18.5*	1.9	133.0**	6.7	86	2		
			0.875	13.5	1.4	113.5**	5.7	82	2		

Appendix A (Continued)

Lab number	Assay number	CytB ^a	Schedule: treatment + recovery (h)	Solvent	Concentration (µg/ml) ^b	Micromonocultured cells ^c		Micromonocultured cells ^c		Survival (%)	Number of cultures analysed	Others ^d	Acc. ^e	Pos. ^f
						Number per 1000 cells	Induction factor	Number per 1000 cells	Induction factor					
Lab 1	Assay 2	-	3 + 21	Medium	1.75	15.0	1.6	169.5**	8.5	67	2			
					1.4	13.5	1.4	tox	tox	39	2	T		
					MMC 0.2	19.0*	2.0	199.0**	10.0	83	2			
					0	7.5	1.0	15.5	1.0	100	2		Y	POS
					0.17	43.0**	5.7	155.0**	10.0	104	2			
					0.26	56.5**	7.5	211.5**	13.6	101	2			
					0.39	74.0**	9.9	231.0**	14.9	73	2			
					0.587	102.5**	13.7	342.5**	22.1	66	2			
					0.875	122.5**	16.3	319.0**	20.6	54	2			
					MMC 0.1	22.0**	2.9	275.5**	17.8	92	2			
					0	17.0	1.0	na	na	100	2		Y	POS
					62.5	176.0**	10.4	na	na	69	2			
					125	138.0**	8.1	na	na	53	2			
					250	123.5**	7.3	na	na	46	2		T	
					500	74.0**	4.4	na	na	37	2			
					MMC 0.2	171.0**	10.1	na	na	79	2			
					0	19.5	1.0	na	na	100	2		Y	POS
0.88	184.5**	9.5	na	na	93	2								
62.5	241.5**	12.4	na	na	18	2		T						
125	189.0**	9.7	na	na	20	2		T						
250	150.5**	7.7	na	na	13	2		T						
MMC 0.2	190.0**	9.7	na	na	91	2								
0	18.0	1.0	na	na	100	2		Y	POS					
3.5	121.5**	6.8	na	na	62	2								
15	113.5**	6.3	na	na	38	2		T						
62.5	90.5**	5.0	na	na	40	2								
250	29.5*	1.6	na	na	25	2		T						
500	24.5	1.4	na	na	27	2		T						
MMC 0.1	87.0**	4.8	na	na	49	2								
0	25.0	1.0	na	na	100	2		Y	POS					
3.5	282.0**	11.3	na	na	63	2								
15	288.0**	11.5	na	na	43	2								
62.5	290.5**	11.6	na	na	32	2		T						
125	278.0**	11.1	na	na	31	2		T						
MMC 0.1	173.5**	6.9	na	na	59	2								
0	13.0	1.0	24.0	1.0	100	2		Y	POS					
3.5	26.0**	2.0	147.5**	6.1	76	2								
30	30.0**	2.3	233.5**	9.7	38	2		T						

62.5	38.5**	3.0	208.0**	8.7	23	2	T		
250	34.0**	2.6	tox	tox	74	1			
500	21.0	1.6	tox	tox	20	1	T		
MMC 0.2	32.0**	2.5	119.0**	5.0	88	2			
0	11.0	1.0	19.5	1.0	100	2		Y	POS
0.29	65.0**	5.9	208.5**	10.7	93	2			
0.88	87.0**	7.9	272.5**	14.0	95	2			
3.5	122.0**	11.1	302.5**	15.5	92	2			
MMC 0.1	23.5**	2.1	165.5**	8.5	94	2			
0	12.0	1.0	na	na	100	2		Y	POS
0.11	20.0	1.7	na	na	84	2			
0.45	29.0**	2.4	na	na	77	2			
0.9	41.0**	3.4	na	na	80	2			
1.8	41.5**	3.5	na	na	86	2			
3.6	54.5**	4.6	na	na	93	2			
15.6	86.0**	7.2	na	na	79	2			
31.2	104.0**	8.7	na	na	86	2			
62.5	148.0**	12.3	na	na	94	2			
250	181.0**	15.1	na	na	46	2			
500	249.0**	20.8	na	na	17	2	T		
MMC 0.2	43.0*	3.6	na	na	nt	2		Y	POS
0	13.0	1.0	na	na	100	2			
0.11	15.0	1.2	na	na	69	2			
0.23	19.0	1.5	na	na	100	2			
0.45	22.5*	1.8	na	na	100	2			
0.9	17.0	1.3	na	na	94	2			
1.8	41.5**	3.2	na	na	66	2			
3.6	51.0**	3.9	na	na	93	2			
7.8	47.5**	3.7	na	na	60	2			
15.6	86.5**	6.7	na	na	55	2			
31.2	97.5**	7.5	na	na	46	2			
62.5	139.5**	10.8	na	na	46	2			
250	214.5**	16.5	na	na	12	2	T		
500	203.0**	15.6	na	na	7	2	T		
MMC 0.2	43.0**	3.3	na	na	nt	1			
0	7.5	1.0	na	na	100	2		Y	POS
0.11	114.5**	15.3	na	na	81	2			
0.23	154.0**	20.5	na	na	80	2			
0.45	158.0**	21.1	na	na	63	2			
0.9	148.5**	19.8	na	na	73	2			
1.8	200.5**	26.7	na	na	79	2			
3.9	128.5**	17.1	na	na	33	2	T		
7.8	115.5**	15.4	na	na	36	2	T		
15.6	84.5**	11.3	na	na	35	2	T		
31.2	90.5**	12.1	na	na	31	2	T		

Appendix A (Continued)

Lab number	Assay number	CytB ^a	Schedule: treatment + recovery (h)	Solvent	Concentration (µg/ml) ^b	Micronucleated mononucleated cells ^c Number per 1000 cells	Induction factor	Micronucleated binucleated cells ^c Number per 1000 cells	Induction factor	Survival (%)	Number of cultures analysed	Others ^d	Acc. ^e	Pos. ^f
					62.5	35.0**	4.7	na	na	17	2	T		
					125	18.0**	2.4	na	na	15	2	T		
					250	7.0	0.9	na	na	18	1	T		
					500	7.0	0.9	na	na	10	1	T		
					MMC 0.1	190.0**	25.3	na	na	58	1			
		+	3+20	Medium	0	nt	nt	6.0	1.0	100	1		Y	POS
					0.11	nt	nt	54.0**	9.0	103	1			
					0.23	nt	nt	54.0**	9.0	105	1			
					0.45	nt	nt	62.0**	10.3	101	1			
					0.9	nt	nt	53.0**	8.8	99	1			
					1.8	nt	nt	83.0**	13.8	101	1			
					3.9	nt	nt	124.0**	20.7	93	1			
					7.8	nt	nt	175.0**	29.2	91	1			
					15.6	nt	nt	185.0**	30.8	80	1			
					31.2	nt	nt	227.0**	37.8	63	1			
					62.5	nt	nt	309.0**	51.5	65	1			
					MMC 0.2	nt	nt	105.0**	17.5	95	1			
		+	24+20	Medium	0	nt	nt	10.0	1.0	100	2		Y	POS
					0.0017	nt	nt	15.0	1.5	93	1			
					0.0034	nt	nt	16.0	1.6	92	1			
					0.0068	nt	nt	25.0**	2.5	90	1			
					0.0137	nt	nt	27.0**	2.7	95	1			
					0.0275	nt	nt	47.0**	4.7	86	1			
					0.055	nt	nt	80.0**	8.0	90	1			
					0.11	nt	nt	163.5**	16.4	98	2			
					0.23	nt	nt	215.0**	21.5	92	2			
					0.45	nt	nt	236.5**	23.7	94	2			
					0.9	nt	nt	301.5**	30.2	82	2			
					1.8	nt	nt	309.5**	31.0	78	2			
					3.9	nt	nt	428.0**	42.8	65	1			
					7.8	nt	nt	504.0	50.4	57	1			
					MMC 0.1	nt	nt	321.0**	32.1	99	1			
Lab 2	Assay 2	-	3+21	Medium	0	9.8	1.0	na	na	100	2		Y	POS
					15.6	120.0**	12.3	na	na	75	2			
					31.2	172.5**	17.7	na	na	54	2			
					62.5	162.0**	16.6	na	na	51	2			
					125	157.5**	16.2	na	na	50	2			
					250	136.0**	13.9	na	na	30	2	T		
					MMC 0.2	109.5**	11.2	na	na	59	2			

Appendix A (Continued)

Lab number	Assay number	CytB ^a	Schedule: treatment + recovery (h)	Solvent	Concentration (µg/ml) ^b	Micronucleated cells ^c		Survival (%)	Number of cultures analysed	Others ^d	Acc. ^e	Pos. ^f
						mononucleated cells ^c Number per 1000 cells	Induction factor					
		–	24 + 0	Medium	0	11.0	1.0	100	1		Y	POS
					0.01	6.0	0.5	78	1			
					0.03	8.0	0.7	60	1			
					0.1	24.0*	2.2	50	1			
					0.3	57.0**	5.2	43	1			
					1	17.0	1.5	36	1	T		
					3	10.0	0.9	33	1	T		
					10	15.0	1.4	24	1	T		
					MMC 0.1	106.0**	9.6	40	1			
		–	24 + 24	Medium	0	11.0	1.0	100	1		Y	POS
					0.01	8.0	0.7	79	1			
					0.03	13.0	1.2	73	1			
					0.1	35.0**	3.2	66	1			
					0.3	55.0**	5.0	33	1	T		
					1	156.0**	14.2	18	1	T		
					3	12.0	1.1	12	1	T		
					10	17.0	1.5	6	1	T		
					MMC 0.1	213.0**	19.4	40	1			
		+	3 + 20	Medium	0	12.0	1.0	100	1		Y	POS
					0.3	12.0	1.0	120	1			
					1	24.0	2.0	118	1			
					3	4.0	0.3	112	1			
					10	8.0	0.7	79	1			
					30	20.0	1.7	55	1			
					100	24.0	2.0	26	1	T		
					MMC 0.2	24.0	2.0	107	1			
		+	24 + 20	Medium	0	4.0	1.0	100	1		Y	POS
					0.003	12.0	3.0	104	1			
					0.01	12.0	3.0	101	1			
					0.03	8.0	2.0	106	1			
					0.1	50.0*	12.5	115	1			
					0.3	tox	tox	116	1			
					1	tox	tox	116	1			
					MMC 0.1	256.0**	64.0	91	1			
Lab 1	Assay 2	–	3 + 21	Medium	0	7.5	1.0	100	2		Y	POS
					1	10.5	1.4	97	2			
					3	26.0**	3.5	75	2			
					10	39.0**	5.2	47	2			
					30	31.5**	4.2	29	2	T		
					MMC 0.2	142.0**	18.9	30	2	T		

Appendix A (Continued)

Lab number	Assay number	CytB ^a	Schedule: treatment + recovery (h)	Solvent	Concentration (µg/ml) ^b	Micronucleated cells ^c		Micronucleated cells ^c		Survival (%)	Number of cultures analysed	Others ^d	Acc. ^e	Pos. ^f
						Number per 1000 cells	Induction factor	Number per 1000 cells	Induction factor					
		–	24+0	Medium	0	9.0	1.0	na	na	100	1		Y	POS
					0.00975	11.0	1.2	na	na	89	1			
					0.0195	13.0	1.4	na	na	100	1			
					0.039	16.0	1.8	na	na	114	1			
					0.078	14.0	1.6	na	na	65	1			
					0.156	27.0**	3.0	na	na	62	1			
					0.3125	103.0**	11.4	na	na	69	1			
					MMC 0.1	107.0**	11.9	na	na	64	1			
		–	24+24	Medium	0	13.0	1.0	na	na	100	1		Y	POS
					0.00975	6.0	0.5	na	na	111	1			
					0.0195	36.0**	2.8	na	na	102	1			
					0.039	20.0	1.5	na	na	111	1			
					0.078	35.0**	2.7	na	na	57	1			
					0.156	110.0**	8.5	na	na	45	1			
					0.3125	143.0**	11.0	na	na	35	1	T		
					MMC 0.05	137.0**	10.5	na	na	99	1			
		+	3+20	Medium	0	0.0	0.0	15.0	1.0	100	1		Y	POS
					0.625	0.0	0.0	18.0	1.2	105	1			
					1.25	8.0	8.0	35.0**	2.3	104	1			
					2.5	4.0	4.0	58.0**	3.9	106	1			
					5	2.0	2.0	106.0**	7.1	83	1			
					10	4.0	4.0	92.0**	6.1	58	1			
					20	12.0*	12.0	67.0**	4.5	44	1			
					MMC 0.2	20.0**	20.0	156.0**	10.4	103	1			
		+	24+20	Medium	0	4.0	1.0	22.0	1.0	100	1		Y	POS
					0.00975	6.0	1.5	5.0	0.2	108	1			
					0.0195	2.0	0.5	21.0	1.0	105	1			
					0.039	14.0	3.5	32.0	1.5	111	1			
					0.078	26.0**	6.5	50.0**	2.3	109	1			
					0.156	161.3**	58.3	184.0**	8.4	115	1			
					0.3125	233.3**	50.0	456.0**	20.7	124	1			
					MMC 0.05	40.0**	10.0	123.0**	5.6	106	1			
Lab 2	Assay 2	–	3+21	Medium	0	7.5	1.0	na	na	100	2		Y	POS
					2.5	33.5**	4.5	na	na	101	2			
					5	54.5**	7.3	na	na	68	2			
					10	52.5**	7.0	na	na	53	2			
					MMC 0.2	75.0**	10.0	na	na	60	2			

Urethane Lab 1	Assay 1		3+45	Medium		7.5	1.0	na	na	100	2	Y	POS
-		0	3+45	Medium	0	7.5	1.0	na	na	100	2	Y	POS
		5			5	57.5**	7.7	na	na	77	2		
		10			10	91.5**	12.2	na	na	93	2		
		20			20	91.0**	12.1	na	na	65	2		
		30			30	237.0**	31.6	na	na	52	2		
		MMC 0.2			MMC 0.2	102.5**	13.7	na	na	105	2		
-		0	24+0	Medium	0	9.5	1.0	na	na	100	2	Y	POS
		0.078			0.078	51.0**	5.4	na	na	67	2		
		0.156			0.156	145.5**	15.3	na	na	84	2		
		0.3125			0.3125	92.0**	9.7	na	na	90	2		
		0.625			0.625	68.0**	7.2	na	na	60	2		
		MMC 0.1			MMC 0.1	149.0**	15.7	na	na	40	2		
-		0	24+24	Medium	0	10.0	1.0	na	na	100	2	Y	POS
		0.00975			0.00975	13.5	1.4	na	na	108	2		
		0.0195			0.0195	14.5	1.5	na	na	99	2		
		0.039			0.039	10.0	1.0	na	na	106	2		
		0.078			0.078	23.0**	2.3	na	na	80	2		
		0.156			0.156	67.0**	6.7	na	na	97	2		
		0.3125			0.3125	153.5**	15.4	na	na	45	2		
		MMC 0.1			MMC 0.1	176.5**	17.7	na	na	92	2		
+		0	3+20	Medium	0	3.0	1.0	12.0	1.0	100	2	Y	POS
		2.5			2.5	12.0*	4.0	54.5**	4.5	102	2		
		5			5	13.0*	4.3	107.0**	8.9	85	2		
		10			10	22.0**	7.3	141.5**	11.8	53	2		
		MMC 0.2			MMC 0.2	25.4**	8.5	230.0**	19.2	96	2		
+		0	24+20	Medium	0	1.0	1.0	14.5	1.0	100	2	Y	POS
		0.039			0.039	14.0**	14.0	32.5**	2.2	105	2		
		0.078			0.078	16.0**	16.0	120.5**	8.3	105	2		
		0.156			0.156	422.9**	422.9	338.5**	23.3	104	2		
		0.3125			0.3125	nt	nt	619.5**	42.7	105	2		
		MMC 0.1			MMC 0.1	157.0**	157.0	643.0**	44.3	97	2		
-		0	3+21	Medium	0	0.5	1.0	na	na	100	2	Y	NEG
		1000			1000	0.0	0.0	na	na	104	2		
		2000			2000	1.0	2.0	na	na	111	2		
		3500			3500	0.0	0.0	na	na	72	2		
		5000			5000	1.5	3.0	na	na	59	2		
		MMC 0.2			MMC 0.2	12.0**	24.0	na	na	nt	2		
-		0	3+45	Medium	0	1.0	1.0	na	na	100	2	Y	NEG
		1000			1000	2.0	2.0	na	na	119	2		
		2000			2000	2.0	2.0	na	na	130	2		
		3500			3500	1.0	1.0	na	na	116	2		
		5000			5000	1.5	1.5	na	na	123	2		
		MMC 0.2			MMC 0.2	9.5**	9.5	na	na	nt	2		

Appendix A (Continued)

Lab number	Assay number	CytB ^a	Schedule: treatment + recovery (h)	Solvent	Concentration ($\mu\text{g/ml}$) ^b	Micromononucleated cells ^c Number per 1000 cells	Induction factor	Micromonucleated cells ^c Number per 1000 cells	Induction factor	Survival (%)	Number of cultures analysed	Others ^d	Acc. ^e	Pos. ^f
		–	24+0	Medium	0	1.5	1.0	na	na	100	2		Y	NEG
					1000	1.5	1.0	na	na	120	2			
					2000	0.5	0.3	na	na	125	2			
					3500	2.0	1.3	na	na	71	2			
					5000	2.5	1.7	na	na	82	2			
					MMC 0.1	7.5**	5.0	na	na	nt	2			
		–	24+24	Medium	0	0.0	0.0	na	na	100	2		Y	NEG
					1000	1.5	1.5	na	na	63	2			
					2000	0.5	0.5	na	na	73	2			
					3500	2.0	2.0	na	na	48	2			
					5000	2.5	2.5	na	na	53	2			
					MMC 0.1	32.0**	32.0	na	na	nt	2			
		+	3+20	Medium	0	5.5	1.0	2.5	1.0	100	2		Y	EQ
					1000	2.4	0.4	14.5**	5.8	101	2			
					2000	10.6	1.9	8.5*	3.4	98	2			
					3500	6.0	1.1	9.0*	3.6	97	2			
					5000	4.0	0.7	9.5**	3.8	100	2			
					MMC 0.2	5.0	0.9	60.0**	24.0	nt	2			
		+	24+20	Medium	0	6.5	1.0	3.5	1.0	100	2		Y	POS
					1000	3.0	0.5	7.5	2.1	93	2			
					2000	7.0	1.1	8.5	2.4	93	2			
					3500	5.0	0.8	11.0**	3.1	80	2			
					5000	3.5	0.5	7.5	2.1	75	2			
					MMC 0.1	23.0**	6.5	285.5**	161.0	nt	2			
Lab 1	Assay 2	–	3+21	Medium	0	1.5	1.0	na	na	100	2		Y	NEG
					1000	0.5	0.3	na	na	125	2			
					2000	0.5	0.3	na	na	108	2			
					3500	0.5	0.3	na	na	183	2			
					5000	1.5	1.0	na	na	71	2			
					MMC 0.2	12.5**	8.3	na	na	nt	2			
		–	3+45	Medium	0	1.5	1.0	na	na	100	2		Y	NEG
					1000	0.0	0.0	na	na	97	2			
					2000	2.0	1.3	na	na	69	2			
					3500	0.5	0.3	na	na	101	2			
					5000	2.0	1.3	na	na	60	2			
					MMC 0.2	11.5**	7.7	na	na	nt	2			

Lab 2	Assay 2	+	24+24	Medium	0	1.0	0.5	1.0	na	1.0	na	1.0	na	100	2	T	Y	NEG
		-	24+24	Medium	0	1.0	0.5	1.0	na	1.0	na	1.0	na	100	2			
					1000	0.5	0.5	0.5	na	0.5	na	0.5	na	69	2			
					2000	0.5	0.5	0.5	na	0.5	na	0.5	na	66	2			
					3500	1.5	1.5	1.5	na	1.5	na	1.5	na	43	2			
					5000	2.0	2.0	2.0	na	2.0	na	2.0	na	24	2	T		
					MMC 0.1	88.5**	88.5**	88.5	na	88.5	na	88.5	na	nt	2			
		+	3+20	Medium	0	4.5	0.1	1.0	4.5	1.0	4.5	1.0	1.0	100	2		Y	NEG
					1000	0.5	0.1	0.1	4.5	1.0	4.5	1.0	1.0	94	2			
					2000	3.5	0.8	0.8	4.5	1.0	4.5	1.0	1.0	97	2			
					3500	2.5	0.6	0.6	5.0	1.1	5.0	1.1	1.1	99	2			
					5000	4.5	1.0	1.0	3.0	0.7	3.0	0.7	0.7	98	2			
					MMC 0.2	8.5	1.9	1.9	65.0**	14.4	65.0**	14.4	14.4	nt	2			
		+	24+20	Medium	0	6.0	1.0	1.0	7.0	1.0	7.0	1.0	1.0	100	2		Y	NEG
					1000	0.0	0.0	0.0	0.5	0.1	0.5	0.1	0.1	83	2			
					2000	0.5	0.1	0.1	1.0	0.1	1.0	0.1	0.1	85	2			
					3500	2.0	0.3	0.3	5.0	0.7	5.0	0.7	0.7	73	2			
					5000	1.0	0.2	0.2	9.5	1.4	9.5	1.4	1.4	62	2			
					MMC 0.1	72.5**	12.1	12.1	403.5**	57.6	72.5**	12.1	57.6	nt	2			
		-	3+21	Medium	0	19.5	1.0	1.0	na	1.0	na	1.0	na	100	2		Y	NEG
					625	22.5	1.2	1.2	na	1.2	na	1.2	na	101	2			
					1250	13.0	0.7	0.7	na	0.7	na	0.7	na	130	2			
					2500	18.0	0.9	0.9	na	0.9	na	0.9	na	93	2			
					5000	16.5	0.8	0.8	na	0.8	na	0.8	na	85	2			
					MMC 0.2	167.5**	8.6	8.6	na	8.6	na	8.6	na	52	2			
		-	24+0	Medium	0	17.5	1.0	1.0	na	1.0	na	1.0	na	100	2		Y	NEG
					625	23.5	1.3	1.3	na	1.3	na	1.3	na	123	2			
					1250	19.0	1.1	1.1	na	1.1	na	1.1	na	134	2			
					2500	14.0	0.8	0.8	na	0.8	na	0.8	na	79	2			
					5000	12.0	0.7	0.7	na	0.7	na	0.7	na	63	2			
					MMC 0.1	88.0**	5.0	5.0	na	5.0	na	5.0	na	40	2			
		-	24+24	Medium	0	29.0	1.0	1.0	na	1.0	na	1.0	na	100	2		N	na
					625	19.5	0.7	0.7	na	0.7	na	0.7	na	95	2			
					1250	17.0	0.6	0.6	na	0.6	na	0.6	na	86	2			
					2500	24.5	0.8	0.8	na	0.8	na	0.8	na	66	2			
					5000	64.5**	2.2	2.2	na	2.2	na	2.2	na	38	2	T		
					MMC 0.1	81.0**	2.8	2.8	na	2.8	na	2.8	na	17	2	T		
		+	3+20	Medium	0	9.0	1.0	1.0	22.0	1.0	22.0	1.0	1.0	100	2		Y	EQ
					625	58.5**	6.5	6.5	38.5**	1.8	38.5**	1.8	1.8	76	2			
					1250	37.0**	4.1	4.1	29.5	1.3	29.5	1.3	1.3	102	2			
					2500	41.5**	4.6	4.6	34.5*	1.6	34.5*	1.6	1.6	101	2			
					5000	32.5**	3.6	3.6	33.5*	1.5	33.5*	1.5	1.5	101	2			
					MMC 0.2	28.5**	3.2	3.2	252.0**	11.5	252.0**	11.5	11.5	25	2	T		
		+	24+20	Medium	0	6.0	1.0	1.0	9.5	1.0	9.5	1.0	1.0	100	2		Y	EQ
					625	5.5	0.9	0.9	16.5	1.7	16.5	1.7	1.7	91	2			
					1250	8.5	1.4	1.4	14.0	1.5	14.0	1.5	1.5	87	2			

Appendix A (Continued)

Lab number	Assay number	CytB ^a	Schedule: treatment + recovery (h)	Solvent	Concentration (µg/ml) ^b	Micronucleated mononucleated cells ^c		Micronucleated binucleated cells ^c		Survival (%)	Number of cultures analysed	Others ^d	Acc. ^e	Pos. ^f
						Number per 1000 cells	Induction factor	Number per 1000 cells	Induction factor					
Lab 3	Assay 1	-	3+21	Medium	2500	7.0	1.2	17.0	1.8	73	2			
					5000	5.0	0.8	19.5*	2.1	60	2			
					MMC 0.1	55.5**	9.3	209.0**	22.0	29	2	T		
					0	8.0	1.0	na	na	100	2		Y	
					1250	4.5	0.6	na	na	102	2			
					2500	9.0	1.1	na	na	94	2			
					5000	9.5	1.2	na	na	83	2			
					MMC 0.05	138.0**	17.3	na	na	94	2			
					0	10.5	1.0	na	na	100	2		Y	
					1250	7.0	0.7	na	na	93	2			
					2500	17.5	1.7	na	na	95	2			
					5000	13.0	1.2	na	na	95	2			
MMC 0.05	161.0**	15.3	na	na	85	2								
0	10.0	1.0	na	na	100	2		Y						
1250	11.0	1.1	na	na	98	2								
2500	10.0	1.0	na	na	94	2								
5000	7.5	0.8	na	na	71	2								
MMC 0.05	136.5**	13.7	na	na	82	2								
0	13.0	1.0	na	na	100	2		Y						
1250	15.0	1.2	na	na	106	2								
2500	16.0	1.2	na	na	97	2								
5000	8.0	0.6	na	na	75	2								
MMC 0.05	166.5**	12.8	na	na	89	2								
0	3.0	1.0	10.0	1.0	100	2		Y						
1250	4.5	1.5	7.0	0.7	98	2								
2500	1.0	0.3	10.0	1.0	89	2								
5000	3.5	1.2	13.5	1.4	54	2								
MMC 0.25	70.5**	23.5	287.5**	28.8	73	2								
0	1.5	1.0	9.0	1.0	100	2		Y						
1250	2.5	1.7	8.5	0.9	99	2								
2500	3.5	2.3	7.0	0.8	95	2								
5000	1.0	0.7	14.0	1.6	93	2								
MMC 0.05	23.5**	15.7	137.5**	15.3	93	2								
Diethylstilboestrol	Assay 2	-	3+21	Ethanol	0	16.5	1.0	na	na	100	2		Y	
					7.5	12.0	0.7	na	na	96	2			

Appendix A (Continued)

Lab number	Assay number	CytB ^a	Schedule: treatment + recovery (h)	Solvent	Concentration ($\mu\text{g/ml}$) ^b	Micronucleated mononucleated cells ^c		Micronucleated binucleated cells ^c		Survival (%)	Number of cultures analysed	Others ^d	Acc. ^e	Pos. ^f
						Number per 1000 cells	Induction factor	Number per 1000 cells	Induction factor					
		–	24+0	Ethanol	0	3.5	1.0	na	na	100	2		Y	POS
					0.05	32.5**	9.3	na	na	107	2			
					0.25	30.5**	8.7	na	na	91	2			
					0.5	40.0**	11.4	na	na	85	2			
					5	33.0**	9.4	na	na	28	2			
					MMC 0.1	165.5**	47.3	na	na	50	2	T		
		–	24+24	Ethanol	0	3.0	1.0	na	na	100	2		Y	POS
					0.05	91.0**	30.3	na	na	104	2			
					0.125	91.0**	30.3	na	na	110	2			
					1	64.0**	21.3	na	na	72	2			
					5	75.0**	25.0	na	na	40	2			
					MMC 0.1	198.0**	66.0	na	na	60	2			
		+	3+20	Ethanol	0	0.0	0.0	0.0	0.0	100	2		Y	POS
					0.5	0.5	0.5	0.0	0.0	91	2			
					1	1.0	1.0	0.5	0.5	91	2			
					3	2.0	2.0	4.0*	4.0	84	2			
					5	0.5	0.5	4.5**	4.5	90	2			
					7	5.0**	5.0	9.0**	9.0	49	2			
					MMC 0.2	6.0**	6.0	60.0**	60.0	89	2			
		+	24+20	Ethanol	0	3.0	1.0	1.5	1.0	100	2		Y	POS
					0.05	5.0	1.7	11.0**	7.3	73	2			
					0.125	1.0	0.3	3.0	2.0	80	2			
					0.25	0.5	0.2	19.5**	13.0	80	2			
					0.5	4.0	1.3	13.5**	9.0	69	2			
					0.75	3.5	1.2	18.5**	12.3	61	2			
					1	0.0	0.0	17.0**	11.3	59	2			
					MMC 0.2	1.5	0.5	106.0**	70.7	73	2			

^a (+) With cytochalasin B; (–) without cytochalasin B.

^b MMC: positive control, mitomycin C.

^c Number of micronucleated cells given for 1000 cells; induction factor: when the number of micronucleated cells was 0 in the control, it was set to 1 to calculate the induction factor for treated cultures (see text for the formula). NS: not statistically higher than controls (Yates Chi-square test); nt: not tested; nd: not determined; na: not applicable; tox: not evaluated due to cytotoxicity. * Statistically higher than controls at $p < 0.05$. ** Statistically higher than controls at $p < 0.01$; (–) lower than the control: statistical analysis not done.

^d p: precipitate; low cells: low number of cells; mmm: multi-micronucleated cells; T: genotoxicity data obtained at survivals clearly below 40% were taken into account for genotoxicity evaluation positive results obtained only at such concentrations were discarded; negative results obtained at such concentrations when lower concentrations gave positive results were not considered; g: no evaluation of survival but as the highest concentration was 5000 $\mu\text{g/ml}$ and as manitol showed no cytotoxicity, the assay was used for genotoxicity evaluation.

^e Y: assay accepted; N: assay not accepted (see text).

^f POS: assay concluded as positive; NEG: assay concluded as negative; EQ: only one positive concentration in the range or no concentration–effect relationship; na: not appropriate.

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In vivo erythrocyte micronucleus assay

III. Validation and regulatory acceptance of automated scoring and the use of rat peripheral blood reticulocytes, with discussion of non-hematopoietic target cells and a single dose-level limit test

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Abstract

The *in vivo* micronucleus assay working group of the International Workshop on Genotoxicity Testing (IWGT) discussed new aspects in the *in vivo* micronucleus (MN) test, including the regulatory acceptance of data derived from automated scoring, especially with regard to the use of flow cytometry, the suitability of rat peripheral blood reticulocytes to serve as the principal cell population for analysis, the establishment of *in vivo* MN assays in tissues other than bone marrow and blood (for example liver, skin, colon, germ cells), and the biological relevance of the single-dose-level test.

Our group members agreed that flow cytometric systems to detect induction of micronucleated immature erythrocytes have advantages based on the presented data, e.g., they give good reproducibility compared to manual scoring, are rapid, and require only small quantities of peripheral blood. Flow cytometric analysis of peripheral blood reticulocytes has the potential to allow monitoring of chromosome damage in rodents and also other species as part of routine toxicology studies. It appears that it will be applicable to humans as well, although in this case the possible confounding effects of splenic activity will need to be considered closely. Also, the consensus of the group was that any system that meets the validation criteria recommended by the IWGT (2000)

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should be acceptable. A number of different flow cytometric-based micronucleus assays have been developed, but at the present time the validation data are most extensive for the flow cytometric method using anti-CD71 fluorescent staining especially in terms of inter-laboratory collaborative data. Whichever method is chosen, it is desirable that each laboratory should determine the minimum sample size required to ensure that scoring error is maintained below the level of animal-to-animal variation.

In the second IWGT, the potential to use rat peripheral blood reticulocytes as target cells for the micronucleus assay was discussed, but a consensus regarding acceptability for regulatory purposes could not be reached at that time. Subsequent validation efforts, combined with accumulated published data, demonstrate that blood-derived reticulocytes from rats as well as mice are acceptable when young reticulocytes are analyzed under proper assay protocol and sample size.

The working group reviewed the results of micronucleus assays using target cells/tissues other than hematopoietic cells. We also discussed the relevance of the liver micronucleus assay using young rats, and the importance of understanding the maturation of enzyme systems involved in the processes of metabolic activation in the liver of young rats. Although the consensus of the group was that the more information with regard to the metabolic capabilities of young rats would be useful, the published literature shows that young rats have sufficient metabolic capacity for the purposes of this assay. The use of young rats as a model for detecting MN induction in the liver offers a good alternative methodology to the use of partial hepatectomy or mitogenic stimulation. Additional data obtained from colon and skin MN models have been integrated into the data bases, enhancing confidence in the utility of these models.

A fourth topic discussed by the working group was the regulatory acceptance of the single-dose-level assay. There was no consensus regarding the acceptability of a single dose level protocol when dose-limiting toxicity occurs. The use of a single dose level can lead to problems in data interpretation or to the loss of animals due to unexpected toxicity, making it necessary to repeat the study with additional doses. A limit test at a single dose level is currently accepted when toxicity is not dose-limiting.

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1. Introduction

The International Workshop on Genotoxicity Test Procedures (IWGTP), first held in February 1993 in Melbourne, Australia in conjunction with the 6th International Conference on Environmental Mutagens, has developed consensus on a number of major methodological issues related to regulatory genetic toxicology assays [1]. The conclusions reached at the Melbourne IWGTP meeting by the expert working group on the micronucleus assay have been summarized by Hayashi et al. [2]. The second IWGTP was held in Washington D.C., March 25–26, 1999, and the report of this meeting is complimentary to that of the first report of the Melbourne meeting. In the 2nd IWGTP, the *in vivo* micronucleus test working group discussed a number of issues, including integration of the repeated-dose micronucleus assay into general toxicology studies, the use of automated scoring techniques, micronucleus scoring in other tissues, and methods for differentiating micronuclei derived from acentric chromosome fragments and from centromeric chromosomes [3].

In the 4th International Workshop on Genotoxicity Testing (IWGT), in San Francisco on September 8 and 9, 2005 (an *in vivo* micronucleus assay working group meeting did not take place at the 3rd IWGT, in Plymouth, 2002) a half-day-meeting was held to consider new developments related to the use of the *in vivo* micronucleus test and aimed:

- (1) to clarify whether automated methods such as those employing flow cytometry or image analysis are sufficiently well validated to allow the acceptance of such data by the regulatory authorities,
- (2) to determine whether the use of rat peripheral blood reticulocytes as the primary endpoint evaluated is sufficiently well validated to allow the acceptance of such data by the regulatory authorities,
- (3) to review the current status and any new data related to the *in vivo* micronucleus assay using tissues other than bone marrow and peripheral blood (for example liver, skin, colon, etc.),
- (4) to evaluate the acceptability of the single-dose-assay not only at the limit dose (i.e., 2000 mg/kg) but also at other single dose levels when defined by other specific rationales.

1.1. Automated scoring for regulatory use

One of the most important characteristics of the rodent micronucleus assay is the simplicity of scoring its endpoint, namely identification of a small membrane-bound chromatin-containing body in the cytoplasm of the erythrocyte from which the main nucleus has been eliminated during the last stage of hematopoiesis. The characteristics of the micronucleus (a smooth-boundaried body that stains strongly and specifically for chromatin) lend themselves well to automated scoring by image analysis or flow cytometry.