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Reference Standards for Flow Cytometry and Application in Comparative Studies of Nuclear DNA Content[1]

Terrence R. Tiersch, Robert W. Chandler, Stephen S. Wachtel,[2] and Sherman Elias

Ecological Research Center, Department of Biology, Memphis State University, Memphis, Tennessee 38152 (T.R.T.); Division of Reproductive Genetics, Department of Obstetrics and Gynecology, University of Tennessee, Memphis, Tennessee 38105 (R.W.C., S.S.W., S.E.)

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Nuclear DNA mass in cells from a reference species can be used to obtain high-resolution estimates of DNA mass from a target species. In our study of DNA mass in cells from 45 selected species, representing each of the major vertebrate classes, we have obtained values of from 1.5 to 110.0 pg of DNA. Because values in or near this range would be expected in the study of nuclear DNA mass in vertebrates and other organisms, the species in this report can provide a useful catalogue of references for comparative studies of DNA.

Key terms: Vertebrate genome, DNA mass, DNA Index, propidium iodide, zero shift error, standardization

Flow cytometry can be used to obtain high-resolution estimates of nuclear DNA content (8,12,27). Much of the work in this area has been confined to clinical studies in the human, but the technology has been extended to organisms such as pocket gophers (24), triploid trout (25), side-necked turtles (6), sex-reversed horses (16), and oysters (1).

In these and similar studies, the DNA content of target cells is quantified relative to a standard DNA content in cells from a reference species (13,14,28). Standards may be used as internal references, when target cells and reference cells are mixed together and assayed simultaneously, or as external references, when target cells and cells from the reference species are analyzed independently. When external references are used, the flow cytometer must be checked for equilibration between analyses.

The standard can be used for several calculations. The standard can be assigned a known DNA mass, against which picogram quantities of nuclear DNA from target cells can be estimated directly (15). Alternatively, a standard could be used as an internal reference in the analysis of individual samples, following which the reference is cancelled during estimation of a DNA Index. An internal reference would also be cancelled during the calculation of picogram quantities of nuclear DNA relative to a separate standard that has a known DNA mass (and a known relationship to the internal reference). These last two calculations do not require knowledge of the precise DNA mass of the internal reference.

Because the use of reference standards provides a relative measure of DNA content, it is crucial that the reference cells and the target cells have a similar DNA content to minimize possible zero shift error (28) and, in the case of an internal reference, that the values for the reference and target cells do not overlap.

In order to gather information relating to the selection of reference standards for use in flow cytometry, we have surveyed a wide spectrum of taxa representing each of the major vertebrate classes. Here we present the results of our survey in 45 species. These species were selected because of their broad distribution and general accessibility, and because they provide an array of fluorescence peaks spanning the range of DNA masses that might be encountered in comparative studies.

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[2]Address reprint requests to Dr. Stephen Wachtel, Department of Obstetrics and Gynecology, University of Tennessee, 711 Jefferson Ave., Memphis, TN 38105.

Table 1
Taxonomic Classification, Common Names and Sources of Animals Used for Analysis of Nuclear DNA Content

Class	Species	Common name	No.	Source
Agnatha	*Eptatretus stouti*	Pacific hagfish	12	A. Gorbman, Bamfield, BC
	Petromyzon marinus	Sea lamprey	10	G. Klar, Marquette, MI
Chondrichthyes	*Dasyatis sabina*	Atlantic stingray	1	A. Foster, Suwanee River, FL
Osteichthyes	*Polyodon spathula*	Paddlefish	7	J. Tidwell, Frankfort, KY
	Oncorhynchus mykiss[a]	Rainbow trout	9	D. Rosell, Norfork, AR
	Salmo trutta	Brown trout	3	Norfork, AR
	Cyprinus carpio	Common carp	5	Auburn and Marion, AL
	Ctenopharyngodon idella	Grass carp	21	C. Goudie, Webster, FL
	Ictalurus punctatus	Channel catfish	50	G. Carmichael, Stoneville, MS
	Arius felis	Hardhead catfish	1	A. Foster, Suwanee River, FL
	Gambusia affinis	Mosquitofish	50	Memphis, TN
	Xiphophorus species (9)	Platyfish, Swordtails	95	K. Kallman, Brooklyn, NY
	Lepomis species (2)	Bluegill, Green sunfish	10	Stewart County, TN
	Micropterus salmoides	Largemouth bass	5	N. Colbert, Hardeman County, TN
Amphibia	*Acris crepitans*	Northern cricket frog	2	Stewart County, TN
	Rana catesbeiana	Bullfrog	5	Stewart County, TN
	Bufo americanus	American toad	6	Stoneville, MS
	Bufo woodhousei	Fowler's toad	4	Stewart County, TN
	Notophthalmus viridescens	Red-spotted newt	2	Stewart County, TN
	Ambystoma opacum	Marbled salamander	6	C. Figiel, Aiken County, SC
	Ambystoma maculatum	Spotted salamander	4	C. Figiel, Stewart County, TN
	Cryptobranchus alleganiensis	Hellbender salamander	2	M. Jackson
Reptilia	*Trionyx spiniferus*	Spiny soft-shelled turtle	17	B. Gutzke, Austin, TX
	Trachemys scripta[b]	Red-eared slider turtle	11	G. Carmichael, Stoneville, MS
	Terrapene carolina	Eastern box turtle	1	Shelby County, TN
	Nerodia sipedon	Northern watersnake	3	Stewart County, TN
	Crotalus horridus	Canebrake rattlesnake	1	B. Gutzke
Aves	*Gallus domesticus*	Domestic chicken	25	Collierville, TN
	Chrysolophus pictus	Golden pheasant	20	J. Price, Fayette County, TN
	Cacatua moluccensis	Moluccan cockatoo	2	M. Douglass, Memphis, TN
Mammalia	*Didelphis virginiana*	Opossum	1	N. Parker, Marion, AL
	Canis familiaris	Domestic dog	2	J. Jenkins, Memphis, TN
	Equus caballus	Domestic horse	5	Collierville, TN
	Sus scrofa	Domestic swine	20	Memphis, TN
	Bos taurus	Domestic cattle	5	Collierville, TN
	Homo sapiens	Domestic human	50	Memphis, TN

[a]Formerly *Salmo gairdneri.*
[b]Formerly *Pseudemys scripta.*

MATERIALS AND METHODS

Most of the 140 species that we surveyed were collected in the south central United States. The source of the 45 species selected for this report and their common names are listed in Table 1. Whole blood was collected in standard ACD solution, or in phosphate-buffered saline (PBS) in the absence of divalent cations. The blood was refrigerated (4°C) until use. Cells were prepared for analysis according to the method of Krishan (17) as modified in Murphy et al. (18). Blood cells were suspended in 0.5 ml of a solution containing 25 μg propidium iodide, 0.1% sodium citrate, 25 μl RNAase in buffer (1 mg/ml), and 0.1% Triton X-100. Cells from any of six internal reference standards (*Gambusia affinis, Ictalurus punctatus, Gallus domesticus, Sus scrofa, Homo sapiens,* or *Rana catesbeiana*) were combined and analyzed simultaneously with cells from each of the species being surveyed. Cells from *G. affinis, I. punctatus,* and *G. domesticus* were frozen in PBS containing 8% dimethyl sulfoxide in liquid nitrogen and stored at −80°C (14,29). Aliquots of cells from each species were thawed and tested with fresh cells of *S. scrofa* and *H. sapiens* to provide day-to-day comparisons among the values for the reference species. The DNA content of the cells was estimated by use of the EPICS V flow cytometer (Coulter Electronics, Hialeah, FL), with argon-ion laser operated at a wavelength of 488 nm at 300 mW in light-stabilized mode. Filters included two 515 nm long-pass, 560 nm dichroic, and a 570 nm long-pass in front of the photomultiplier tube.

Nuclear DNA content was estimated in relation to an assigned value of 7.0 pg DNA in fresh male human leukocytes. This value is based on absorption cytophotometry of Feulgen-stained cells (2–5) and on 24 determinations in cells from various normal human tissues in 11 separate studies cited in Shapiro (23). The mean of these latter values (23) was 7.00 ± 1.65 (S.D.); discounting a single value of 11.06, the mean of values for leukocytes in three studies was 7.13 ± 0.43. Furthermore Rasch et al. (21) derived a value of approximately 2.5 pg for DNA mass in the chicken, based on their own study and 28 other studies cited by them. This is the

same value that we derive for blood cells of the chicken, using 7.0 in the human male as a constant.

Fresh human leukocytes from one of us (R.W.C.) were used as an internal reference for the direct calculation of a picogram quantity of DNA, when cells of the target species possessed a nuclear DNA content that was similar to, but did not overlap with, the human value. When the human cells and cells of the target species had widely disparate DNA values, cells from another species were used as an internal reference. In this case picogram values were calculated by the following formula:

$$Pg = 7.0\ (X/S)\ (S/H)$$

were X is the fractional mode channel (FMC) for the fluorescence peak of cells from the target species, H is the FMC for the male human, and S is the FMC of the fluorescence for cells of the species selected for use as an internal reference. Thus the picogram value of DNA in the target cells is directly proportional to the ratio of FMC of the target cells to the FMC of the human standard.

RESULTS

The species that we studied are organized according to DNA content in Table 2. The range of values in the table covers the several orders of magnitude of nuclear DNA mass (approximately 1–200 pg DNA per diploid nucleus) that exists in vertebrates (7,19). By absorption microspectrophotometry of Feulgen-stained nuclei, Rasch (22) obtained similar values for DNA mass in species such as chicken, carp, rainbow trout, and human.

Variability was negligible among repeated measurements of the same samples within this system. With regard to the ratios between the fluorescence values obtained for any two of the six internal reference standards, the standard deviations were consistently less than 0.005.

We routinely calibrate the flow cytometer by analysis of 3, 4, or 5 reference standards simultaneously, including fresh leukocytes of the human and domestic pig, and any or all of three frozen standards. Mixtures of greater numbers of species are possible, given adequate separation between the fluorescence peaks (Fig. 1).

Table 2
DNA Content in 45 Species

DNA content[a]	Common name (No.)	Std[b]
1.5[c]	Platyfish, Swordtails (95)	Ch
1.5 ± 0.02	Mosquitofish (50)	Ch
2.0 ± 0.01	Channel catfish (50)	Ch
2.0 ± 0.02	Bluegill (5), Green sunfish (5)	Ch
2.0 ± 0.02	Largemouth bass (5)	Ch
2.0 ± 0.01	Diploid Grass carp (7)	Ch
2.4 ± 0.04	Golden pheasant (20)	C, H
2.5 ± 0.04	Domestic chicken (25)	C, H
3.0 ± 0.01	Triploid Grass carp (14)	Ch
3.1 ± 0.06	Moluccan cockatoo (2)	S
3.4 ± 0.01	Common carp (5)	Ch
3.5—	Canebrake rattlesnake (1)	S
3.9 ± 0.03	Paddlefish (7)	S
4.0 ± 0.02	Northern watersnake (3)	S
4.2 ± 0.07	Sea lamprey (10)	Ch
4.5—	Hardhead catfish (1)	Ch
5.3 ± 0.02	Red-eared slider turtle (11)	Ch, S
5.4 ± 0.04	Spiny soft-shelled turtle (17)	H
5.4 ± 0.15	Pacific hagfish (12)	Ch, S
5.5 ± 0.05	Rainbow trout (9)	Ch
5.5—	Eastern box turtle (1)	Ch, H
5.6 ± 0.05	Domestic dog (2)	H
5.9 ± 0.06	Brown trout (3)	Ch
6.0 ± 0.07	Domestic swine (20)	H
6.3 ± 0.07	Domestic horse (5)	H[d]
7.0 ± 0.08	Human (50)	S
7.3 ± 0.07	Domestic cattle (5)	S
8.3 ± 0.13	Northern cricket frog (2)	H
8.3—	Opossum (1)	H
10.0 ± 0.03	American toad (6)	H
10.0 ± 0.06	Fowler's toad (4)	H
10.1—	Atlantic stingray (1)	S
15.0 ± 1.48	Bullfrog (5)	H
67.0 ± 1.68	Marbled salamander (6)	F
70.0 ± 0.91	Red-spotted newt (2)	F
76.0 ± 2.44	Spotted salamander (4)	F
110.0 ± 2.89	Hellbender salamander (2)	H

[a]Mean pg DNA ± S.D. per diploid nucleus. Values in this table are the means of values for male and female; see Discussion.

[b]Std = internal standard: C, channel catfish; Ch, chicken; F, bullfrog; H, human; S, swine.

[c]This was a mean value; nine species of *Xiphophorus* were studied (16 stocks); among the different stocks DNA estimates varied from 1.48 pg to 1.56 pg (26).

[d]Human female used as internal standard.

DISCUSSION

General references that provide a survey of nuclear DNA content in various organisms, such as Shapiro (23) or Hinegardner and Rosen (11), can provide a preliminary basis for selection of an internal standard. However, the measurements obtained by biochemical techniques or other methods of estimating DNA mass do not always agree with the measurements obtained by flow cytometry. It is useful to evaluate the cells of several species as potential references for study of the particular species under investigation.

Variations in nuclear DNA content within a species may often exceed the variations observed between species (10) [Sherwood and Patton (24) reported within-population variation of 35% in pocket gophers, for example], and chromosomal morphology may differ from individual to individual within a species. Variations in nuclear DNA content within a species have accordingly been linked to chromosomal polymorphisms, and thereby, to varying levels of constitutive heterochromatin (9,20,24). Strains produced by artificial selection, via breeding practices utilized in species of commercial importance, may differ in nuclear DNA content. Hybridization, whether naturally occurring or human-induced, may also affect DNA content.

Alternative DNA values may be observed for males and females, especially in mammals and birds in which

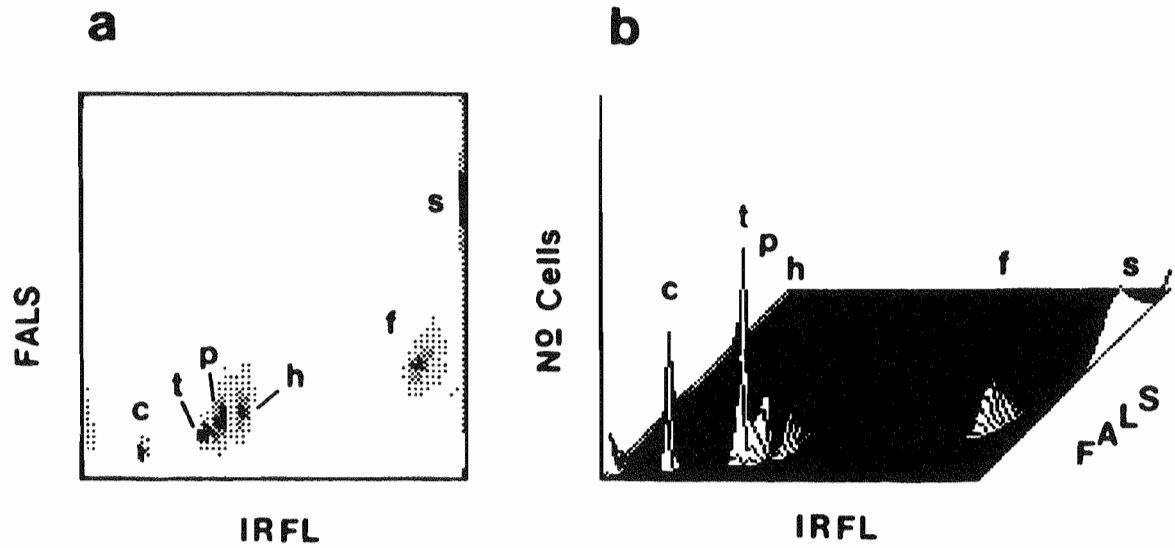

FIG. 1. Two-dimensional (a) and three-dimensional (b) representations of simultaneous analysis by two-parameter flow cytometry of blood cells from six species: c, domestic chicken; t, red-eared slider turtle; p, domestic pig; h, human male; f, bullfrog; s, marbled salamander. The values for nuclear DNA content range from 2.5 pg (c) to 67.0 pg (s). FALS, forward angle light scatter; IRFL, integrated red fluorescence (linear propidium iodide fluorescence).

the heteromorphic X/Y (Z/W) sex chromosomes occur (8,28 and our unpublished observations in 35 species of birds). Chromosome number is not always a reliable indicator of DNA content. For example, the female muntjac *(Muntiacus reevesi),* a small deer native to Southeast Asia, has a nuclear DNA content similar to that of humans yet possesses only 6 chromosomes, whereas the horse, which possesses 64 chromosomes, has 10% less DNA than the human has (16). Variations in DNA values can occur within individuals. For instance, mosaicism can exist in an organism, and different tissues, treated in the same manner, can exhibit differential fluorescence intensity (26,27).

Fluorochromes possess specific binding affinities for the different bases in DNA. Thus, use of a particular stain can affect the level of fluorescence detected during analysis (15). The binding kinetics of stains also may affect fluorescence intensity (12). Should cells be frozen for later use, fluorescence can shift during storage (our unpublished observation). Shifts in the zero level adjustment of the flow cytometer can affect the DNA values calculated in relation to an internal standard. Although the simultaneous use of two internal reference standards can correct for this (28), we check the zero calibration level frequently during each use.

In conclusion, reference standards facilitate the calculation of nuclear DNA content. As flow cytometry is extended to the study of additional nonhuman organisms, in evolutionary studies, for example, catalogues of novel reference standards will become increasingly valuable. The roster of species provided here is intended to assist in the selection of references for use in comparative studies.

LITERATURE CITED

1. Allen SK: Flow cytometry: Assaying experimental polyploid fish and shellfish. Aquaculture 33:317–328, 1983.
2. Allison DC, Ridolpho PF, Rasch EM, Rasch RW, Johnson TS: Increased accuracy of absorption cytophotometric DNA values by control of stain intensity. J Histochem Cytochem 29(10):1219–1228, 1981.
3. Atkin NB, Mattinson G, Beçak W, Ohno S: The comparative DNA content of 19 species of placental mammals, reptiles, and birds. Chromosoma 17:1–10, 1965.
4. Bachmann K: Genome size in mammals. Chromosoma 37:85–93, 1972.
5. Bachmann K, Harrington BA, Craig JP: Genome size in birds. Chromosoma 37:405–416, 1972.
6. Bickham JW, Tucker PK, Legler JM: Diploid-triploid mosaicism: An unusual phenomenon in side-necked turtles (Platemys platycephala). Science 227:1591–1593, 1985.
7. Cavalier-Smith T: Nuclear volume control by nucleoskeletal DNA, selection for cell volume and cell growth rate, and the solution of the DNA C-value paradox. J Cell Sci 34:247–278, 1978.
8. Elias S, Chandler RW, Wachtel S: Relative DNA content of interphase leukocytes by flow cytometry: A method for indirect diagnosis of chromosomal abnormalities with potential for prenatal diagnosis. Am J Obstet Gynecol 158:808–818, 1988.
9. Gold JR, Amemiya CT, Ellison JR: Chromosomal heterochromatin differentiation in North American cyprinid fishes. Cytologia 51:557–566, 1986.
10. Gold JR, Amemiya CT: Genome size variation in North American minnows (Cyprinidae). II. Variation among 20 species. Genome 29:481–489, 1987.
11. Hinegardner R, Rosen DE: Cellular DNA content and the evolution of teleostean fishes. Am Nat 106:621–644, 1972.
12. Hoehn H, Johnston P, Callis J: Flow-cytogenetics: Sources of DNA content variation among euploid individuals. Cytogenet Cell Genet 19:94–107, 1977.
13. Iversen OE, Laerum OD: Trout and salmon erythrocytes and human leukocytes as internal standards for ploidy control in flow cytometry. Cytometry 8:190–196, 1987.
14. Jakobsen A: The use of trout erythrocytes and human lympho-

cytes for standardization in flow cytometry. Cytometry 4:161–165, 1983.
15. Johnson OW, Utter FM, Fabinovitch PS: Interspecies differences in salmonid cellular DNA identified by flow cytometry. Copeia 1987:1001–1009, 1987.
16. Kent M, Chandler R, Wachtel S: DNA analysis by flow cytometry. Cytogenet Cell Genet 47:88,89, 1988.
17. Krishan A: Rapid cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J Cell Biol 66:188–193, 1975.
18. Murphy WM, Emerson LD, Chandler RW, Moinuddin SM, Soloway MS: Flow cytometry versus urinary cytology and the evaluation of patients with bladder cancer. J Urol 136:815–819, 1986.
19. Olmo E, Capriglione T, Odierna G: Genome size evolution in vertebrates: Trends and constraints. Comp Biochem Physiol [B] 92(3):447–453, 1989.
20. Pathak S, Hsu TC, Arrighi FE: Chromosomes of Peromyscus (Rodentia, Cricetidae) IV. The role of heterochromatin in karyotypic evolution. Cytogenet Cell Genet 12:315–326, 1973.
21. Rasch EM, Barr HJ, Rasch RW: The DNA content of sperm of *Drosophila melanogaster*. Chromosoma 33:1–18, 1971.
22. Rasch EM: DNA "standards" and the range of accurate DNA estimates by Feulgen absorption microspectrophotometry. In: Advances in Microscopy, Cowden RR, Harrison FW (eds). Alan R. Liss, Inc., New York, 1985, pp 137–166.
23. Shapiro HS: Deoxyribonucleic acid content per cell of various organisms. In: Handbook of Biochemistry and Molecular Biology, Fasman GD (ed). CRC Press, Cleveland, 1976, vol 2, pp 284–306.
24. Sherwood SW, Patton JL: Genome evolution in pocket gophers (Genus Thomomys). Chromosoma 85:163–179, 1982.
25. Thorgaard GH, Rabinovitch PS, Shen MW, Gall GAE, Propp J, Utter FM: Triploid rainbow trout identified by flow cytometry. Aquaculture 29:305–309, 1982.
26. Tiersch TR, Chandler RW, Kallman KD, Wachtel SS: Estimation of nuclear DNA content by flow cytometry in fishes of the genus *Xiphophorus*. Comp Biochem Physiol (in press).
27. Vindeløv LL, Christensen IJ, Jensen G, Nissen NI: Limits of detection of nuclear DNA abnormalities by flow cytometric DNA analysis. Results obtained by a set of methods for sample-storage, staining and internal standardization. Cytometry 3:332–339, 1983.
28. Vindeløv LL, Christensen IJ, Nissen NI: Standardization of high-resolution flow cytometric DNA analysis by the simultaneous use of chicken and trout red blood cells as internal reference standards. Cytometry 3:328–331, 1983.
29. Vindeløv LL, Christensen IJ, Keiding N, Spang-Thomsen M, Nissen NI: Long-term storage of samples for flow cytometric DNA analysis. Cytometry 3:317–322, 1983.