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A CONTROLLED PORE GLASS BEAD ASSAY FOR THE MEASUREMENT OF CYTOPLASMIC AND NUCLEAR GLUCOCORTICOID RECEPTORS

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Summary—An assay for the quantitation of cytoplasmic and nuclear glucocorticoid receptors in lymphoid tissue has been developed using controlled pore glass (CPG) beads. Soluble receptor–^{3}H-steroid complex (cytosol or nuclear extract) is adsorbed quantitatively within the crevasses of porous glass beads. Excess labeled steroid as well as most non-specifically bound steroid is easily washed away, leaving the hormone–receptor complex retained by the beads. Bound ^{3}H-steroid is eluted with ethanol and measured for radioactivity. This procedure which is simple, rapid, and highly reproducible is carried out using frozen samples (stable for many months) containing as few as 1×10^7 cells. A comparison of the CPG assay to dextran coated charcoal and a whole cell assay demonstrates that CPG and dextran coated charcoal give equivalent measurements of cytosolic receptor concentration, while the CPG and whole cell assays provide equivalent values for total receptor content.

INTRODUCTION

Many techniques have been used for the measurement of glucocorticoid receptors in normal and neoplastic cells. Most methods used routinely utilize either a whole cell [1–4] or a cytosol assay [5–7]. Recently, Iacobelli *et al.*[8] compared the use of whole-cell assay techniques to that of cell-free cytosolic assays. In that report the cytosol assay was found to consistently underestimate total receptor binding compared to the whole cell assay in cells with low receptor content. Some whole-cell assay techniques, while often giving higher values, do not provide information concerning the localization of the receptor between cytoplasm and nucleus [1–3]. Because the distribution of receptor between cytoplasm and nucleus may lead to a better understanding of hormone responsiveness, whole-cell assay techniques have been developed which provide this information. The use of low temperatures, necessary to preserve *in vivo* distribution of receptor, however, may lead to an underestimation of total receptor content [2–4]. An additional problem limiting whole cell assays is the high level of non-specific binding which occurs in the cell preparations [8]. Thus, an assay which does not underestimate total receptor content while preserving *in vivo* cytoplasmic and nuclear ratios with low non-specific binding would be useful.

We have recently examined the use of Controlled Pore Glass (CPG) beads to assay both cytosolic and nuclear glucocorticoid receptors. In this report we describe the development of a rapid and simple method which affords a measurement of total receptor sites, as well as the distribution of receptor between cytoplasm and nucleus‡ under cell free conditions. A comparison of the CPG assay is made to a conventional dextran coated charcoal (DCC) cytosolic assay [5] as well as to the whole cell assay (WCA) described by Munck *et al.*[4]. The CPG assay was found to provide equivalent measurements of total receptor binding compared to the whole cell assay, while maintaining the native distribution of receptor between cytoplasm and nucleus.

EXPERIMENTAL

Materials

[^{3}H]Triamcinolone acetonide (sp. act. 31–37 Ci/mmol), [^{3}H]corticosterone (50 Ci/mmol) and [^{3}H]-dexamethasone (50 Ci/mmol) were purchased from New England Nuclear Corp. (Boston MA); controlled pore glass beads (120/200 mesh, 544 Å mean pore dia) were purchased from Electro-Nucleonics (Fairfield, NJ); unlabeled steroids, Tris, and dithiothreitol (DTT) were from Sigma Chemical Co. (St Louis, MO). All other reagents were analytical grade. Stock solutions of all steroids were made in absolute ethanol and stored at 4°C. Buffers used are defined as

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‡We use the terms cytosolic and nuclear receptor to mean that portion of the total receptor which is solubilized by consecutive extraction with low and high salt buffers respectively.

follows: PBS, Phosphate buffered saline, pH 7.4 (Gibco Laboratories, Grand Island, NY); HBSS, Hanks balanced salt solution without Mg^{+2} or Ca^{+2}, pH 7.4 (Gibco); T_{10}, 10 mM Tris (pH 7.4); TK (7.4), 10 mM Tris, 400 mM KNO_3 (pH 7.4); TK (8.4), 10 mM Tris, 400 mM KNO_3 (pH 8.4), TEDGM, 10 mM Tris, 1 mM EDTA, 2.5 mM DTT, 10% glycerol, 20 mM Na_2MoO_4 (pH 7.4); TEDGMK, TEDGM plus 500 mM KCl adjusted to pH 8.4; TEDGMS, TEDGM plus 500 mM NaSCN adjusted to pH 8.4.

Preparation of CPG beads and columns

CPG bead suspensions were prepared by combining equal volumes of T_{10} buffer containing 1 mM sodium azide and clean dry beads. Trapped air was removed *in vacuo*. CPG columns were constructed from 2.5 ml glass hypodermic syringes, each fitted with a 16 gauge stainless steel needle with the point cut off to 2.5 cm in length. The beads were held in place by tight fitting glass fiber discs cut from Whatman glass microfiber paper (GF/A) above and below the bed of beads. A 200 μl column of beads was obtained by adding 400 μl of the 1:1 CPG suspension. Columns were washed with 2 ml of TK (7.4) buffer and cooled to 4°C before use. Used beads and columns were cleaned by soaking in concentrated acid (85% H_2SO, 15% HNO_3) for 48 h at room temperature or 2 h at 70°C, followed by a thorough rinse with water.

Isolation of rat thymocytes

The thymus glands from ten 22-day old female rats killed by decapitation, were removed by blunt dissection, minced, and gently homogenized by hand in HBSS containing 5 mM glucose in a loose fitting glass homogenizer. The homogenate was filtered through a 60-mesh stainless steel screen to remove connective tissue. HBSS buffer was added to bring the total volume to 125 ml, giving a suspension containing about 4×10^7 cells per ml. Viability was always greater than 86% as determined by trypan blue exclusion. Aliquots of the suspension containing 1×10^8 cells were centrifuged, the supernatant fraction decanted, and the pellets stored in liquid nitrogen until use.

Isolation of human lymphocytes

Lymphocytes were isolated from human volunteers as follows: 40–80 ml of peripheral blood anticoagulated with citrate was obtained by venopuncture, diluted 1:1 with HBSS and separated on a Ficoll–Hypaque density gradient. Cells were washed 4 times at 4°C with HBSS plus 1% fetal calf serum and suspended at 1×10^6 cells per ml in HBSS. Aliquots of the suspension containing 1×10^8 cells were centrifuged, the supernatant fraction decanted, and the pellets stored in liquid nitrogen until use. Viability was always greater than 95% as determined by trypan blue exclusion.

Determination of cytosolic and nuclear receptor by CPG

To a frozen pellet of rat thymocytes (or human lymphocytes) was added TEDGM buffer cooled to 4°C (1.0 ml per 1×10^8 cells) containing 50 nM [^{3}H]triamcinolone acetonide plus or minus 10 μM unlabeled triamcinolone acetonide. As the pellet thawed, the suspension was vortexed to a homogeneous consistency. After 2 h at 4°C, each mixture was transferred to a polyallomer tube and centrifuged at 250,000 ***g*** for 30 min. After an additional 2 h at 4°C, three replicate 100 μl aliquots of each supernatant fraction were placed on 0.2 ml columns of CPG beads cooled to 4°C. Each column was then washed with 20 ml of cold (4°C) TK (7.4) buffer to remove the excess steroid. This was done by attaching a 20 ml plastic syringe as a reservoir. When washing was complete, the reservoir and 16 gauge needle were removed, the columns warmed to room temperature, and bound steroid eluted with two 1 ml portions of absolute ethanol into vials for measurement of radioactivity.

The pellets obtained from the preparation of cytosol was suspended in 0.5 ml of TEDGM buffer at 4°C, transferred to clean test tubes and centrifuged for 10 min at 4800 ***g***. The wash is repeated once. The pellets were then suspended in 0.5 ml of TEDGMK or TEDGMS containing 50 nM [^{3}H]triamcinolone acetonide plus or minus 10 μM unlabeled triamcinolone acetonide and vigorously mixed. Equivalent results were obtained using TEDGMK or TEDGMS buffer as well as extraction of the pellet with ethanol. After 1 h at 4°C, the nuclear suspension was centrifuged at 250,000 ***g*** for 30 min. Four replicate 100 μl aliquots of each supernatant fraction were placed on CPG columns and treated as described above. Disintegrations per minute (dpm) obtained from cells extracted with a 200-fold excess of unlabeled steroid was used to define non-saturable (non-specific) binding and was subtracted from total bound dpm to yield specific receptor binding. All measurements of dpm are corrected for quenching by ethanol.

Determination of cytosolic receptor by Dextran Coated Charcoal (DCC) assay

A suspension of DCC was prepared by dissolving 0.01 g of T-70 dextran in 10 ml of 1.5 mM $MgCl_2$ followed by addition of 0.1 g of charcoal (Norit-A). Supernatent containing light particles which float after low speed centrifugation (200 ***g***) was removed by aspiration and replaced by an equal volume of buffer. Cytosol receptor concentration was assayed as follows: duplicate 150 μl aliquots of cytosol, obtained as described above, and labeled with [^{3}H]triamcinolone were added to pellets of DCC obtained from 225 μl of DCC suspension. The suspension of cytosol and DCC was continuously agitated for 20–30 min at 4°C followed by centrifugation at 4800 ***g*** for 10 min.

A 100 μl aliquot from each tube was removed for measurement of radioactivity, taking care not to disturb the DCC pellet.

Determination of cytosolic and nuclear receptor by Whole Cell Assay (WCA)

The procedure used has been described in detail by Munck *et al.*[4, 18]. The only modification of this procedure entailed the radioactive measurement of nuclear pellets with or without washing once with buffer.

RESULTS

Optimal conditions for the CPG assay

Glucocorticoid receptors have been shown to be stabilized by a wide variety of reagents including glycerol [9], sulfhydryl-protecting agents [10, 11], molybdate [12], cation chelators [13], phosphatase inhibitors [14], and ATP [15], as well as endogenous heat stable factors [15, 16]. For CPG analysis optimal buffer composition consisted of 10 mM Tris, 1 mM EDTA, 2.5 mM DTT, 10% glycerol, 20 mM Na_2MoO_4 (TEDGM, pH 7.4). The addition of 100 units per ml of trasylol (a protease inhibitor) or the substitution of thioglycerol for DTT provided no advantage.

Because the CPG assay depends on extensive washing of the beads with buffer to remove free and non-specifically bound steroid, the dissociation rate of the labeled steroid–receptor complex is a significant factor. Thymocytes incubated in the presence of [^{3}H]triamcinolone acetonide consistently showed a 2-fold increase in measured ^{3}H-steroid binding compared to those incubated in the presence of [^{3}H]dexamethasone. In a representative experiment the mean $\pm$SD was 1655 ± 142 vs 820 ± 70 binding sites/cell respectively. Similarly, an 8-fold increase was found when [^{3}H]triamcinolone acetonide was compared to [^{3}H]corticosterone (2111 ± 211 vs 275 ± 66 binding sites/cell). This difference is consistent with the reported dissociation rates for these steroids [17]. A concentration of 50 nM [^{3}H]triamcinolone acetonide was found to provide maximum steroid receptor binding.

In order to determine the minimum number of cells which could be routinely assayed on CPG, rat thymocytes were frozen in varying aliquots ranging from 1×10^8 to 1×10^6 cells. The results of CPG analysis are shown in Table 1. Minimal variation (<9%) occurred between 1×10^8 and 1×10^7 cells for both cytosolic and nuclear receptor values, but the assay failed to measure the receptor content of 1×10^6 cells.

Table 2. Assay variation using CPG analysis

Total dpm* $\pm$ SD	(*n*)	Non-saturable dpm* $\pm$ SD	(*n*)
1. 1423 ± 148	(8)	5. 51 ± 13	(8)
2. 1459 ± 52	(4)	6. 52 ± 17	(4)
3. 1446 ± 53	(4)		
4. 1448 ± 45	(4)		

*Values are given in dpm above background. (*n*) = Number of replicate CPG columns from each sample.

CPG assay variation

The intra and inter assay variation between identical samples measured by CPG is shown in Table 2. Six samples were extracted with 1 ml TEDGM. Samples 1–4 contained 50 nM [^{3}H]triamcinolone acetonide for measurement of total bound steroid and samples 5–6 contained the same plus 10 μM unlabeled triamcinolone acetonide for measurement of non-specific binding. Samples 1, 2–4 and 5–6 were handled by different investigators. The coefficient of variation for measurements of total bound dpm varied from 3.1–10.4% within each sample and less than 1% between samples. The large variation in sample 1 was due to one of 8 replicate CPG columns being 22% lower than the average. The coefficient of variation for measurements of non-specific binding varied from 25–33% within each sample and less than 1% between samples. The relatively large variation in non-specific binding within each sample is due to the very low dpm measured, but because it represents only 3.5% of the total binding, the actual error in net dpm is very small. Identical samples assayed on four different days over the course of 2 months contained 1784 ± 100 receptor binding sites/cell (mean $\pm$SEM), demonstrating the stability of frozen samples over time.

Comparison of CPG assay to DCC and WCA assay

To compare DCC to the CPG assay, cytosol was prepared from frozen cells in the presence of 50 nM [^{3}H]triamcinolone acetonide and assayed by each method. The results of four seperate measurements (shown in Table 3) demonstrate that while similar values of receptor binding are obtained with each assay, non-specific binding is significantly less ($P < 0.01$) in the CPG assay. A comparison of values

Table 1. Limits of the CPG assay

Cell number	Cytosolic receptor sites/cell* $\pm$SD	Nuclear receptor sites/cell* $\pm$SD	Total receptor sites/cell
100×10^6	2389 ± 140	86 ± 3	2475
50×10^6	2218 ± 25	90 ± 9	2308
25×10^6	2507 ± 107	119 ± 4	2626
10×10^6	2278 ± 14	116 ± 13	2394
1×10^6	0	0	0

*Mean $\pm$ SD of 2 experiments each measured in triplicate.

Table 3. Comparison of DCC and CPG assays

Assay	(n)	Total dpm ± SD	Non-saturable dpm ± SD	Receptor binding sites/cell ± SD
DCC:	(4)	3846 ± 225	464 ± 44	2478 ± 145
CPG:	(4)	3721 ± 174	41 ± 17	2697 ± 127

(n) = Number of samples, each measured in triplicate for CPG and in duplicate for DCC.

from the WCA and CPG assays is shown in Fig. 1. When the total receptor content (T′) is calculated from WCA measurements of cytosolic (C) plus unwashed nuclear pellet receptor (N′) and compared to the CPG total receptor content (T_{CPG}), the CPG assay appears to underestimate the receptor content. When the nuclear pellet obtained in the WCA is washed once with buffer, a substantial amount of labeled steroid is lost from the pellet. When the total receptor content (T), via WCA, is calculated from the cytosolic (C) plus washed nuclear pellet receptor (N) identical values are obtained with the two assays. This result was obtained in 4 consecutive experiments on different cell populations.

Selective measurement of cytosolic and nuclear receptor on CPG

When frozen cells were assayed by CPG prior to exposure to exogenous steroid and elevated temperatures, greater than 95% of the glucocorticoid receptors were found in the cytosol. Recently Kaufman *et al.*[18] have reported the loss of nuclear receptor into the supernatent of cells lysed in a hypotonic medium after a freeze thaw cycle. To demonstrate the selective extraction of cytosolic receptor by TEDGM as well as the ability of TEDGMS to extract nuclear receptor, freshly isolated rat thymocytes were divided into three groups. One group of cells was isolated immediately by centrifugation while the other two identical groups were incubated at 0 and 20°C respectively with 50 nM [^{3}H]triamcinolone acetonide plus or minus a 200-fold excess of unlabeled steroid for 30 min. Following incubation the cells were cooled to 0°C, isolated by centrifugation and all 3 groups of cells frozen in liquid nitrogen. When receptor binding was measured by CPG analysis the results shown in Fig. 2 were obtained. Cells frozen without incubation had 85% of the total receptor content in the cytosol. Cells incubated at 0°C showed only a slightly increased nuclear content, while cells incubated at 20°C had only 20% of the total receptor in the cytosol with 80% in the nuclear extract. The total receptor content remained essentially constant.

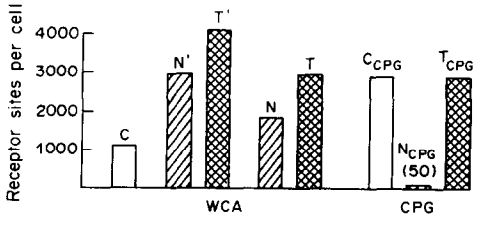

Fig. 1. Comparison of WCA and CPG assays. Rat thymocytes were incubated with 50 nM [^{3}H]triamcinolone acetonide plus or minus 10 μM unlabeled triamcinolone acetonide at 37°C for 30 min. Aliquots of cells were lysed in hypotonic $MgCl_2$ (1.5 mM) at a 6-fold dilution containing DCC for cytosolic receptor measurements and at 60-fold dilution for nuclear receptor measurements. Before counting the pellets, one of two sets of duplicate nuclear pellets was washed once in lysing buffer. Triplicate CPG measurements were made on frozen cells without prior exposure to steroid or temperatures above 4°C. Bars are labeled as follows: C, cytosolic receptor (WCA); N, nuclear receptor from washed pellet (WCA); N′, nuclear receptor from unwashed pellet (WCA); T, total receptor (N + C); T′, total receptor (N′ + C); C_{CPG}, cytosolic receptor on CPG; N_{CPG}, nuclear receptor on CPG; T_{CPG}, total receptor ($C_{CPG} + N_{CPG}$).

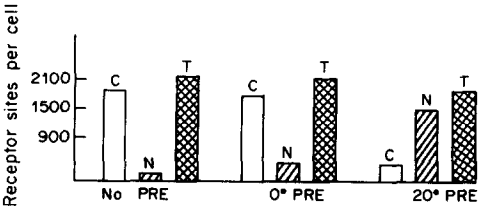

Fig. 2. Measurement of nuclear translocation by CPG assay. Freshly isolated rat thymocytes were preincubated at 20°C (20°C PRE) or 0°C (0°C PRE) in HBSS with 50 nM [^{3}H]triamcinolone acetonide prior to freezing. Once frozen, the cells were assayed on CPG as usual and compared to one set not preincubated (No PRE). Bars are labeled as follows: C, cytosolic receptor; N, nuclear receptor; T, total receptor. Each bar represents the mean of 4 measurements.

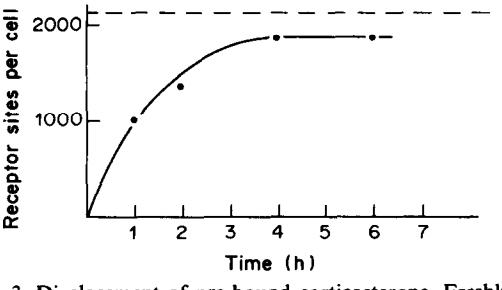

Fig. 3. Displacement of pre-bound corticosterone. Freshly isolated rat thymocytes were preincubated at 0°C for 1 h in HBSS with 50 nM unlabeled corticosterone prior to freezing. The frozen pellets were then extracted with TEDGM buffer containing 50 nM [^{3}H]triamcinolone acetonide. At the times indicated, measurements of receptor binding were made in triplicate on CPG columns. The control value for a 2.5 h extraction of cells not preincubated with corticosterone is indicated by the dashed line (– –).

Table 4. CPG analysis of human lymphocyte glucocorticoid receptors

Subject	Cystolic receptor sites/cell	Nuclear receptor sites/cell	Total receptor sites/cell
1	2728	33	2761
2	1865	35	1900
3	4711	86	4797
4	1854	67	1921
5	1802	33	1835
6	2667	56	2723
7	1453	0	1453
8	1101	15	1116
9	1953	0	1953
10	2502	0	2502
	2264 ± 1004*	33 ± 30	2296 ± 1023

*Mean ± SD.

Displacement of endogenously bound steroid

To simulate the effect of receptor occupation by endogenous steroid on the CPG assay, freshly isolated cells were incubated in HBSS with 50 nM unlabeled corticosterone at 0°C for 1 h. After incubation the cells were removed from suspension by centrifugation, the incubation medium decanted, and the cells frozen in liquid nitrogen. The frozen pellet was extracted with TEDGM and then 50 nM [^{3}H]-triamcinolone acetonide was added. At various times duplicate aliquots were removed and assayed for receptor binding on CPG. The results of one such experiment (Fig. 3) indicate that just over 4 h are required for maximum binding to occur. In the absence of endogenous steroid, maximum binding is achieved by 2 h (data not shown).

Measurement of human lymphocyte glucocorticoid receptors

The CPG assay developed using rat thymocytes as a model cell preparation has been applied to the quantification of glucocorticoid receptors in human lymphocytes. Peripheral lymphocytes were isolated from whole blood (anticoagulated with citrate) on Ficoll–Hypaque gradients and frozen in liquid nitrogen. Cytosolic and nuclear extracts were prepared as described above. Results on 10 normal human volunteers are shown in Table 4. The observed values are consistent with those reported by others [2, 3, 19].

DISCUSSION

We have previously reported the development of a controlled pore glass (CPG) bead assay for the determination of occupied and unoccupied estrogen receptors in breast cancer [20], as well as the use of CPG in measuring estrogen receptor content in ovarian epithelial carcinomas [21]. Due to the inherent instability of the glucocorticoid receptor, significant modifications of the original assay technique were required. For the determination of estrogen receptors, it is advantageous to adsorb the unoccupied cytosolic receptor to the CPG beads, where they react more efficiently with labeled hormone than in cytosol itself. In the measurement of glucocorticoid receptors, instability of the unoccupied receptor necessitates binding of the hormone to the receptor prior to adsorption onto the beads.

To measure glucocorticoid receptors using the CPG assay it was necessary to use a steroid hormone ligand with a low dissociation rate from the receptor to prevent loss of bound hormone from the column. Preliminary experiments were carried out using tritiated dexamethasone which dissociates more slowly from the receptor than naturally occurring hormones like cortisol or corticosterone [17]. Initial findings were promising but somewhat erratic suggesting that the dissociation rate of dexamethasone from the receptor was too rapid to provide dependable results. With tritiated triamcinolone acetonide as the hormone ligand, it was shown that steroid concentrations of 50 nM or higher provide optimal results giving consistent values twice those obtained using dexamethasone. When significant amounts of endogenously bound steroid are present, exchange with labeled steroid at 4°C requires just over 4 h for quantitative measurements.

When frozen cells were extracted with 1.0 ml of TEDGM buffer per 1×10^8 cells and 100 μl aliquots applied to 0.2 ml columns of CPG beads, all of the solubilized receptor–steroid complex was retained by the beads. In support of this, the ^{3}H-steroid washed off the CPG columns with buffer after applying cell extracts could be completely adsorbed by DCC and none of the eluted ^{3}H-steroid was retained by fresh CPG beads.

With the experimental protocols described, glucocorticoid receptor measurements using the CPG bead technique were compared with the DCC procedure and equivalent results obtained. Non-saturable binding was considerably less in the CPG assay (1–2%) compared to DCC (12%). In initial studies comparing CPG and WCA measurements of receptor binding, the WCA assay measured greater ^{3}H-steroid binding compared to CPG. This discrepancy was eliminated when the thymocyte nuclei, obtained in the whole cell assay, were washed once with buffer prior to the measurement of bound ^{3}H-steroid, which resulted in a loss of labeled steroid from the nuclei. When the nuclei were washed with buffer, non-specific binding decreased from 40–50%

to about 10%. When the supernatent from the washed nuclei was applied to fresh CPG columns, no ^{3}H-steroid was retained by the beads. Treatment of the supernatent with DCC resulted in total adsorption of all ^{3}H-steroid by the charcoal. In contrast, TEDGMS extracts of washed nuclear pellets, when applied to CPG columns, provided equivalent quantities of nuclear receptor compared to direct measurements of washed whole nuclear pellets (±6%). These facts suggest that non-specifically bound or free steroid was removed from the nuclear pellets. However, we have not ruled out the possibility that loss of an unstable receptor–steroid complex occurred under these conditions.

The WCA and CPG assays provide equivalent numbers of total receptor content within a population of cells. When these cells were assayed by CPG, using temperatures below 4°C and cell-free conditions, greater than 90% of the receptors were found in the cytoplasmic extract, a distribution which we believe reflects the native distribution of receptor at the time of isolation. The CPG assay therefore provides a valuable tool for assessment of receptor localization as well as total receptor content.

The value of the CPG assay derives from its reproducibility, simplicity and convenience. Triplicate measurements on the same sample consistently agree within 5–8% providing excellent replication. Non-saturable binding represents 1–5% in the CPG assay while accounting for 20–40% in many whole cell assays and 10–20% in the DCC assay. Because the CPG assay can be carried out on frozen samples containing as few as 1×10^7 cells, which are stable for many months, and requires no cell culture techniques, it is more convenient than assays performed on fresh samples which depend on the integrity of live cells.

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