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DIMETHYL SULFOXIDE AS SOLUBILIZATION AGENT IN THE RADIOIMMUNOASSAY FOR THE DETECTION OF POLYCHLORINATED DIBENZO-P-DIOXINS.

by
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MANAGEMENT PERSPECTIVE

This study was undertaken as part of a larger effort to develop screening tests for the detection of trace organic contaminants, such as dioxins. Such screening tests should facilitate a dramatic reduction in sample loads in routine analytical laboratories by eliminating samples that are contaminant free from further time consuming conventional analysis. This report addresses some initial problems associated with the hapten labelling reaction used in the dioxin screening assay. The described labelling procedure should enable other analysts to repeatably produce the key labelled dioxin reagent. Some inherent weaknesses of the present labelled hapten are discussed and an alternative hapten which should result in a vastly improved assay is proposed. The described modifications to the RIA for PCDDs should improve the departments ability to screen samples for this important contaminant class.

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PERSPECTIVE-GESTION

L'étude présentée ici fait partie d'un ensemble de travaux qu'on a entrepris afin de mettre au point des épreuves de sélection pour détecter les contaminants organiques à l'état de traces comme les dioxines. Avec de telles épreuves, il devrait être plus facile de réduire dans une très grande mesure le nombre d'échantillons nécessitant une analyse de routine, car ceux qui ne renferment pas de contaminants devraient être éliminés, de sorte qu'on s'épargne l'analyse classique plus longue. Dans le rapport présenté ici, on traite de certains des premiers problèmes qu'a posé la réaction de marquage des haptènes utilisée dans l'épreuve de sélection de la dioxine. Avec la méthode de marquage décrite, il devrait être possible de produire à volonté le principal réactif marqué pour la détection de la dioxine. On traite aussi de certaines des lacunes inhérentes à l'haptène marqué actuellement employé et l'on propose un autre haptène dont l'utilisation devrait permettre d'améliorer considérablement l'épreuve. Dans sa forme modifiée, l'épreuve radio-immunologique de détection des PCDD devrait permettre aux ministères de sélectionner les échantillons en vue de la détection de cet important type de contaminant.

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ABSTRACT

Immunoassays are potentially valuable tools for use in screening environmental samples for a broad range of contaminants, such as polychlorinated dibenzo-p-dioxins (PCDDs). The performance of the RIA for PCDDs was characterized using 4 solubilization systems: Cutscum, Triton, horse serum, and dimethyl sulfoxide (DMSO). The DMSO based assay appeared to perform best at low PCDD levels. The effects of assay incubation time and hapten storage conditions on the DMSO based assay were assessed. The separation of bound from unbound radioactivity was accelerated without adversely affecting assay performance. Further assay development through the use of an improved hapten is considered.

RÉSUMÉ

Les épreuves immunologiques peuvent être des outils précieux pour sélectionner les échantillons d'origine environnementale dans la détection de toute une gamme de contaminants comme les polychlorodibenzo-p-dioxines (PCDD). On a évalué l'épreuve radio-immunologique des PCDD avec 4 systèmes de solubilisation : Cutscum, Triton, sérum de cheval et diméthylsulfoxyde (DMSO). L'épreuve au DMSO a semblé donner de meilleurs résultats pour la détection de PCDD à faible concentration. On a étudié l'influence de la durée d'incubation et des conditions de conservation de l'haptène dans l'épreuve au DMSO. On a pu accélérer la séparation des produits radioactifs liés des produits non liés sans compromettre les résultats de l'épreuve. On envisage de perfectionner encore davantage l'épreuve en utilisant un haptène amélioré.

1.0 INTRODUCTION

It should prove possible to reduce the overloads common to many organic residue laboratories through the judicious use of screening tests, such as immunoassays. Immunoassays are usually proposed as complements to, rather than replacements for, conventional analytical procedures (Mumma and Brady, 1987).
The quantification steps most commonly used in immunoassays measure either radioactivity (radioimmunoassay: RIA) or enzyme activity (enzyme immunoassay: EIA). In a typical RIA procedure, the analyte competes for antibody binding sites with radio-labelled (usually ^{125}I) analyte (hapten). The quantity of analyte in a sample is inversely proportional to the amount of bound radioactivity (Hunter, 1973). EIA procedures, which come in a variety of formats, employ enzyme markers, for the same purpose (Tijssen, 1985).

Being notoriously time consuming and expensive to analyze, the polychlorinated dibenzo-p-dioxins (PCDDs) are suitable candidates for detection using screening assays; RIA (Albro et al., 1979) and EIA (Stanker et al., 1987) techniques have been developed for this purpose. The use of RIA to detect PCDDs in environmental samples poses difficulties that are related to the analyte's properties -in particular its extreme hydrophobicity, usually ultra-low environmental levels, and the nature and variety of matrices with which it is associated.

The present report deals with the former two problems by comparing several alternative dioxin solubilization procedures, and their effect on assay performance at low levels. Some ways in which assay efficiency can be improved without adversely affecting performance are also considered. Hapten associated problems and potential solutions are described.

2.0 MATERIALS AND METHODS

2.1 Antiserum

The polyclonal antibodies used (ALB 5), were raised in rabbits against an antigen that had been prepared by conjugating 1-amino-3,7,8-trichlorodibenzo-p-dioxin to bovine thyroglobulin by means of an adipamide linkage.

2.2 Labelled Hapten

The hapten (1-N-(5-iodovaleramido)-3,7,8-trichlorodibenzo-p-dioxin) which is capable of being bound by the anti-dioxin antibodies, but is not itself antigenic, was rendered radioactive by exchanging its iodo group for ^{125}I. Unlabelled hapten (10 ug) dissolved in approximately 250 uL of dry acetone was injected into a vial of $Na^{125}I$ (5mCi Amersham, IMS300, carrier free). Vial and contents were incubated at $50^{0}C$ for 66 h. Labelled hapten was extracted in $CHCl_3$, and purified on silica gel.

2.3 Assay Procedure

Antibodies (sufficient to bind 40% of the added radioactivity) and sample extract were incubated together for 40 minutes at 37 °C. Labelled hapten (7000 cpm) was added to the assay tubes. The tubes were vortex mixed, and re-incubated at 37 °C for 1 h, and then at 4 °C for 64 h. A second antibody procedure, using goat anti-rabbit gamma-globulin, was used to separate bound from free tracer. The bound radioactivity was quantified using a gamma radiation counter (LKB 1271). A reduction in the amount of radioactivity bound is proportional to the quantity of analyte present. Analyte concentration was interpolated from a standard curve.

2.4 Accelerated Separation Procedure

After addition of the second antibody, the assay tubes were incubated at 4°C for 2 hours. 1 mL of PEG reagent (4 g of polyethylene glycol (Sigma P-2139, MW 8000) dissolved in 100 mL PBS) was then added to each tube. The tubes were then vortex mixed and centrifuged at 2000 rpm for 30 minutes. The sedimented radioactivity was counted.

3.0 EXPERIMENTAL

3.1 Labelling Reaction

The hapten is unstable in dry acetone at room temperature. Dry storage at -80^{0}C under nitrogen prevented this short term instability: TLC revealing a single spot (R_f0.71).
In an initial labelling experiment, a large proportion (up to 75%) of the organically bound radioactivity eluted from the silica gel column in the first 50 mL of eluent was volatile. The volatile radioactivity was presumably a labelled portion of the hapten's aliphatic side chain. In later experiments, finer eluent fractionation (10 mL) indicated that the bulk of the volatile radioactivity was associated with the early organic fractions (Table 1). Reduced volatility was associated with fractions 7-9, which also contained the bulk of the bindable radioactivity; this radioactivity had a high affinity for the anti-dioxin antibodies and yielded typical assay calibration curves (Fig. 1).

Subsequently, chromatographic eluent was collected in 10 mL fractions, counted, examined for the presence of volatile components, and challenged with anti-dioxin antibodies: 80% binding was normally obtained using the desired fractions.

Table 1. Labelled hapten preparation: fractionation of eluent from chromatography column

Fraction	Activity (cpm)	Blow-down Loss (%)	B/T %
1	$1.26*10^6$	-	-
2	$4.81*10^6$	-	-
3	$8.8*10^7$	99	1
4	$4.5*10^8$	98	2
5	$4.8*10^8$	99	15
6	$2.0*10^8$	94	66
7	$5.0*10^7$	23	88
8	$6.1*10^7$	0	85
9	$4.0*10^7$	13	61
10	$8.1*10^6$	-	-
11	$2.3*10^6$	-	-

3.1.1 Elimination of Hapten Breakdown Products.

In an effort to eliminate the production of radioactive impurities a low hydroxide (10^{-5}M) ^{125}I preparation (New England Nuclear NEZ033L) was evaluated. The bindable radioactivity was eluted in a single peak. No peaks of unbindable or volatile

organically bound radioactivity were recovered. The labelled hapten, so produced, yielded typical assay calibration curves (Fig. 1).

3.1.2 Problems with Present Labelling Reaction.

The RIA for PCDDs uses an aliphatic dioxin derivative (1-N-(5-iodovaleramido)-3,7,8-trichlorodibenzo-p-dioxin), in which the cold iodide is replaced by ^{125}I, as the labelled hapten. However, that hapten is unstable, it tends to cyclize, which results in variable yields from the labelling reaction; it also tends to gradually lose iodide during storage, which causes reduced reaction yields. The labelled hapten's low specific activity, approximately 65 Ci/mmol out of a theoretically possible 2175 Ci/mmol, when combined with the inability to separate unlabelled from labelled hapten, adversely affects assay performance. Furthermore, the labelling reaction requires the use of a relatively high level (5 mCi) of ^{125}I in a three day exchange reaction.

3.2 PCDD Solubilization Systems

PCDDs are extremely hydrophobic, whereas antibodies require an aqueous medium to function correctly. Thus, for a successful assay, the dioxin molecules must be solubilized, without unduly interfering with antibody binding. Various surfactants

and solvents can be used for this purpose. The following solvents were evaluated with negative results: acetonitrile, N-methylpyrrolidone, dimethylformamide, dimethyl acetamide, hexamethylphosphoramide, sulfolane, and dimethyl sulfone. A solvent concentration of 33.3% (v/v) was used in the previous experiment in an effort to maximize sample capacity, which can be a limiting factor in the RIA for PCDDs. Assay calibration curves obtained using the following solubilization systems are presented in Figure 1: Cutscum (0.5 % (w/v)) -30 minutes ultrasonic treatment; Triton X-305 (0.5 % (w/v)) - 30 minutes ultrasonic treatment; Horse serum (5.6 % (w/v)) - no ultrasonic treatment; dimethyl sulfoxide (DMSO) (33.3 % (v/v)) - 30 minutes ultrasonic treatment.

The Triton assay had a better response at low concentrations than the Cutscum assay which had a wider working range. The Horse serum assay was equivalent to the Triton assay at low concentrations, but deteriorated rapidly above 500 pg. The DMSO assay had the best response at low concentrations and the steepest slope, but the narrowest working range.

3.2.1 Assay Precision

Precision profiles for the four assay systems (Figure 2) were prepared. A set of replicated 2,3,7,8-TCDD standards (*10) was analyzed in a single experiment. The estimated 2,3,7,8-TCDD content of each standard was interpolated from the assay calibration curve. The CV% was calculated (CV% = (SD/Mean)*100) and plotted against the quantity of 2,3,7,8-TCDD.

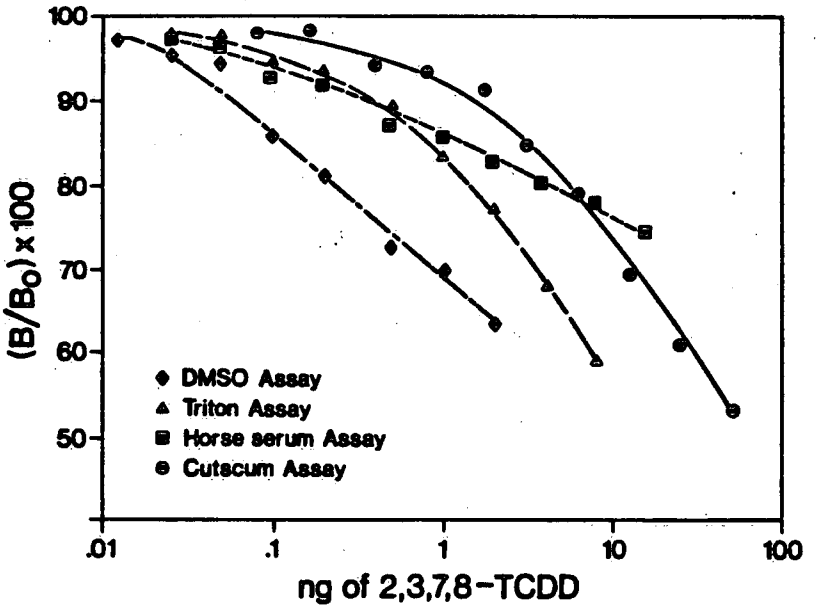

Fig. 1. Assay calibration curves prepared using four solubilization systems

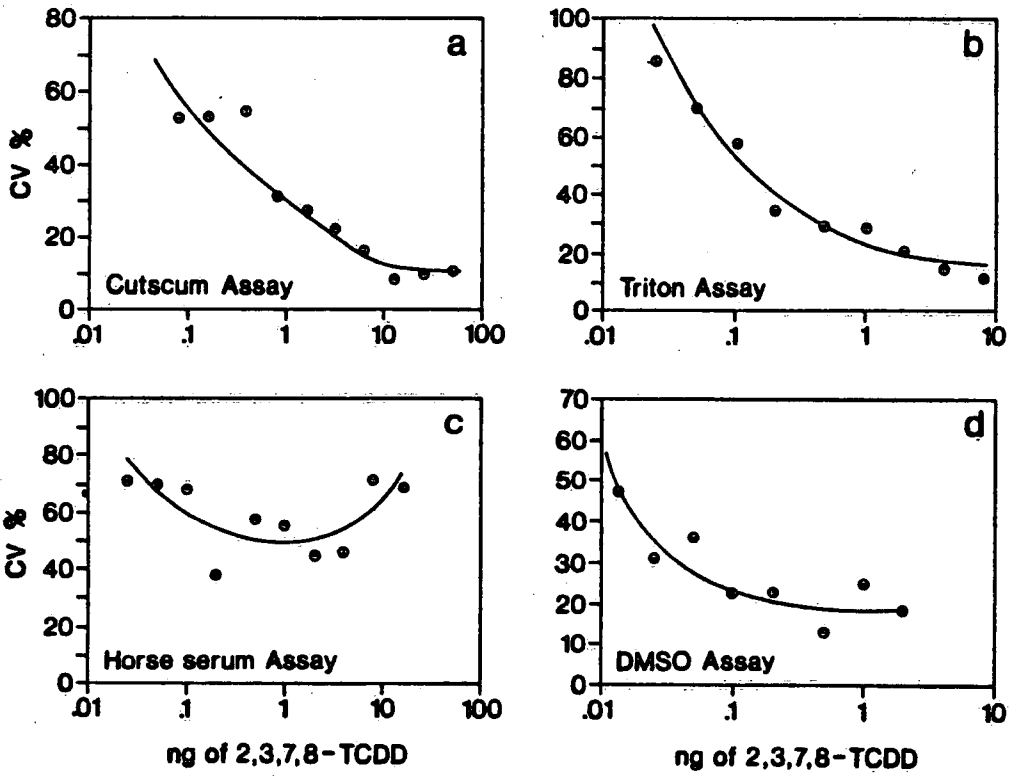

Fig. 2. Precision profiles for four solubilization systems

3.2.2 Assay Sensitivity

Assay sensitivity, defined as the precision of measurement of zero dose (Ekins, 1983), was estimated by plotting SD or log (SD) against analyte concentration, and extrapolating the curve to intercept the Y axis at zero dose (Table 2).

3.2.3 Minimum Detectable Concentration

The minimum detectable concentration (MDC) (Table 2), which provides an estimate of the least quantity of analyte that can be reliably determined in practice, is a 'black box' value derived by the RIACALC2 (LKB/WALLAC) software using the following formula: MDC = Concentration at (Y(0) + 3dY/R); dY is the larger of the SD or the Response Error (RER) at zero dose; Y(0) = fitted cpm response at 0 dose; dY = estimated error of Y(0); R = number of replicates of the zero standard.

The use of the R term can cause the MDC values to be low biased. An alternative estimate, the MDCb, based on the uncorrected error associated with zero dose, is also presented in Table 3.

It is apparent that the DMSO based assay is more sensitive, precise, and had lower MDC and MDCb values than the other assays (Figure 2 and Table 2).

Table 2. Performance characteristics using four solubilization systems

	Solubilization System			
	Cutscum	Triton	Serum	DMSO
Precision (CV% Mid)	29-8	30-13	60-38	20
Sensitivity (pg)	110	38(40)[1]	35	9
MDC[2] (pg)	428	55(61)	32	15
MDCb[3] (pg)	330	114	105	27

(1): Mean of 2 determinations; higher value in parentheses.

(2): MDCA: RIACALC2 estimate.

(3): MDCB: 3*SD(0).

3.3 Stability of Labelled Hapten in DMSO

After 7 days dark storage at room temperature, the

labelled hapten was unbindable by the ALB 5 antibodies. Dark storage at 4 °C decreased the proportion of radioactivity bound in the absence of analyte (Table 3); the effect increased with time and was apparently similar for uninterrupted and interrupted storage. After 7 days storage, a marginal deterioration in assay response, which appeared to increase slightly, though inconsistently, with increased storage duration, was observed. The decrease level of bound radioactivity observed after 21 days storage could adversely affect counting precision at high TCDD levels (low counts).

Table 3. Stability of labelled hapten in DMSO

	DAYS AT 4 °C						
TCDD (pg)	0	7	14	21	7 F/T*1[2]	14 F/T*2	21 F/T*3
0	46.0[3]	35.0	30.0	25.0	36.0	29.0	24.0
50	91.3[1]	92.7	92.7	93.5	92.4	93.1	93.6
100	87.7	89.5	89.1	91.5	90.8	91.2	90.2
200	85.4	85.3	86.8	87.3	83.4	86.3	87.7
500	76.2	77.1	76.6	79.7	79.5	75.3	79.5

(1) $(B/B_0)*100$; replication level: *5
(2) Number of freeze/thaw events
(3) (B/T)*100

3.4 Effect of Incubation Time on Assay Performance

Increasing the incubation period from 24 to 48 h slightly improved low end (12.5 - 200 pg) assay response (Fig. 3). A further increase in incubation time to 72 h negatively affected assay response; this apparent effect requires verification and further investigation. The differences between the curves were slight. Although a 48 h incubation period may be optimal, 24 h incubation provides an adequate response when time saving is important.

3.5 Accelerated Separation Procedure

The accelerated separation procedure, which reduces assay execution time by 4 hours, did not adversely affect assay response (Fig. 4a). The deviation of the 50 and 100 pg points from the accelerated assay's fitted curve, are probably explainable by experimental variation; the curve for the normal system being exceptionally well behaved. The normal assay produced unusually precise data at low analyte levels (Fig. 4b): the level of precision described in Figure 2 being more typical. From 100 - 2000 pg both systems were comparably precise.

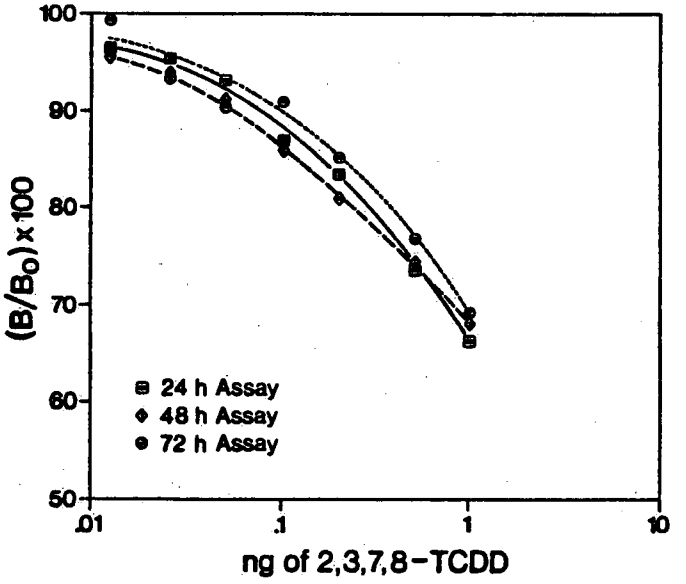

Fig. 3. Effect of incubation time on assay performance

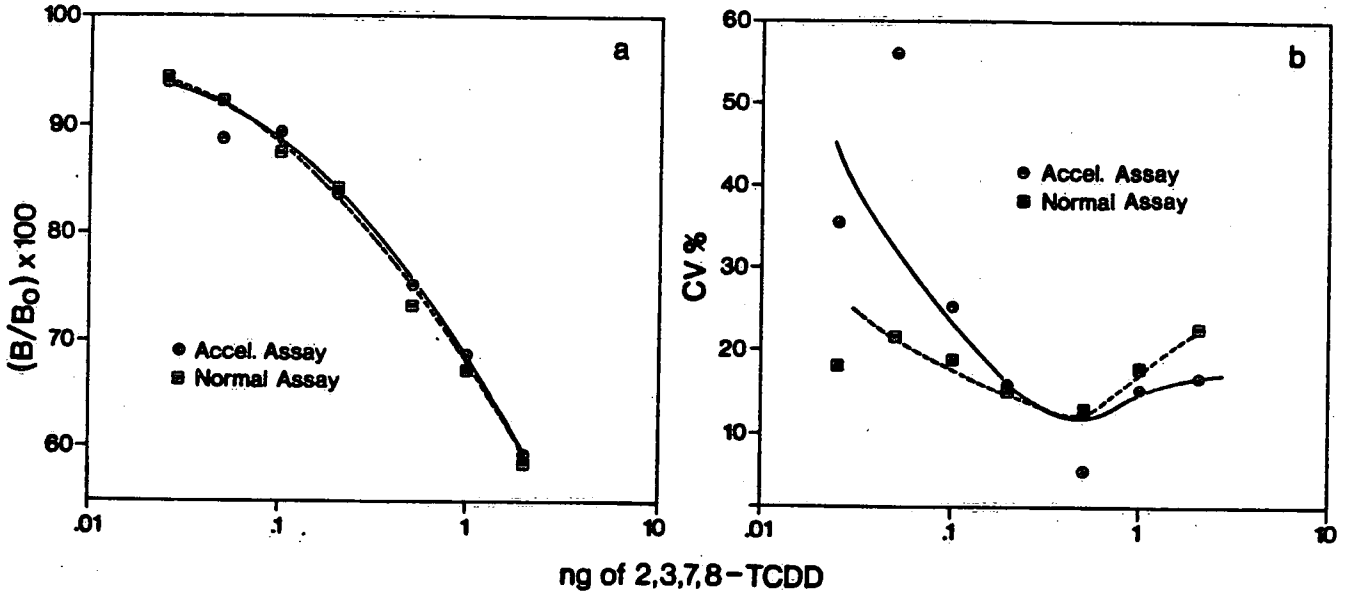

Fig. 4. Assay performance using accelerated and normal separation procedures; (a) calibration curves; (b) precision profiles

3.10 DISCUSSION

Routine use of RIA to detect trace PCDD contaminants in environmental matrices is dependant on further assay development and optimization, designed to improve reliability and performance at low analyte levels. Improvements in assay sensitivity and precision are related to several factors: the labelled hapten's specific activity and immuno-purity; the solubilization system's effectiveness; antiserum avidity, and the mechanics of the individual assay steps.

The present hapten's shortcomings negatively affect assay performance and reliability. The hapten's low specific activity means that unlabelled hapten molecules compete with PCDDs for antibody binding sites, so reducing assay responsiveness. A promising approach would be to label a tyramine derivative of 3,7,8-TCDD with ^{125}I which would generate a product with high specific radioactivity that could be readily separated from unlabelled hapten. Several important assay improvements would result.

When evaluated using laboratory standards, the DMSO based assay had a steeper calibration curve, and was more sensitive and precise than the assays based on the other solubilization systems. Use of DMSO in the RIA eliminates a solvent removal step and simplifies the assay calibration curve's preparation: 2,3,7,8-TCDD standards can be prepared and stored, in the short term, in DMSO. A further advantage is the reduction of assay incubation time by 48h. However, care must be exercised when storing the labelled hapten in DMSO; storage for longer than 5 days at 4 ^{o}C is inadvisable. Previously reported data from fish analyses (Sherry

Triton X-305. The DMSO based assay may, however, prove practicable with other matrices.

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