Quantitation of Targets for PCR by Use of Limiting Dilution

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ABSTRACT

We describe a general method to quantitate the total number of initial targets present in a sample using limiting dilution, PCR and Poisson statistics. The DNA target for the PCR was the rearranged immunoglobulin heavy chain (IgH) gene derived from a leukemic clone that was quantitated against a background of excess rearranged IgH genes from normal lymphocytes. The PCR was optimized to provide an all-or-none end point at very low DNA target numbers. PCR amplification of the N-ras *gene was used as an internal control to quantitate the number of potentially amplifiable genomes present in a sample and hence to measure the extent of DNA degradation. A two-stage PCR was necessary owing to competition between leukemic and non-leukemic templates. Study of eight leukemic samples showed that approximately two potentially amplifiable leukemic IgH targets could be detected in the presence of 160 000 competing non-leukemic genomes.*

The method presented quantitates the total number of initial DNA targets present in a sample, unlike most other quantitation methods that quantitate PCR products. It has wide application, because it is technically simple, does not require radioactivity, addresses the problem of excess competing targets and estimates the extent of DNA degradation in a sample.

INTRODUCTION

Quantitation of DNA or RNA by the PCR is a problem that is presently under active investigation by many workers. Nearly all methods reported to date have used co-amplification of reporter DNA in the same tube and some form of quantitation of the amplified material (3,4,11). It is assumed that the efficiency of amplification of the reporter DNA is the same as that of the target DNA, and calculation of the amount of target DNA initially present is based on the amount of reporter DNA added or originally present and on the ratio of the quantities of target and reporter DNA as determined in the amplified material by various methods.

We have been using the rearranged immunoglobulin heavy chain (IgH) gene as target DNA in the PCR to study patients with acute lymphoblastic leukemia (ALL) in order to detect and quantitate a minor population of leukemic cells within a larger population of normal lymphoid and non-lymphoid cells (1,2). In a particular patient, all leukemic cells will have the same rearranged IgH gene that can act as a genetic marker to distinguish leukemic cells from normal non-lymphoid cells and T lymphocytes, which have not rearranged their IgH genes, and from normal B lymphocytes, which have undergone various and different rearrangements of their IgH genes. Quantitation of the unique rearrangement of the leukemic clone poses two problems. First, only a few copies may be present in a tissue sample taken from a patient in remission following treatment. Second, germ-line IgH genes from cells, other than B lymphocytes, and rearranged IgH genes from normal B lymphocytes will be present and, owing to homology with the leukemic sequence, may compete with it in the PCR. Our approach to quantitation has differed from that of other workers in avoiding use of reporter DNA and quantitation of amplified material. Rather, we have used the principle of limiting dilution, which is based on the use of a qualitative all-or-none end point and on the premise that one or more targets in the reaction mixture give rise to a positive end point. Accurate quantitation is achieved by performing multiple replicates at serial dilutions of the material to be assayed. At the limit of dilution, where some end points are positive and some are negative, the number of targets present can be calculated from the proportion of negative end points by using Poisson statistics.

In this paper we illustrate the use of limiting dilution analysis to quantitate a target for the PCR, using the rearranged IgH gene from a leukemic clone as an example. We also discuss four related issues: optimization of the PCR to detect one or a few DNA targets; the effect of excess competing targets in the PCR; interference in the PCR by primers for an unrelated DNA segment; and the problem of DNA degradation in the sample. Although data from a small number of patients are included in this paper, the clinical and biological information obtained by limiting dilution analysis from a large number of patients with ALL will be described elsewhere (unpublished data).

MATERIALS AND METHODS

DNA Samples

PBL1 DNA was obtained from normal blood cells separated by Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway) to contain predominantly normal lymphocytes, and Ho DNA was from a bone marrow sample of a patient with ALL obtained at diagnosis. Ho DNA provided a source of specific leukemic IgH targets and normal *N-ras* targets, and PBL1 DNA provided a source of normal IgH and *N-ras* targets. Ho DNA contained virtually 100% leukemic cells, whereas PBL1 was estimated to contain approximately 15% normal B lymphocytes.

The optimization experiments relied on DNA concentration of the Ho DNA sample (90 ng/µl) to estimate the number of PCR targets present in the dilutions. Due to the small amount of material available, an OD_{260} was not possible. The concentration of the DNA was obtained by ethidium bromide spotting against known DNA standards (Reference 8, Appendix E, p.6). Because one human diploid cell contains 6 pg of DNA (5), 1 µg of PBL1 DNA would contain approximately 3.3×10^5 *N-ras* genes, approximately 2.4×10^4 rearranged IgH genes and 3×10^5 unrearranged IgH genes.

DNA from 7 other patients with ALL was extracted from fresh bone marrow aspirate samples for patients 1, 2, 3 and 7, from frozen Ficoll-Paque separated lymphocytes for patient 4 and from stained, fixed bone marrow slides for patients 5 and 6. The DNA concentration of samples 1, 2, 3, 4 and 7 was determined by OD_{260} and for patients 5 and 6 by ethidium bromide spotting.

PCR

PCRs (7) contained 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, pH 8.8, 10 mM β-mercaptoethanol, 200 µg/ml gelatin, 2 mM $MgCl_2$, 0.1 mM each of deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate and deoxythymidine triphosphate, 100 ng of each primer, varying amounts of template DNA (0.45 pg–1 µg), and 0.4 U of *Taq* DNA Polymerase (AmpliTaq®: Perkin-Elmer, Norwalk, CT) in a volume of 25 µl, overlaid with 25 µl light mineral oil. The samples were subjected to an initial 5 min denaturation at 94°C followed by varying numbers of cycles of 1 min annealing at 55°C, 1 min extension at 72°C and 1 min denaturation at 94°C. A final 20 min extension at 72°C was performed at the end of each round of PCR.

In all experiments, negative controls containing no template DNA were subjected to the same procedures to detect any possible contamination.

PCR Primers

Primers were synthesized on an Applied Biosystems Model 371 automated synthesizer (Foster City, CA). Consensus primers (2) used to amplify all IgH genes in the first round of PCR were FR3A - 5′ ACACGGC(C/T)(G/C)-TGTATTACTGT 3′ for the 3′ end of the V region; LJH - 5′ TGAGGAGACG-GTGACC 3′ for the 3′ end of the J region.

Patient-specific primers (1) sited between LJH and FR3A were used to amplify Ho IgH genes only in the second round for the Ho DNA: Ho1 - 5′ TGT-GCGAAAGAATCTCTGCC 3′ for the 3′ end of V; Ho2 - 5′ CCAGTAGTCA-AGGGTGGGTA 3′ for the 5′ end of J.

Patient-specific IgH primers for the other seven patients will be published elsewhere.

Specific primers were used for *N-ras* in both first and second rounds. First round: 231 (intron 1) - 5′ AAGCTTTAAAGTACTGTAGAT 3′; NB12 (exon 1) - 5′ CTCTATGGTGG-GATCATATTCA 3′. Second round: NA12 (exon 1) - 5′ ATGACTGAGTA-CAAACTGGTGGTG 3′ that lies between 231 and NB-12 and in the second round is used with NB-12.

For single-round PCR, *N-ras* was amplified by 231 and NB12 and Ho IgH by Ho1 and Ho2. For two-round PCR, *N-ras* was amplified by 231 and NA12 followed by NB12 and NA12 and leukemic IgH genes by FR3A and LJH, followed by patient-specific primers in the second round for patients 1–7. PCR products were electrophoresed on 6% polyacrylamide gels in 0.5× TBE buffer (44.5 mM, Tris-HCl, 44.5 mM boric acid, 1 mM EDTA) at 260 V for 1.5 h, stained in (0.5 µg/ml) ethidium bromide, and visualized using short wavelength UV light.

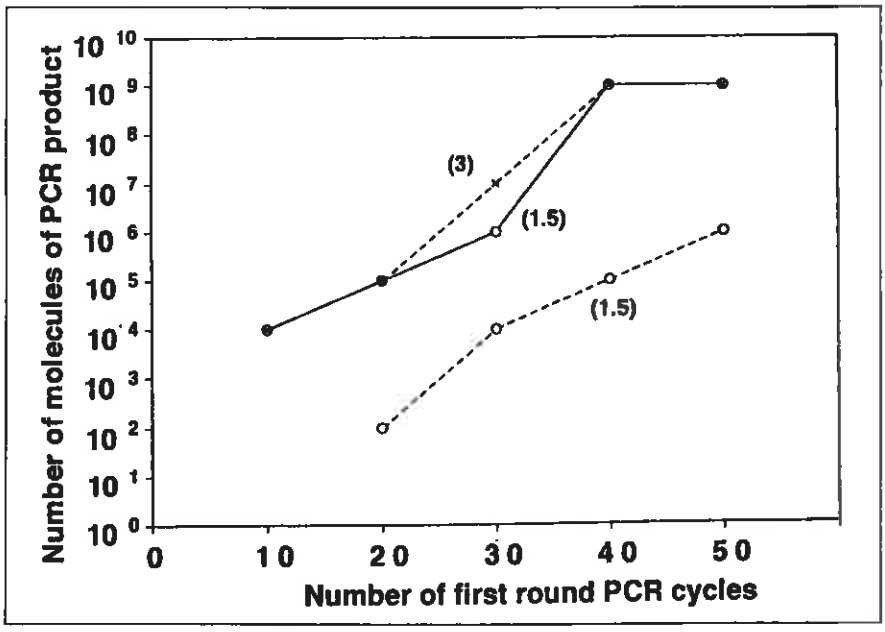

Figure 1. Number of *N-ras* and Ho IgH PCR products produced by a first-round PCR after varying PCR cycle number, in the presence/absence of competing PBL1 DNA. The numbers in parentheses indicate the average number of original PCR targets expected in a single sample of Ho-diluted DNA. *N-ras* PCR product (-PBL1), ---×---; Ho IgH PCR product (+PBL1), ---o---; Ho IgH PCR product (-PBL1), —o—.

Quantitation

Threefold dilutions of samples were prepared in water or PBL1 DNA and 10 replicates of each dilution were analyzed using the optimal PCR protocol presented in this paper. The mean number of targets required to give a PCR product was determined using the method of Taswell (10), which finds the Poisson distribution that best fits the data and provides an estimate (χ^2 test) of how well the data conform to that Poisson distribution.

Table 1. Sensitivity of Detection (Copies) of *N-ras* or IgH in the Presence or Absence of Competing Template

Number of Copies Added*		Number of Positives in 10 Tubes		
IgH	*N-ras*	*N-ras*	IgH (-PBL1)	IgH (+PBL1)
37.50	75.00	10	10	10
11.25	22.5	10	10	10
3.75	7.5	9	6	3
1.125	2.25	6	3	1
0.375	0.75	4	0	1
0.113	0.225	-	1	0
Mean No. copies detected		2.6	3.3	6.1

*Estimated from DNA concentrations

RESULTS

Preliminary Experiments

Although one or a few *N-ras* or IgH targets could be detected by an optimized single-stage PCR, a mixing experiment showed that the addition of 1 µg of PBL1 DNA to provide excess competing non-leukemic templates decreased detection of Ho templates to 18% of that otherwise obtainable. A two-round PCR strategy using nested primers was therefore developed to improve sensitivity and specificity of amplification.

Second-Round PCR — Number of Cycles Required

Serial dilutions were made to produce aliquots that contained varying numbers of Ho IgH targets in 1 µg of PBL1 DNA. Each aliquot was amplified for 45 cycles in the first round, and 10^{-4} to 10^{-9} dilutions of the product were made in water to produce a dilution containing one or a few copies of amplified DNA as starting templates for a second round of 20–50 cycles. For IgH targets, amplified product could be detected from the 10^{-4} dilution after 20 cycles, from the 10^{-5} dilution after 30 cycles and from the 10^{-6} and 10^{-7} dilutions after 40 cycles. Amplified product could not be detected for the 10^{-8} and 10^{-9} dilutions. These results indicated that approximately 10^7 IgH targets for the second round were being produced by the first-round PCR and suggested that 45 cycles should be adequate for second-round amplification.

First-Round PCR — Number of Cycles Required and Effect of Competing Templates

An initial experiment was performed using varying numbers of Ho IgH targets in 1 µg PBL1 DNA, 20–50 first-round PCR cycles, a 1:1000 dilution between the first and second round PCR and 45 second-round PCR cycles. In this experiment, 30 first-round cycles were sufficient to enable an average of 1.5 IgH targets to be detected.

The quantitative aspects of the first round were then studied in more detail in order to determine both the optimal number of cycles and also the appropriate dilution to use between the first and second rounds of the PCR. Serial 10-fold dilutions of Ho DNA were made in water or in 1 µg of PBL1 DNA. Between 20 and 50 cycles of first-round PCR were performed, each aliquot of amplified material was diluted 10^{-2} to 10^{-12} in water and second-round PCR of 45 cycles was performed on each dilution.

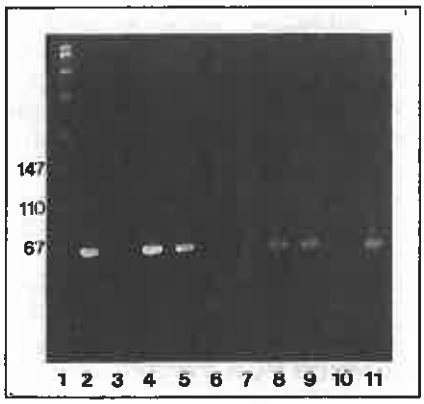

Figure 2. An ethidium bromide stained polyacrylamide gel of PCR products. Lane 1, molecular weight DNA markers; lanes 2–11, amplified products from 10 replicate dilutions of Ho DNA, each containing 3.75 IgH targets.

The results are shown in Figure 1. Because the 45-cycle, second-round PCR gives detectable amplified DNA from one or a few targets, the maximum dilution of the first-round material that still leads to amplification in the second round indicates the approximate number of copies produced in the first-round amplification. A dilution of Ho DNA containing an average starting target number of 1.5 (IgH) and 3 (*N-ras*) genomes, in the first-round PCR, resulted in a plateau of 10^9 copies after 40 cycles of amplification. However, when other IgH targets were present, having been provided by dilution of starting material in PBL1 DNA, amplification of Ho DNA was less efficient and only 10^6 Ho IgH were produced after 50 cycles of amplification.

Based on these results, we decided to use 45 cycles for first-round amplification and a 10^{-3} dilution of amplified material between the first and second round. These conditions would be expected to produce approximately 1000 targets as starting material for the second round.

Quantitation by Limiting Dilution Analysis and Poisson Statistics

Serial dilutions of Ho DNA each involving 10 replicates were analyzed by the optimized, two-round PCR (45 cycles, 10^{-3} dilution, 45 cycles). Each tube was scored as positive or negative for amplification (Figure 2). The detailed results of one such experiment

Table 2. Sensitivity of Detection (Copies) in Diagnostic ALL Patient Samples Using Two-Round PCR

	Minimum Mean No. of Copies Detected			Ratio *N-ras*/IgH	
Patient	*N-ras*	IgH (-PBL1)	IgH (+PBL1)	-PBL1	+PBL1
Ho[a,c]	2.1	3.3	6.0	0.64	0.35
1[b]	1.2	9.7	3.8	0.12	0.32
2	1.2	1.3	2.1	0.92	0.57
3	1.6	0.7	0.9	2.28	1.78
4	1.3	1.5	3.5	0.87	0.37
5	86	211	614	0.41	0.14
6	541	550	621	0.98	0.87
7	23	3	22	7.67	1.05
Geometric mean of ratios				0.90	0.52

[a]10 replicates of dilutions studied.

[b]5 replicates of dilutions studied for patients 1–7.

[c]Geometric mean of all experiments in Ho are shown.

are shown in Table 1. In two experiments, 1.7 and 2.6 copies of *N-ras* could be detected, and in three experiments, 3.5, 6.1 and 10 copies of Ho IgH could be detected in the presence of 1 μg of PBL1 DNA. The data in each experiment were consistent with a Poisson distribution (χ^2 test, $p > 0.05$ for each).

We studied sensitivity of detection in 7 additional diagnostic ALL patient samples. The results of all 8 samples are summarized in Table 2, and the integrity of these DNA samples was investigated by electrophoresis on a 1.3% agarose gel (Figure 3). In this table, the results in the columns referring to minimum mean number of copies detected are calculated from the total number of copies present as based on the estimated DNA concentration. High sensitivity for detection of *N-ras* and IgH genes was observed in 5 patients and lesser sensitivity in 3, patients 5, 6 and 7. Because the number of *N-ras* copies detected depends on both the total number of copies which are present and the proportion which are amplifiable, the ratios of the number of *N-ras* targets detected to the number of IgH targets detected give the proportion of potentially amplifiable IgH targets that were actually amplified. In the absence of competing nonleukemic IgH targets, it proved possible to amplify a mean of 90% of the leukemic targets, and in the presence of competing targets, it proved possible to amplify a mean of 52%, i.e., to detect approximately two ($^1/_{0.52}$) potentially amplifiable leukemic targets.

Electrophoresis confirmed extensive DNA degradation in samples from patients 5 and 6 but not in patient 7, in whom the results, although somewhat variable, suggested a lesser degree of degradation. The DNA seen in Figure 3 for patient 7, although faint, appears to be largely intact.

Factors Affecting Amplification Efficiency

Competing IgH targets. The effect of these in reducing amplification of the specific target has already been illustrated (Figure 1 and Table 1).

Competition between primer pairs. Several methods for quantitation of PCR targets rely on the use of two primer pairs in the same tube (3,4,11). To see whether this interferes with the efficiency of amplification and whether *N-ras* and IgH targets could be amplified in the same tube, we quantitated low numbers of IgH targets in a two-round PCR by amplifying using IgH primers either alone or together with *N-ras* primers and in the absence or presence of PBL1 DNA. In the absence of PBL1 DNA, co-amplification with *N-ras* primers had no effect on the minimum mean number of IgH targets detectable (3.2 targets). However, in the presence of PBL1 DNA, IgH primers alone were able to detect mean target numbers of 3.5 in one experiment and 10 in a second, whereas with co-amplification with *N-ras* primers, the IgH primers could only detect mean target numbers of 18 or 79. This effect of *N-ras* primers presumably resulted from the concurrent amplification of the 10^5-fold excess of *N-ras* targets provided by the PBL1 DNA.

DISCUSSION

We have presented a general method for quantitation of targets by PCR using the principle of limiting dilution and use of Poisson statistics. For this approach, the PCR needs to be optimized so that amplification will take place in an "all-or-none" fashion, and one or a few starting targets will give a positive result. When the optimal conditions are known, target concentration can be estimated by Poisson statistics applied to the results from replicate tubes taken at the limit of dilution.

Limiting dilution analysis is widely used for quantitation in cell biology but is not commonly used for molecular quantitation. In 1990 Simmonds et al. (9) reported its use to detect and quantitate single HIV molecules. We were unaware of their report when we developed the method presented herein, and review of the PCR literature suggests that the general approach is either not known or is misunderstood. Several other workers have performed serial dilutions in their PCR studies and have used the term "limiting dilution." However, replicates have not been performed, the results have not been analyzed by Poisson statistics and, importantly, the PCR has not been verifiably all-or-none because the reaction has been stopped after an arbitrary number of cycles, and there has been no knowledge of the number of targets that leads to either a positive or a negative reaction. Single serial dilution with use of an arbitrary end point does not constitute a proper limiting dilution analysis and gives only semi-quantitative results.

Some areas of research require a method to quantitate a small number of

targets against a background of highly homologous targets, e.g., detection of specific mutations in a population of normal cells. The biological problem in our study was the detection of rare leukemic cells in a large population of normal cells, which in molecular terms became the problem of detection of a rare unique IgH sequence against a background of numerous other IgH sequences. The two-stage PCR system that was developed proved capable of detecting approximately two ($^1/_{0.52}$) potentially amplifiable leukemic IgH sequences against a background of approximately 160 000 total genomes. These genomes would provide a vast excess of sequences that would compete with the leukemic IgH sequences for the PCR primers because they would contain approximately 2.4×10^4 rearranged IgH genes from normal B lymphocytes and 3×10^5 germ-line IgH genes, each containing multiple V and J segments.

Quantitation of PCR targets by limiting dilution can be compared with other methods for quantitation which use an added internal or external standard, which is carried through the amplification, and which involve some form of quantitation of the amplified product(s) (3,4,11). Quantitation by limiting dilution does not require the use of an added standard, with the inherent assumptions involved, and the end point is simple, nonquantitative and nonradioactive. Furthermore, the end point is based on an all-or-none signal derived from the terminal plateau phase of the PCR, and the technique is therefore relatively robust, being able to cope with wide variations in amplification efficiency without affecting the estimation of DNA target number. One potential disadvantage is the possibility of contamination, particularly if a two-stage PCR is performed. We use the precautions recommended by Kwok et al. (6) to minimize the risk of contamination. Replicate negative controls are used but also, in effect, dilutions below the critical limit of dilution act as additional negative controls.

When molecules are quantitated by PCR, it is necessary to express the results in terms of a denominator such as mass of DNA studied or number of cells from which DNA was extracted. The assumption is that the extraction process does not modify the DNA. However, chemical modifications to DNA, such as strand breakage, depurination or formation of adducts, may render the DNA incapable of acting as a template for the PCR. Quantitation by limiting dilution may have a unique advantage in overcoming the potential problem of DNA modification because it is possible to also quantitate an endogenous gene yielding a PCR product of similar size which is present in known number in all cells and which also undergoes the extraction procedure. In the present study the *N-ras* gene was selected as the endogenous gene to correct for degradation. The 8 patients studied fell into 2 groups (Table 2). In 5 patients, all or nearly all of the *N-ras* targets could be amplified, whereas in 3 patients a lesser proportion of targets could be amplified. This suggested the presence of a variable degree of DNA degradation in these 3 patients, and this was confirmed in the 2 most obvious cases by electrophoresis (Figure 3). Nevertheless, as seen in Table 2, in all 8 patients there was an approximately constant ratio between the number of amplifiable IgH targets and the number of amplifiable *N-ras* targets. These data suggest that the number of amplifiable *N-ras* genes, rather than the DNA concentration, is the best indicator of the number of amplifiable genomes present, that virtually all potentially amplifiable leukemic IgH genes are amplified in the absence of competing non-leukemic IgH genes and that approximately half of the leukemic IgH genes are amplified in the presence of competing genes. Quantitation in the presence of DNA degradation can thus be performed based on these data. Degradation can be a significant problem especially where fresh samples are unavailable and to our knowledge this is the first reported PCR approach that enables correction to be made for it.

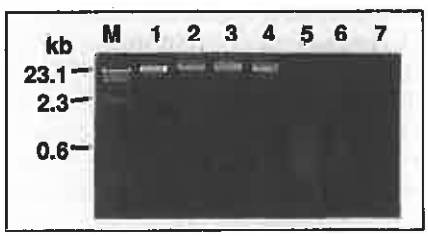

Figure 3. Extracted DNA from diagnostic ALL patient samples. Approximately 250 ng of DNA were loaded onto the gel except for patient 7 where only 24 ng were available. M - molecular weight markers with the sizes given in kb. Lanes 1–7, patients 1–7.

Limiting dilution quantitation is simple, requires few manipulations of samples and has widespread application. Our usual approach is to perform a preliminary serial dilution experiment to determine the approximate point at which some amplifications are likely to be negative and some positive and then perform a detailed experiment, perhaps 40 tubes in all, involving multiple replicates of dilutions around this point. If quantitation is to be performed in terms of the number of amplifiable targets of another gene such as *N-ras*, it should be noted that separate amplification reactions must be performed for each gene and that the variance of the final value will be contributed to additively by the variance of the estimation for each gene. More replicates will therefore be required for a given level of precision.

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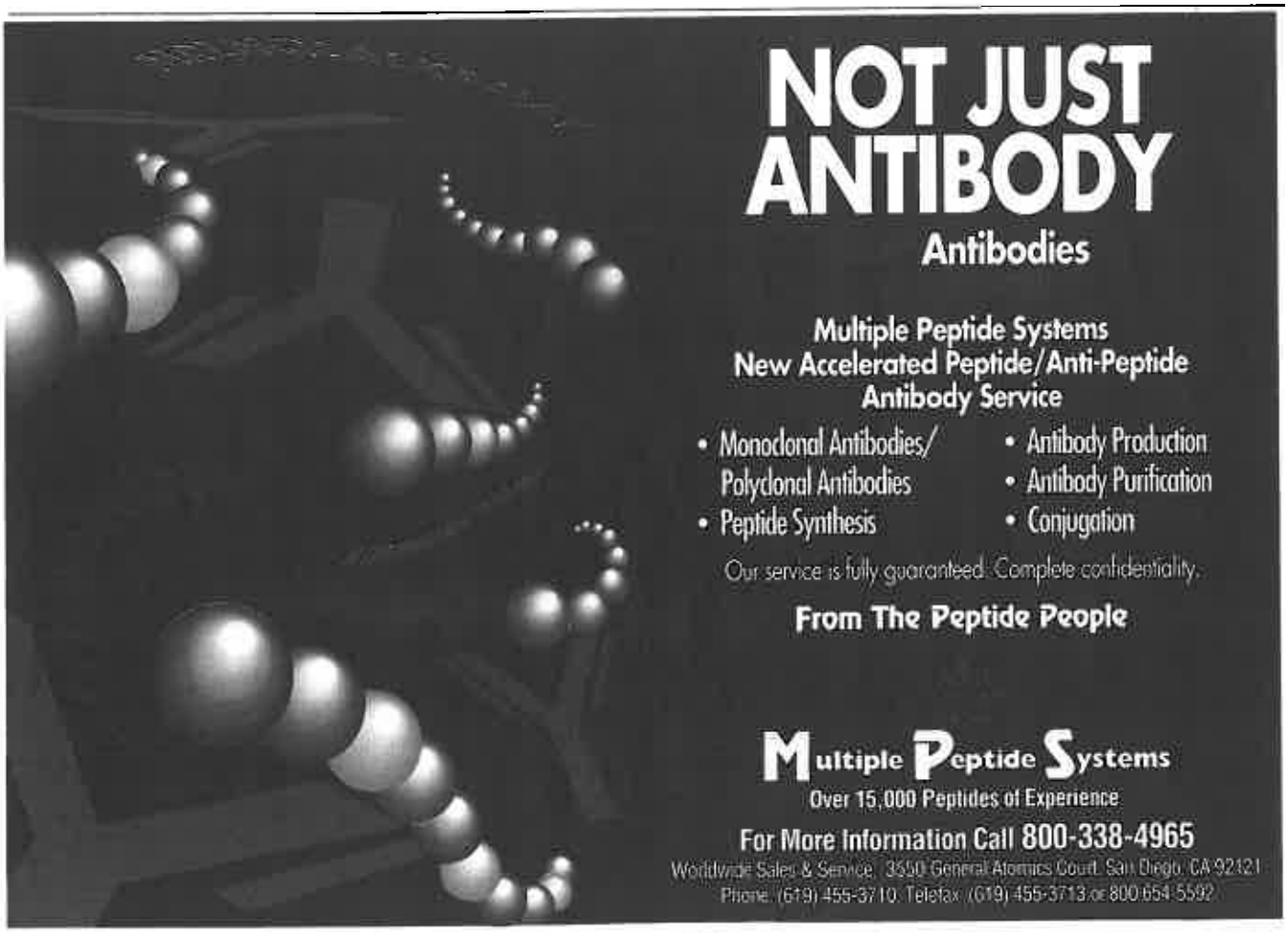

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