The DNAQuant™ DNA Quantitation System for Sensitive Detection of dsDNA

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The accurate determination of DNA concentration in a sample is important for many procedures in molecular biology. However, the methods used for this determination are often not sensitive nor specific for double-stranded DNA (dsDNA), and they can be greatly affected by contaminants in the sample. In this report, we present a new DNA measurement system that is specific for the detection of linear, dsDNA and is not affected by many of the materials that affect other systems. The DNAQuant™ DNA Quantitation System[(a)] is able to detect and quantitate picogram levels of DNA, making it more sensitive than spectrophotometric or gel analysis methods. DNA concentration is determined using three coupled enzymatic reactions to generate a light signal directly proportional to the amount of DNA present (Figure 1). We present the precision, accuracy and linearity of the system, and we describe the use of the system for the measurement of dsDNA, including plasmid and genomic DNA.

Introduction

Many techniques in molecular biology depend upon the accurate measurement of the amount of DNA in a solution. For example, successful experiments involving the transfection of DNA into mammalian cells are often dependent upon knowing the ratio between the DNA to be transfected and the lipid that is used to facilitate the transfection. If the amount of DNA is not known, comparisons cannot be made between results obtained in different experiments designed to optimize the transfection process.

Many of the methods used to determine the concentration of DNA in a solution are subject to interference by materials commonly encountered in biological samples or do not have the required sensitivity. For example, the absorbance of a solution at 260nm is often used to measure DNA in solution. However, the presence of significant levels of RNA, free nucleotides or protein in the solution will also contribute to the absorbance at 260nm, resulting in an overestimate of the DNA concentration. Gel electrophoresis followed by staining of the DNA with fluorescent chemicals such as ethidium bromide is also used to estimate the amount of DNA in a sample. This technique can be accurate and sensitive but suffers if the DNA is dilute and separated into multiple bands on the gel. This can happen because the DNA is composed of fragments of different sizes or multiple forms, such as a plasmid species that is present in relaxed circular, supercoiled and linear forms.

The difficulty encountered in accurately determining the level of DNA in a sample increases when only a small amount of sample is available for analysis. In some cases, there is insufficient sample to use the techniques described above, or doing so requires that the entire sample be used simply to determine the amount of DNA present.

In this report, a new system for the determination of DNA concentration in a sample is described that is sensitive and relatively immune to possible effects of many of the chemicals commonly used in molecular biology techniques. It will detect picogram amounts of dsDNA.

The DNAQuant™ DNA Quantitation System

The basis for the measurement of DNA using the DNAQuant™ DNA Quantitation System includes 1) the use of a series of enzymes to produce an amount of ATP dependent upon the amount of DNA present, followed by 2) the generation of a light signal dependent upon the concentration of ATP using the Luciferase/Luciferin Reagent reaction (Figure 1).

The first two coupled reactions are pyrophosphorylation and transphosphorylation (Figure 1). The pyrophosphorylation reaction is the reverse of the DNA polymerization reaction and is catalyzed by T4 DNA Polymerase. Conditions are used to allow the T4 DNA Polymerase to reverse the polymerization reaction, adding pyrophosphate to the 3′-terminal DNA bases to release deoxynucleoside triphosphates (dNTPs). The transphosphorylation reaction is catalyzed by the enzyme Nucleoside Diphosphate Kinase (NDPK). In this reaction, the terminal phosphate from the dNTPs is transferred to ADP to form ATP.

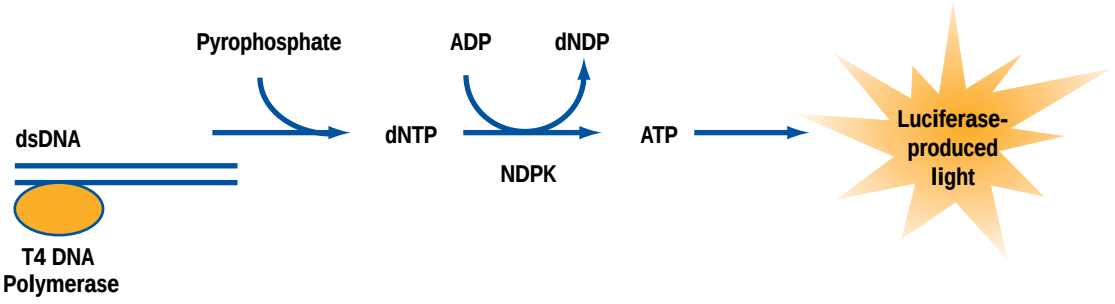

Figure 1. Detection of double-stranded DNA using the DNAQuant™ DNA Quantitation System.

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Table 1. Limits of Detection and Quantitation of the DNAQuant™ DNA Quantitation Assay.

Mass of DNA (pg)	Net Light Units	SD	Average SD	3X Average SD	6X Average SD	Detectable*	Quantifiable*
40	37.2	4.09	2.17	6.5	13.0	Yes	Yes
30	24.6	0.44	0.34	1.0	2.1	Yes	Yes
20	14.4	1.26	0.75	2.3	4.5	Yes	Yes
15	14.6	4.30	2.27	6.8	13.6	Yes	Yes
10	7.3	0.33	0.29	0.9	1.7	Yes	Yes
5	3.2	0.14	0.19	0.6	1.2	Yes	Yes
2	1.1	0.30	0.27	0.8	1.6	Yes	No
0	0	0.25					

*We define Detectable and Quantifiable as follows: If the difference between the light units for the sample and the negative control (Net Light Units) is greater than three times the average of their standard deviations, the sample is said to be detectable. If the difference is greater than six times the average of the standard deviations, the sample is considered to be quantifiable.

The amount of ATP produced by these reactions is measured using a highly sensitive and quantitative Luciferase/Luciferin Reagent reaction system, which produces a light signal within a wide dynamic range proportional in intensity to the amount of ATP present in the solution.

Since this series of reactions is dependent upon T4 DNA Polymerase recognizing DNA as a substrate for the pyrophosphorylation reaction, linear double-stranded DNA is required. The system does not produce signals from closed, circular DNA unless it is first linearized. Single-stranded DNA also does not generate a signal unless it forms a double-stranded segment of DNA, such as a dimer or hairpin structure. RNA is also not a substrate.

Linearity and Sensitivity of the DNAQuant™ System

The DNAQuant™ DNA Quantitation System is more sensitive than spectrophotometry or gel analysis and is able to detect picogram amounts of DNA (Figure 2). Reactions containing the indicated mass of DNA were performed in triplicate. The light units for the replicates were averaged, and the data was analyzed by least mean squares analysis. As shown in Figure 2, Panel B, the data shows good agreement with the best-fit line (coefficient of determination, $R^2 > 0.98$).

We recommend that the assay be used in the linear range of 20–1,000pg of total DNA added per reaction (Figure 2, Panel B). This means that samples should contain between 10–500pg/µl DNA, and 2µl is then added to each 20µl reaction. More concentrated samples need to be diluted before addition to the reactions (Figure 2, Panel C).

The sensitivity of the system is shown in Table 1, which contains the results obtained with the most dilute DNA samples from Figure 2, Panel A. The sensitivity limit for quantitation in this example was 5pg of DNA. Table 1 indicates the typical limits of detection and quantitation for this assay.

Measurement of DNA using the DNAQuant™ System

To test the accuracy of the system, various DNA marker fragments at concentrations between 20–50µg/ml were diluted 1:100 for quantitation by the DNAQuant™ System. They were also quantitated spectrophotometrically, and then the two values for concentration were compared (Table 2).

The DNAQuant™ System is specific for linear DNA. However circular plasmid DNA can be measured after linearization with restriction enzymes (Table 3). A sample of plasmid DNA, pGEM®-3Zf(–) Vector[b] (Cat.# P2261), was diluted and digested using several restriction enzymes. After digestion, the DNA was quantitated using the DNAQuant™ System assay. An absorbance reading was also made on the original

Table 2. Accurate Concentration Determinations Using the DNAQuant™ DNA Quantitation System.

DNA	Ratio of Pyro:A_{260}*	Size Range (bp)	Cat.#
25bp DNA Step Ladder	1.05	25–300 + 1,800	G4511
50bp DNA Step Ladder	1.12	50–800	G4521
100bp DNA Step Ladder	0.98	100–4,000	G6951
200bp DNA Step Ladder	1.09	200–6,600	G6961
1kb DNA Ladder	0.94	250–10,000	G5711
1kb DNA Step Ladder	0.92	1,000–10,000	G6941
ϕX174 DNA *Hin*f I Markers	1.03	24–726	G1751
ϕX174 DNA *Hae* III Markers	1.14	72–1,375	G1761
pGEM® DNA Markers[b]	0.96	36–2,645	G1741

*Ratio of the concentration determined by the DNAQuant™ System assay to the concentration according to the spectrophotometric method. ϕX174 *Hin*f I DNA of known concentration was used to create the standard curve for the pyrophosphorylation assay. For the spectrophotometric method, a conversion factor of 1 A_{260} = 50µg/ml was used.

Table 3. Accurate Measurement of Plasmid DNA Using the DNAQuant™ DNA Quantitation System.

Restriction Enzyme	Concentration Ratio (DNAQuant™:A_{260})	Type of End	Number of Fragments
*Bam*H I	1.03	5′ overhang	1
Xba I	0.83	5′ overhang	1
*Sau*3A I	1.03	5′ overhang	15
Sac I	1.04	3′ overhang	1
Sph I	0.84	3′ overhang	1
Pvu I	0.88	3′ overhang	2
*Hin*c II	0.99	Blunt	1
Sma I	1.12	Blunt	1
None	0.26	None	0
None	0.15	None	0

pGEM®-3Zf(–) Vector (Cat.# P2261) was suspended in TE buffer to approximately 25–50µg/ml. The actual concentration was determined experimentally using both the DNAQuant™ System and spectrophotometry. For the DNAQuant™ System, 1µl DNA samples were digested for one hour at 37°C using various restriction enzymes. Two microliters of each reaction were then used directly in the DNAQuant™ System assay, performed in triplicate. ϕX174/*Hin*f I Marker DNA (Cat.# G1751) at a spectrophotometrically (260nm) determined concentration was used to create the standard curve. The concentration of the pGEM®-3Zf(–) Vector initial sample was determined to be 36µg/ml spectrophotometrically using a conversion factor of 1 A_{260} unit = 50µg/ml. The ratios of the concentrations determined by the DNAQuant™ System to 36µg/ml are shown.

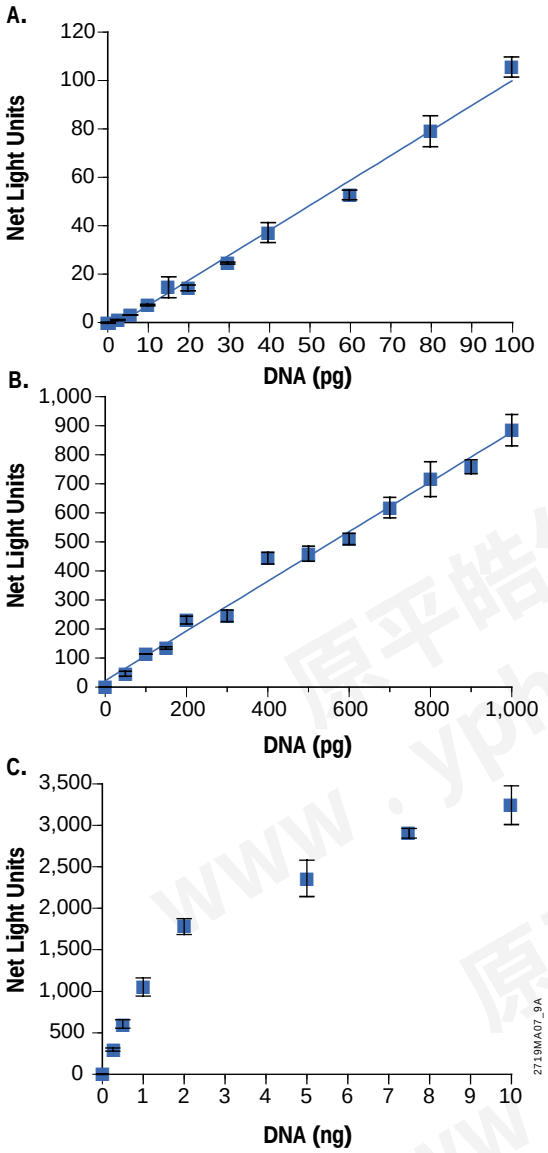

Figure 2. Linearity and sensitivity of the DNAQuant™ DNA Quantitation System. ϕX174 DNA/*Hin*f I Markers (Cat.# G1751) at 1mg/ml (A_{260}) were diluted in 10mM Tris-HCl (pH 7.3) to various concentrations within the linear range of the assay (**Panels A** and **B**). The linear range is 20–1,000pg of total DNA. Two microliters of each dilution were added to the pyrophosphorylation reaction. The total amount of DNA added per reaction is indicated on the X-axis. Light signals were measured using a Turner Luminometer (Promega Cat.# E2041; Turner Designs, TD-20/20, 52% sensitivity setting). Each sample was assayed in triplicate, and error bars represent ±1 SD. The best-fit line was generated by linear regression analysis. See Table 1 for the data in the 0–40pg range of DNA. **Panel C:** Response of the DNAQuant™ DNA Quantitation System in the nonlinear range. ϕX174 DNA/*Hin*f I Markers were diluted as above except to concentrations greater than the linear range of the assay. Two microliters of each dilution were added to the pyrophosphorylation reaction. The total amount of DNA added is indicated on the X-axis. Each sample was assayed in triplicate, and error bars represent ±1 SD.

concentrated sample. Table 3 shows the ratio of these two readings for the pGEM®-3Zf(–) Vector. Undigested DNA gave a low reading presumably due to the fraction of nicked DNA, which was visible on an ethidium bromide-stained gel.

Independence of End Type: The various restriction enzymes used in the above experiment left blunt ends, 5′ overhangs or 3′ overhangs. All were pyrophosphorylated by T4 DNA Polymerase to the same extent (Table 3) and gave equivalent responses. While the polymerase is expected to use DNA segments with either blunt ends or 5′ overhangs as substrates, the 3′→5′ exonuclease activity of the polymerase allows the system to also measure DNA segments with 3′ overhangs, probably after such overhangs are removed by the exonuclease activity of the enzyme.

Size Dependency and Quantitation of Chromosomal DNA: Also note that some enzymes used in Table 3 produce one fragment while others produce multiple smaller fragments. However, the signals were similar for all digests. This suggests that the pyrophosphorylation reaction is dependent upon the mass of DNA present and not upon size of DNA fragments for these digestions. The ability of the system to use DNA fragments of various sizes was examined by digestion of plasmids and by testing DNA fragments of known sizes after separation by electrophoresis on LMP agarose (data not shown). The DNAQuant™ System assay is mass-dependent (size-independent) up to 6,000 bases (Figure 3). This is the largest size specifically tested. The signal was similar whether the plasmid was digested to form one large or three small fragments.

However, when larger DNA (e.g., chromosomal DNA) is used, the light output does increase upon digestion (Figure 4). This suggests that at some point, a sample of larger DNA fragments will not produce the same signal as an equivalent mass of smaller fragments. This indicates that larger fragments must be restricted into smaller pieces for mass-dependent detection and quantitation.

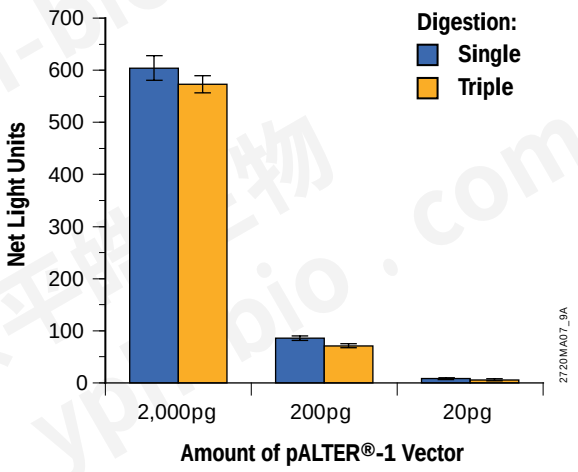

Figure 3. Detection of DNA is size-independent and mass-dependent. pALTER®-1 Vector[b] (Cat.# Q6301), 5,680bp, was used as the starting DNA. A single enzyme digestion was performed with *Eco*R I, which has one cleavage site in the vector; triple digestion was with *Eco*R I, *Sty* I and *Sca* I to yield fragments of 1,386, 1,819 and 2,475bp. Complete digestion was confirmed by gel electrophoresis. DNA was diluted and assayed in triplicate using the DNAQuant™ System assay. Error bars are ±1 SD.

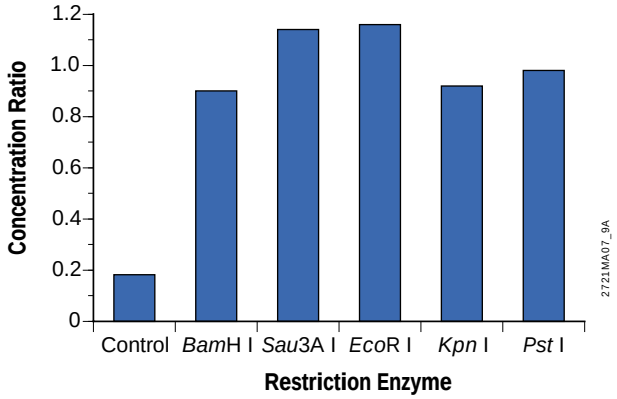

Figure 4. Accurate measurement of chromosomal DNA. Human Genomic DNA (Cat.# G3041) was resuspended to approximately 50µg/ml in TE buffer. The concentration of the DNA was determined experimentally using the DNAQuant™ System by first digesting 1µl of DNA for one hour at 37°C using various enzymes. The DNA was then assayed in triplicate using the pyrophosphorylation assay. The standard curve was generated using ϕX174/*Hin*f I Markers (Cat.# G1751) of known concentration. The concentration was also determined to be 51µg/ml by measuring the absorbance of the sample at 260nm. The ratio of the two concentrations (DNAQuant™ System:A_{260}) was calculated and plotted versus restriction enzyme used in the initial digest.

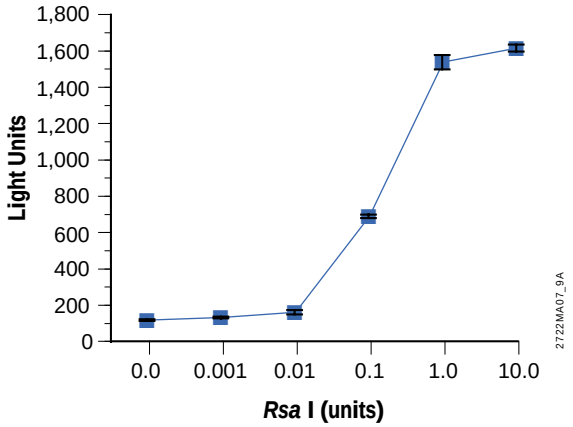

Figure 5. Detection of endonuclease activity. In this experiment the restriction enzyme *Rsa* I (Cat.# R6371) was used to linearize a circular (plasmid) DNA. *Rsa* I at 10 units/µl was serially diluted, and 1µl was added to a 20µl digest containing approximately 20ng of pGEM®-3Zf(+) Vector[b] (Cat.# P2271). After a one-hour incubation at 37°C, the DNA was assayed in triplicate using the DNAQuant™ System. The signal increased with increasing concentration of restriction enzyme, indicating the generation of substrate. The system detected fewer than 0.01 units of *Rsa* I, with a limit of detection of 0.001 units of *Rsa* I, at a final concentration of 5×10^{-5} units/µl in the 20µl digestion. Error bars are ±1 SD.

The effect of RNA and single-stranded DNA (ssDNA) on the signals produced from dsDNA species was examined by quantitating dsDNA in the presence and absence of RNA or ssDNA. Also, RNA and ssDNA were tested alone. Results show that nanogram levels of either RNA or ssDNA do not produce a signal by themselves or interfere with the measurement of 500pg of dsDNA (data not shown; please see Technically Speaking that begins on page 28 of this issue of *Promega Notes*).

Use of the System to Measure Nucleases

The system can be used to measure small amounts of dsDNA. However, due to the unique substrate properties of the system, it can also be used to detect a number of enzymatic activities that modify nucleic acids, such as endonucleases and exonucleases.

Measurement of endonuclease activity is easily accomplished using closed circular plasmid DNA. Since the system relies on the presence of a DNA segment with an "end," no signal is expected from closed circular DNA species such as plasmids unless they are nicked. However, if the DNA is linearized through the use of an endonuclease, the resulting DNA becomes a substrate for the T4 DNA Polymerase, and a signal can be measured. Figure 5 shows the response seen from digestion of plasmid DNA with low levels of the endonuclease *Rsa* I. As expected, the signal increases as the amount of enzyme increases due to generation of appropriate substrate. Low (0.001–0.01 units) levels of endonuclease can be measured in this manner.

As an example of how the system can be used to measure exonuclease activity, samples of Exonuclease III at various concentrations from 0.0175–175 units were incubated with a linear, 500bp DNA segment. The exonuclease reduces the amount of dsDNA and, because the DNAQuant™ System only measures dsDNA, this results in a loss of substrate and signal. As shown in Figure 6, the system allows low levels of exonuclease to be detected. Enzyme at 8.75×10^{-4} units/µl gives a discernable signal in this assay.

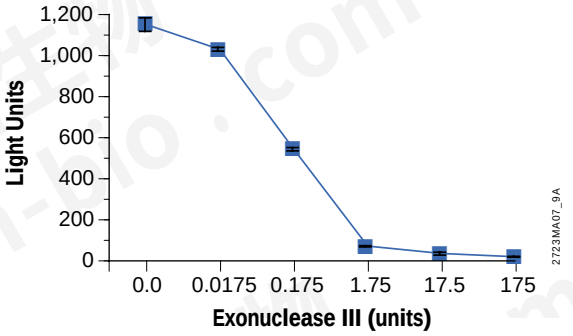

Figure 6. Detection of exonuclease activity. Exonuclease III (Cat.# M1811; 175units/µl) was diluted, and 1µl was added to a 20µl digestion containing approximately 20ng of a 500bp PCR fragment. After a one-hour incubation at 37°C, the DNA was assayed in triplicate using the DNAQuant™ System. A loss of signal was observed with increasing enzyme due to the destruction of DNA substrate. Less than 0.175 units of Exonuclease III was detectable, with a limit of detection of 0.0175 units of Exonuclease III, at a final concentration of 8.75×10^{-4} units/µl in the 20µl digestion. Error bars are ±1 SD.

Summary

The DNAQuant™ DNA Quantitation System is a new system for the detection and quantitation of linear dsDNA. The measurement relies on a series of enzymatic reactions to convert the bases of the DNA into a proportional amount of ATP. The first of these is the pyrophosphorylation reaction, which is the reversal of polymerization. The ATP is measured using the light-emitting luciferase reaction. Though linear DNA is the required substrate, circular plasmid DNA can be linearized and quantitated. Larger fragments such as genomic DNA can also be quantitated, though restriction is required to reduce the fragment size. This system can detect and quantitate picogram levels of DNA, and it is specific for dsDNA, making it more sensitive and selective than other methods commonly used to measure DNA.

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Ordering Information

Product	Size	Cat.#	Price ($)
DNAQuant™ DNA Quantitation System	100 reactions	K4000	148

Related Products

Product	Size	Cat.#	Price ($)
Wizard® PCR Preps DNA Purification System[c]	50 preps	A7170	70
	250 preps	A2180	285
Vac-Man® Laboratory Vacuum Manifold	20 ports	A7231	99
Vac-Man® Jr. Laboratory Vacuum Manifold	2 ports	A7660	12
ϕX174 DNA/*Hin*f I Markers	50µg	G1751	95
Agar*ACE*® Enzyme[d] (0.15–0.30u/µl)	25u	M1741	50
	500u	M1743	500
ENLITEN® Luciferase/Luciferin Reagent	100 assays	FF2021	95
Turner Designs Luminometer Model TD-20/20 Genetic Reporter Instrumentation Package for Stabilized Assays		E2041	4,803

ART® Aerosol Resistant Tips

Product	Size	Cat.#	Price ($)
ART® 20P, Pipet Tip, 20µl	960/pk	DY1071	115
ART® 200, Pipet Tip, 200µl	960/pk	DY1121	115
ART® GEL, Gel Loading Pipet Tip, 100µl	1,000/pk	DY1081	115

[a]Patent Pending.

[b]U.S. Pat. No. 4,766,072.

[c]Licensed under U.S. Pat. No. 5,075,430.

[d]U.S. Pat. No. 5,869,310 and other patents.