



(51) International Patent Classification:

C12Q 1/68 (2006.01) *G01N 27/72* (2006.01)
C12P 19/30 (2006.01) *G01N 33/553* (2006.01)
G01N 24/00 (2006.01) *A61B 5/055* (2006.01)

(21) International Application Number:

PCT/US2012/036505

(22) International Filing Date:

4 May 2012 (04.05.2012)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/483,192	6 May 2011 (06.05.2011)	US
61/495,769	10 June 2011 (10.06.2011)	US
61/496,488	13 June 2011 (13.06.2011)	US

(71) Applicant (for all designated States except US): **THE GENERAL HOSPITAL CORPORATION** [US/US]; 55 Fruit Street, Boston, Massachusetts 02114 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **JOSEPHSON, Lee** [US/US]; 139 Oak Street, Reading, Massachusetts 01867 (US). **ALCANTARA, David** [ES/ES]; Guatemala, 1, 1C, Alcala de Guadaira, E-41500 Sevilla (ES).

(74) Agents: **KUGLER DEYOUNG, Janice** et al.; Fish & Richardson P.C., P.O. Box 1022, Minneapolis, Minnesota 55440-1022 (US).

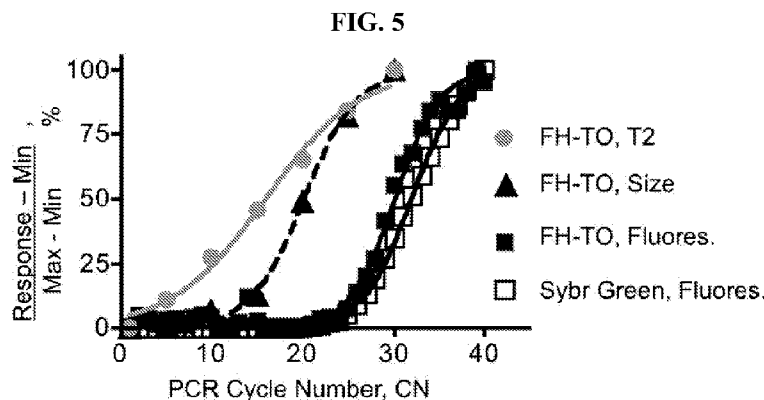
(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

(54) Title: NANOCOMPOSITIONS FOR MONITORING POLYMERASE CHAIN REACTION (PCR)



(57) Abstract: Described are methods for monitoring PCR reactions using surface functionalized magnetic particles (SFMPs), e.g., surface functionalized magnetic nanoparticles, in a PCR reaction mix. In some embodiments, the magnetic resonance (MR) T2 proton relaxation times of the PCR reaction mixture are then determined. Additionally, methods of purifying nucleic acids using SFMP's are provided.

NANOCOMPOSITIONS FOR MONITORING POLYMERASE CHAIN REACTION (PCR)

CLAIM OF PRIORITY

This application claims the benefit of U.S. Provisional Patent Application Serial Nos. 61/483,192, filed on May 6, 2011; 61/495,769, filed on June 10, 2011; and 61/496,488, filed on June 13, 2011. The entire contents of the foregoing are hereby
5 incorporated by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with Government support under National Institutes of Health Grants Nos. R01 EB 011996 and R01 EB 009691. The Government has certain rights in this invention.

10

TECHNICAL FIELD

The present disclosure provides methods for monitoring PCR reactions. In addition, methods of separating and purifying nucleic acids are disclosed.

BACKGROUND

Contamination can be a substantial problem in polymerase chain reactions
15 (PCR). Contamination can occur during sample preparation where, for example, DNA from a human sample preparer can contaminate DNA to be amplified. In addition, post-amplification contamination is also an issue, as PCR reaction generates billions of copies of low-molecular weight DNA. If aerosolized, this low molecular weight DNA rapidly contaminates reagents, instruments, and personnel with
20 templates for subsequent PCR amplifications. Post-amplification contamination is a particular problem when PCR assays for a single genetic element that are run repeatedly and frequently in a facility, such as in clinical labs.

In addition to various types of contamination, the PCR amplification process can yield errors and the probability of these errors increases with increasing numbers
25 of PCR cycles. More sensitive methods of detecting PCR reaction products permit the use of fewer PCR cycles and thus reduce the chance of PCR amplification errors.

Problems of contamination occur via aerosols or micro-droplets and often necessitate the design of air handling and sample handling facilities to avoid this contamination. Because of this, PCR testing often requires a multi-room facility designed specifically to minimize false-positive results, specially trained personnel, careful and strict laboratory procedure, and positive and negative controls. Thus, there is a need for PCR methods that both reduce the chances of contamination and which are more sensitive and reduce the chances of amplification errors.

Common methods of preparing DNA include (i) the classical phenol-chloroform extraction, (ii) solid phase, silica based methods, and (iii) solid phase, charge-based methods. Generally speaking, with all of these methods, the samples (e.g. biological fluids, serum, cells) are first exposed to agents, which denature both DNA and proteins and permit the subsequent separation of DNA from proteins. With phenol-chloroform extraction the DNA and proteins are denatured and separated by partition into various solvents. With solid phase based methods the phosphate groups of DNA bind to those solid phases. Solid phase, charge based methods include INVITROGEN™, “charge switch” kits, where negatively charged nucleic acids bind to a positively charged solid phase and released by changing pH. (Such solid phase methods do not use a DNA binding fluorochrome attached to a solid phase magnetic particle, as does the instant invention, to bind and extract DNA from solution.) In general, with these methods, DNA is purified but any components to which it is bound are lost through the initial denaturation and cell lysis step. These DNA binding components include proteins or elements regulating gene expression. Thus, there exists a need for a facile way of extracting and purifying DNA from a solution which would permit analysis of proteins or other regulatory molecules bound to that DNA. The present methods also address these needs.

SUMMARY

The present invention provides, in part, methods for monitoring the polymerase chain reaction (PCR), using a surface functionalized magnetic particle (SFMP) in a PCR reaction mix. The magnetic resonance (MR) proton relaxation times, such as the T2 relaxation time, of the PCR reaction mix can then be determined. The T2 relaxation time changes based on the binding of the SFMP to DNA and thus can be used to monitor amounts of PCR DNA product produced. For

example, a magnetic particle can be surface functionalized with one or both of two general types of materials: (i) an oligonucleotide or (ii) a nucleic acid (DNA) binding fluorochrome (DBF). These functional groups bind the products of the PCR reaction in either a sequence specific manner (oligonucleotide) or sequence independent manner (DNA binding fluorochrome).

In addition, SFMPs, e.g., synthesized using DNA binding fluorochromes, as described herein can be used to separate and purify DNA from a sample, e.g., from a solution or reaction mixture. The SFMPs, e.g., surface functionalized magnetic particles, are contacted with a solution containing nucleic acids and allowed to bind to the nucleic acids. Using a magnetic field, the SFMPs are withdrawn from solution together with the bound nucleic acids. Through the use of heat, and/or dissociating agents, the DNA is then separated from the magnetic particle and recovered. A particular advantage of the invention is that it does not require the use of denaturing agents, and thus, the methods described herein permit the extraction of DNA, and proteins bound to DNA, which includes proteins which regulate gene expression.

Accordingly, in one aspect the present disclosure provides methods for monitoring amplification of DNA in a polymerase chain reaction (PCR) mixture, the method comprising: providing a PCR reaction mixture comprising: DNA template, deoxynucleotide triphosphates (dNTPs), DNA polymerase, and primers; contacting surface functionalized magnetic particles (SFMPs) comprising a DNA-binding functional group, a magnetic particle, and a linker with the PCR reaction mixture; and determining progress of the PCR reaction (e.g., the concentration of amplified DNA) by measuring proton relaxation time (which is related to the concentration of amplified DNA) in the mixture. The mixture is exposed to conditions for amplification (e.g., thermal cycling), and measurements can be made before, during, or after each cycle of amplification.

In another aspect, the present disclosure provides methods for monitoring amplification of DNA in a polymerase chain reaction (PCR) mixture, the method comprising: providing a PCR reaction mixture comprising: DNA template, deoxynucleotide triphosphates (dNTPs), DNA polymerase, and primers; contacting surface functionalized magnetic particles (SFMPs) comprising a DNA-binding moiety, a magnetic particle, and a linker with the PCR reaction mixture; and

measuring a proton relaxation time, thereby monitoring amplification of DNA. The mixture is exposed to conditions for amplification (e.g., thermal cycling), and measurements can be made before, during, or after each cycle of amplification.

5 In some embodiments, the DNA amplification is monitored by measuring a first proton relaxation time; performing at least one cycle of amplification; and measuring a second proton relaxation time, thereby detecting and/or quantifying the amplification of the DNA.

10 In some embodiments, the DNA-binding moiety is a DNA-binding oligonucleotide, e.g., a DNA-binding fluorochrome, e.g., a DNA-binding fluorochrome selected from the group consisting of: ethidium bromide, propidium iodide, TO-PRO 1, TO-PRO 3, YO-PRO, and 4',6-diamidino-2-phenylindole (DAPI), and bisbenzimidazole (Hoescht 33342).

15 In some embodiments, proton relaxation time is measured by determining one or more of T1, T2, T2*, ratios of T1-T2, T2-T2*, T1-T2* or other measurements. In some embodiments, the measuring is performed using a relaxometer.

In some embodiments, the SFMPs comprise magnetic microparticles or magnetic nanoparticles. In some embodiments, the magnetic microparticles or magnetic nanoparticles comprise ferumoxytol (feraheme, AMAG Pharmaceuticals, Inc.) or a superparamagnetic cross-linked iron-oxide (CLIO) particle.

20 In some embodiments, the measuring is performed at intervals over time, and the proton relaxation times are compared, and changes in proton relaxation times are correlated with the increase in concentration of DNA and template amplification in the sample.

25 In some embodiments, the measuring of the proton relaxation time is conducted with the PCR mixture in a closed tube.

In another aspect, the present disclosure provides a kit for use in the methods described herein comprising: deoxynucleotide triphosphates (dNTPs), DNA polymerase, and a surface functionalized magnetic particle as described herein.

30 In some embodiments, the kits include a surface functionalized magnetic particle (SFMP) comprises a DNA-binding moiety, e.g., a DNA binding fluorochrome selected from the group consisting of: ethidium bromide, propidium iodide, TO-PRO

1, TO-PRO 3, YO-PRO, 4',6-diamidino-2-phenylindole (DAPI) and bisbenzimidazole (Hoescht 33342).

In some embodiments, the SFMP comprises a magnetic microparticle or a magnetic nanoparticle.

5 In some embodiments, SFMP comprises feraheme (ferumoxytol) or a superparamagnetic cross-linked iron-oxide (CLIO) particle.

In another aspect, the present disclosure provides a device for monitoring PCR amplification comprising (i) an automated thermal cycler module capable of alternately heating and cooling, and adapted to receive, at least one reaction vessel containing an amplification reaction mixture comprising a target nucleic acid, reagents for nucleic acid amplification, and a SFMP comprising a DNA-binding functional group; and (ii) a detector module operable to detect a proton relaxation signal without opening the at least one reaction vessel, which proton relaxation signal is related to the amount of amplified nucleic acid in the reaction vessel.

15 By virtue of their design, in some embodiments the methods described herein possess certain advantages and benefits. First, the radiofrequency radiation measurement used by MR for the determination of T2 relaxation times is indifferent to light based interferences in the sample. These can include light scattering dust or soluble fluorescent or light absorbing materials. In fact, the penetrating, radiofrequency measurements permits measurement T2 measurements of the PCR reaction mix from within sealed PCR tubes, avoiding the possibility of post-amplification contamination. Second, the methods described herein provide an increased sensitivity of detection of PCR products, and a way of detecting sequences using fewer PCR cycles. This higher sensitivity, and reduction in the number of PCR cycles needed, reduces the chances of amplification errors. Third, remarkably, the SFMPs described herein do not inhibit the various chemical reactions needed by PCR reaction. These include DNA hybridization (or melting) and the polymerase reaction. Fourth, remarkably, the SFMPs are so stable they survive the numerous, extreme temperature changes that occur through multiple PCR cycles.

30 A fifth advantage of some embodiments of the invention is the fact that the reaction between SFMPs and DNA, as measured by T2, does not exhibit a DNA concentration dependent high dose hook effect. The so-called "hook effect" is a

biphasic response to increasing analyte concentrations, where the signal response first increases and then decreases. With the hook effect a given signal response can be the same for two analyte concentrations, and this is a major disadvantage of methods that exhibit the “hook”. The hook effect is a general characteristic of solution phase reactions between multivalent antibodies and multivalent antigens, and also between multivalent SFMPs and its multivalent target DNA. When the SFMPs described herein form aggregates by reacting with target DNA, a hyperbolic function of T2 versus DNA concentration was obtained. Proton relaxation times, e.g., T2 measurements, unlike conventional light scattering measurements of SFMP/DNA aggregate formation, are free of the high dose hook effect.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1 provides an exemplary structure of a DNA-binding fluorochrome.

FIG. 2 depicts an exemplary scheme for chemical synthesis of a Surface Functionalized Magnetic Particle (SFMP), specifically FH-TO.

FIG. 3 depicts an image of an intercalation model of TO-PRO 1 interacting with DNA.

FIGs. 4A-C are line graphs showing FH-TO/DNA interactions by fluorescence (4A), T2 (4B), and size (4C), over a wide DNA concentration range.

FIGs. 4D and E are line graphs showing T2 (4D) and size (4E) over a low DNA concentration range.

FIG. 4F is a schematic illustration of a low affinity monovalent intercalation of TO-PRO 1 into a double stranded DNA.

FIG. 4G is a schematic illustration of a large extended multivalent aggregate due to multivalent interactions between TO-PRO 1 on the FH-TO surface and
5 polymeric DNA.

FIG. 4H is a schematic illustration of a small, wrapped multivalent aggregate due to multivalent interactions with DNA wrapping around the FH-TO.

FIG. 5 is a line graph showing T2, size, and fluorescence measurements as a function of PCR cycle number.

10 FIG. 6 depicts an image of closed PCR tubes in preparation for MR imaging.

FIG. 7 is an image of PCR reaction tubes as a function of PCR cycle number. In the original, the scale on the left ranges from red at the top to violet at the bottom. In the left-most column, FH-TO/T+, the dots are red, yellow/orange, green, blue-green, and blue, in descending order. The dots in the second and third columns are
15 blue in the original.

DETAILED DESCRIPTION

The present disclosure provides methods for monitoring DNA amplification in a PCR mixture using a surface functionalized magnetic particle (SFMP) and magnetic resonance. For example, when a SFMP binds a product of the PCR reaction, there is
20 change in the relaxation properties, e.g., T2 relaxation times, of the PCR reaction solution, enabling detection or quantification of target sequences. Because magnetic resonance (MR) is a radiofrequency based method, the PCR reaction can be monitored from within an enclosed tube, preventing one of the major problems of the PCR method: post-amplification contamination. The methods described herein
25 include the use of a SFMP, which includes a DNA-binding moiety (DBM), e.g., a DNA-binding fluorochrome or an oligonucleotide, a magnetic particle, and a linker that connects the DBM and the magnetic particle.

In addition, the present disclosure provides methods for separating and purifying nucleic acids using SFMPs. The DNA-binding moiety, e.g., a DNA-binding
30 fluorochrome or an oligonucleotide, is allowed to bind to nucleic acids in solution. When a magnetic field is applied to the solution, the SFMPs together with the bound nucleic acids are removed from the solution. The nucleic acids can then be separated

from the SFMPs using heat or dissociating methods. In addition, when DNA reacts with the magnetic-fluorochromes, it is protected from the actions of DNA cleaving DNases.

5 **Structure of the Surface Functionalized Magnetic Particles (SFMP's)**

The SFMPs described herein include a DNA-binding moiety (DBM), e.g., a DNA-binding fluorochrome or an oligonucleotide, which must: 1) bind to DNA, preferably strongly; 2) allow for covalent attachment to the magnetic particle, in which the covalent attachment does not interfere with DNA binding; and 3) not
10 interfere with the PCR reaction. SFMPs can bind DNA by different mechanisms such as base-pairing, intercalation between the bases or in the major groves or minor groves of the double helix. Also SFMPs can be selected for their binding to nucleic acids other than DNA, e.g., RNA.

15 ***DNA-Binding Moieties***

DBMs suitable for use in the present methods must fulfill the following criteria include: 1) the DBM must exhibit strong binding to DNA, whether the molecule is an intercalator or binds either the major or minor groove of DNA; 2) the DBM must have a location on the molecule where a chemical group can be attached
20 that is amenable to attachment to a magnetic particle, e.g., a chemical group that can be attached to a linker as defined herein; 3) and the DBM must not interfere with DNA replication (i.e., must not inhibit the PCR reaction). There are several ways of determining whether a molecule can act as an effective DNA-binding moiety (DBM).

Methods for selecting strong DNA-binding moieties to use with the methods
25 described herein are known in the art and include selecting a compound that is known to bind DNA. This can be achieved, e.g., by selecting a molecule known to bind DNA, e.g., a DNA binding molecule known in the art. For example, a compound can be selected from a public database, e.g., a database that discloses X-ray crystallographic data of molecules that bind DNA.

30 Other ways of identifying strong DNA-binding moieties include: a) testing molecules (e.g., test compounds) for their DNA-binding properties and measuring a change, e.g., a change in an optical property such as fluorescence, or an NMR shift.

Further methods of selecting strong DNA-binding moieties can include linear dichroism, see e.g., Lincoln et al. *J. Am. Chem. Soc.* 118(11): 2644–2653, 1996. See Mutation Research/Genetic Toxicology and Environmental Mutagenesis 444(1): 181–192, 1999, for a method of determining DBMs using Chinese hamster V79 cells by an adaptation of the bleomycin amplification assay.

Other methods of selecting DNA-binding moieties include, but are not limited to, the selection and amplification from self-assembled combinatorial libraries. See, e.g., Klekota et al. *Tetrahedron Letters* 38: 8639–8642, 1997. See Klekota et al. *Tetrahedron* 55: 11687–11697, 1999 for methods of selecting DBMs using oligo d(A·T)-cellulose resin; Boger et al. *J. Am. Chem. Soc.* 123(25): 5878–5891, 2001.

One of skill in the art can readily identify DBMs suitable for use in the compositions and methods described herein, by methods known in the art, e.g., by methods comprising the following steps:

- 1) selecting a candidate strong DNA-binding moiety;
- 2) chemically attaching a linker to the selected DNA-binding moiety through the methods described herein, and methods that are known in the art of organic chemistry;
- 3) attaching the selected DNA-binding moiety to a magnetic particle as described herein to form a surface functionalized magnetic particle (SFMP);
- 4) contacting the SFMP with a PCR mixture;
- 5) optionally determining whether the SFMP has bound to amplified DNA either through fluorescence changes or NMR methods;
- 6) confirming that the SFMP has not inhibited the PCR reaction by comparing the amplification of a target nucleic acid in the presence and absence of the SFMP.

There are several ways molecules can interact with DNA. For example, DNA-binding moieties may interact with DNA by covalently binding, electrostatically binding, or intercalating. See, e.g., Saher Afshan Shaikh and B. Jayaram “DNA Drug Interaction” available on the world wide web at scfbio-iitd.res.in/doc/preddicta.pdf. In addition, the molecule can base-pair with the DNA, e.g., in the case of molecules comprising one or more nucleotides.

Intercalator DNA-binding Moieties

Intercalation occurs when molecules of an appropriate size and chemical nature fit themselves in between base pairs of DNA. These molecules are mostly polycyclic, aromatic, and planar, and therefore often make good nucleic acid stains.

5 For example, DNA-binding moieties that act as intercalators include, but are not limited to, berberine, ethidium bromide, proflavine, daunomycin, doxorubicin, and thalidomide. See also, e.g., *Anticancer Drug Des.* 4(4): 241-63, 1989. See also Antonini et al. *J. Med. Chem.* 40(23): 3749-3755, 1997 for acridine DNA intercalators and Ferguson et al. *Mutation Research/Fundamental and Molecular*
10 *Mechanisms of Mutagenesis* 623(1-2): 14-23, 2007 for other examples of DNA intercalators.

Minor Groove DNA-binding Moieties

DNA-binding moieties can also interact with DNA by binding to the minor groove of DNA. Examples of known minor groove DNA-binding ligands include, but
15 are not limited to, Netropsin, Distamycin, Hoechst 33258, and Pentamidine.

Other examples of a DNA-binding moieties that bind the minor groove of DNA can include distamycin A, which is characterized by the presence of an oligopeptidic pyrrolecarbonyl frame ending with an amidino moiety that reversibly binds to the minor groove of DNA by hydrogen bonds, van der Waals contacts, and
20 electrostatic interactions with a strong preference for adenine-thymine (AT)-rich sequences containing at least four AT base pairs. See, e.g., Baraldi et al. *Pure Appl. Chem.* 75: 187-194, 2003. See also Dervan et al. *Bioorganic & Medicinal Chemistry* 9: 2215-2235, 2001 for examples of other natural products that can bind DNA.

Further examples include hairpin pyrrole-imidazole (Py-Im) polyamides,
25 which are programmable oligomers that bind the DNA minor groove in a sequence-specific manner with affinities comparable to those of natural DNA-binding proteins (Dervan et al. *Tetrahedron* 63(27): 6146-6151, 2007).

Yet further examples of minor groove DNA-binding moieties include those described in Shaikah et al. *Arch Biochem Biophys.* 429(1): 81-99, 2004.

Major Groove DNA-binding Moieties

30 DNA-binding moieties can also interact with DNA by binding to the major groove of DNA. Examples of DNA-binding moieties that bind the major groove can

include, but are not limited to, Neomycin-Hoechst 33258 Conjugate. See, e.g., *J. Am. Chem. Soc.* 125: 12398-12399, 2003.

Additional exemplary DBMs include sequence specific DBMS, such as oligonucleotides, and non-sequence specific DBMs, such as fluorochromes and small molecules.

DNA-Binding Oligonucleotides

The SFMPs can include oligonucleotides as the DNA-binding moiety. Oligonucleotides that can be used with the methods described herein can be complementary (or identical) to a portion of the sequence of the target that is located between the PCR primers, e.g., have sufficient complementarity to form base pairs with the target sequence with a desired level of affinity. The oligonucleotides can be present in an amount that is orders of magnitude lower in concentration than the PCR primers, and/or can be chemically modified to prevent their participation as primers in the PCR reaction. For example, the oligonucleotides can be modified at the 3' end of the oligonucleotide to prevent extension thereof, or can include 1-2 non-complementary nucleotides to prevent annealing and strand synthesis.

DNA-Binding Fluorochromes

The SFMPs can also include DNA-binding fluorochromes (DBFs) that can be used with the methods described herein. The DBFs are positively charged, low molecular weight organic compounds, e.g., less than about 800 Daltons, and typically contain two parts: 1) an unsaturated ring or rings and, 2) an aliphatic arm. As shown in FIG. 1, the DBF TO-PRO 1 possesses an aliphatic arm with two ends, its first end is attached to the unsaturated ring or rings and its second end is attached to a terminal quaternary nitrogen that is positively charged. The positive charge assists in binding to nucleic acids while the unsaturated ring or rings of the DNA-binding fluorochromes give rise to absorption and emission (Abs/em) of light (fluorescence). Exemplary DBFs are listed in Table 1 below. The rings intercalate between the bases of double-stranded DNA. DBFs can also show weaker binding to single-stranded nucleic acids. The positive charges also assist in tight DNA binding, by interacting with negative phosphate groups on DNA.

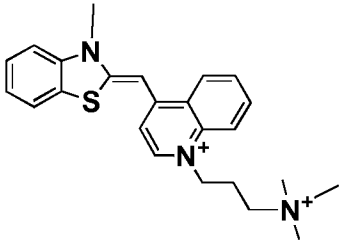
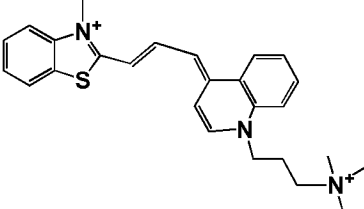
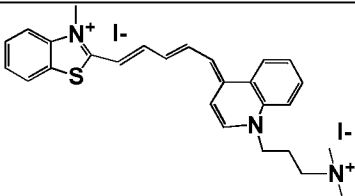
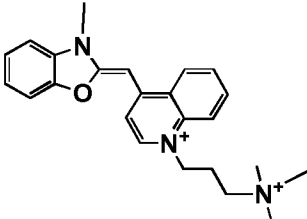
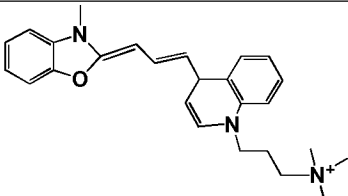
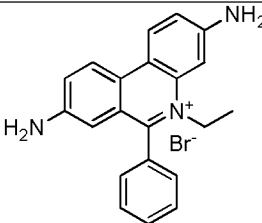
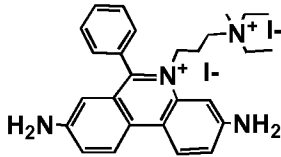
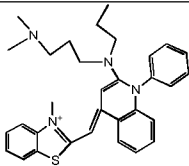
Table 1: Exemplary DBFs and their Optical Properties		
Fluorochrome	VF Structure	Abs/em (nm)
TO-PRO 1		515/531
TO-PRO 3		612/631
TO-PRO 5		745/770
YO-PRO		491/509
YO-PRO 3		612/631
Ethidium Bromide		482/616

Table 1: Exemplary DBFs and their Optical Properties		
Fluorochrome	VF Structure	Abs/em (nm)
Propidium Iodide (PI)		530/625
Sybr Green		490/520

Linkers

The term “linker” as used herein refers to a group of atoms, *e.g.* 5-100 atoms, and may be comprised of the atoms or groups, carbon, amino, alkylamino, oxygen, sulfur, sulfoxide, sulfonyl, amide, and carbonyl. The linker is connected at a first end to the magnetic particle. In addition, the linker is connected at a second end to the DBF through a covalent bond. Examples of covalent bonds include, but are not limited to, an amide bond or a triazole ring. Alternatively, a first linker can be extended with a second linker, to produce a longer and more complex linker, prior to attachment of the reporter group.

Additionally, the linker can be a cleavable linker placed between the DNA-binding moiety and the magnetic particle. After the nucleic acids bind to the SFMP and are removed from solution, a cleaving agent can be used to cleave the cleavable linker, resulting in the release of the nucleic acid from the SFMP. Examples can include TNKase® and linkers containing disulfide bonds (S-S bond). In some embodiments, the cleavable linker can be sensitive to light and can thus be cleaved using light (*e.g.* UV light).

Magnetic Particle

Magnetic particles useful in the methods described herein can be a nanoparticle or a microparticle. The term “nanoparticle” refers to a particle that has a characteristic dimension of less than about 1 micrometer, where the characteristic dimension is the largest cross-sectional dimension of a particle. For example, the

particle may have a characteristic dimension of less than about 500 nm, less than about 250 nm, less than about 150 nm, less than about 100 nm, less than about 50 nm, less than about 30 nm, less than about 10 nm, or less than about 3 nm in some cases.

“Microparticles” with a size of between 1.0 μm and 100 μm can also be employed in the new conjugates.

In some embodiments, the magnetic nanoparticle useful for the methods described here can include feraheme (ferumoxytol), which consists of bioavailable iron oxide surrounded by a carbohydrate shell, *i.e.*, carbomethyldextran. Other examples of magnetic particles can include a paramagnetic nanoparticle, e.g., monocrystalline iron oxide nanoparticle (MION), e.g., cross-linked iron oxide (CLIO) nanoparticles (CLIO has a MION core caged in cross-linked dextran); see, e.g., US 2011/0046004; Josephson et al., *Bioconjug. Chem.*, 10(2):186-91 (1999). In all embodiments, the magnetic particles have a polymer coating to provide chemical functional groups (e.g., electrophilic functional groups such as carboxy groups or nucleophilic groups such as amino groups), and/or sites for further chemical modifications, for attachment to the DNA-binding moiety.

Carboxy functionalized magnetic particles can be made, for example, according to the method of Gorman (see WO 00/61191). In this method, reduced carboxymethyl (CM) dextran is synthesized from commercial dextran. The CM-dextran and iron salts are mixed together and are then neutralized with ammonium hydroxide. The resulting carboxy functionalized nanoparticles can be used for coupling amino functionalized groups, (e.g., a further segment of the functional group or the substrate moiety).

Carboxy-functionalized magnetic particles can also be made from polysaccharide coated nanoparticles by reaction with bromo or chloroacetic acid in strong base to attach carboxyl groups. In addition, carboxy-functionalized magnetic particles can be made from amino-functionalized nanoparticles by converting amino to carboxy groups by the use of reagents such as succinic anhydride or maleic anhydride.

Particle size can be controlled by adjusting reaction conditions, for example, by using low temperature during the neutralization of iron salts with a base as described in U.S. Patent No. 5,262,176. Uniform particle size materials can also be

made by fractionating the particles using centrifugation, ultrafiltration, or gel filtration, as described, for example in U.S. Patent No. 5,492,814.

Magnetic particles can also be synthesized according to the method of Molday (Molday, et al. J. Immunol. Methods, 1982, 52(3):353-67, and treated with periodate to form aldehyde groups. The aldehyde-containing nanoparticles can then be reacted with a diamine (e.g., ethylene diamine or hexanediamine), which will form a Schiff base, followed by reduction with sodium borohydride or sodium cyanoborohydride.

Dextran-coated magnetic particles can be made and cross-linked with epichlorohydrin. The addition of ammonia will react with epoxy groups to generate amine groups, see, e.g., U.S. Patent App. Pub. No. 20030124194 and U.S. Patent App. Pub. 20030092029 Josephson et al., *Angewandte Chemie, International Edition* 40: 3204-3206, 2001; Hogemann et al., *Bioconjug. Chem.*, 11(6): 941-6, 2000; and Josephson et al., *Bioconjug. Chem.*, 10(2):186-91, 1999. This material is known as cross-linked iron oxide or "CLIO" and when functionalized with amine is referred to as amine-CLIO or NH₂-CLIO.

Carboxy-functionalized magnetic particles can be converted to amino-functionalized magnetic particles by the use of water-soluble carbodiimides and diamines such as ethylene diamine or hexane diamine.

Avidin or streptavidin can be attached to the magnetic particles for use with a biotinylated DNA-binding moiety, such as an oligonucleotide. See e.g., Shen et al., *Bioconjug. Chem.*, 1996, 7(3):311-6. Similarly, biotin can be attached to a magnetic particle for use with an avidin-labeled DNA-binding moiety.

Attachment of the Magnetic Particle to a DNA-Binding Moiety- Synthesis of a Surface Functionalized Magnetic Particle

Various methods are known to attach the magnetic particle to the DNA-binding moiety (DBM). In one example, the linker can be attached to the DBM by forming an amide bond between an amine on the magnetic particle and a carboxyl group (or maleimide) located on the DBM. Reagents that can be used include EDC/NHS (N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide) or SPDP (N-succinimidyl 3-[2-pyridyldithio]-propionate) in both aqueous and organic solvents (such as, but not limited to, dichloromethane,

acetonitrile, chloroform, tetrahydrofuran, acetone, formamide, dimethylformamide, pyridines, dioxane, or dimethylsulfoxide). See, e.g., Garanger, et al. Chem Commun (Camb), 4444-4446, 2009, which is incorporated by reference in its entirety, and provides methods for converting the aliphatic quaternary nitrogen on the DNA-binding fluorochrome (DBF) to an NHS-ester group.

One method of synthesizing magnetic particle-fluorochromes is (i) the synthesis of the N-hydroxysuccinimide (NHS) ester of a fluorochrome, (ii) the reaction of the NHS-fluorochrome with an aminated magnetic particle. Syntheses of NHS-Fluorochromes are described in Examples 1-3 and 5-10 of WO 2010/141833, which is incorporated by reference in its entirety. The attachment to magnetic particles is described in WO 2010/141833.

In addition, various methods are known to attach the linker to the DBF using "click chemistry" (see, e.g., the Sigma Aldrich catalog and U.S. Patent No. 7,375,234, which are both incorporated herein by reference in their entireties). Of the reactions including "click" chemistry, one example is the Huisgen 1,3-dipolar cycloaddition of alkynes to azides to form 1,4-disubstituted-1,2,3-triazoles. The copper (I)-catalyzed reaction is mild and very efficient, requiring no protecting groups, and requiring no or only minimal purification in many cases. The azide and alkyne functional groups are generally inert to biological molecules and aqueous environments. Use of the click reaction is also described in WO 2010/141833.

Magnetic particles can be conjugated to oligonucleotides through a variety of other conjugation chemistries known in the art. See U.S. Patent No. 5,512,439; Greg Hermanson "Bioconjugate Techniques," Academic Press, 1996; Gordon Bickerstaff "Immobilization of Enzymes and Cells," Humana Press, 1997.

Characterization of Magnetic Particle-Fluorochromes

Where the DNA binding moiety is fluorescent, the number of fluorochromes attached to magnetic particles can be assessed from fluorochrome absorbance or fluorochrome fluorescence. An optimum number of fluorochromes per magnetic particle is one that maximizes the multivalent effects, which increase the affinity of DNA for the surface of the magnetic particle. However, the charge of the particles should be negative and exhibit a negative zeta potential, to prevent electrostatic binding between negatively charged DNA and a positively charged surface of the

magnetic particle. A wide variety of magnetic particles with varying sizes and compositions can be used.

Methods of Monitoring DNA Amplification

5 Methods for monitoring DNA amplification can include both light scattering spectroscopy and relaxometry. The PCR thermal cycler can be connected to a detector such that the amplification reaction is monitored without opening the PCR tube. The methods include detecting the levels of amplified nucleic acids using light scattering, e.g. resonance light scattering (RLS) technique with a common
10 spectrofluorometer, dynamic light scattering, or quasi-elastic light-scattering spectroscopy. The methods include detecting levels of amplified nucleic acids using magnetic resonance MR, and which uses magnetic nanoparticles (MNPs) as proximity sensors that modulate the transverse relaxation time of neighboring water molecules. This signal can be quantified using MR imagers or NMR relaxometers, including
15 miniaturized NMR detector chips that are capable of performing highly sensitive measurements on microliter sample volumes and in a multiplexed format. In some embodiments, relaxation times (T_2) can be determined by relaxation measurements using a nuclear magnetic resonance benchtop relaxometer. In general, T_2 relaxation time measurements can be carried out at 0.47 T and 40°C (Bruker NMR Minispec,
20 Billerica, MA) using solutions with a total iron content of 10 $\mu\text{g Fe/mL}$. Other devices include the miniaturized NMR (mNMR) detectors described in Lee et al., Nat Med 14, 869-874 (2008); Lee et al., Proc. Natl Acad. Sci. USA 106, 12459–12464 (2009); and Lee et al., Angew. Chem. Int. Ed. Engl. 48, 5657–5660 (2009), as well as those described in WO 2009/045551; Haun et al., Methods Mol Biol 726, 33-49
25 (2011). Sun et al., IEEE ISSCC Digest Tech Papers 488 - 489 (2010); Sun et al., IEEE J Solid-State Circuits 44, 1629 (2009); and Liu et al., IEEE ISSCC Digest Tech Papers 140–141 (2008). All of the foregoing are incorporated herein by reference.

 In some embodiments, T_1 relaxation times can also be measured as a way of monitoring the progress of the PCR reaction. T_1 refers to the mean time for an
30 individual nucleus to return to its thermal equilibrium state of the spins. Once the nuclear spin population is relaxed, it can be probed again, since it is in the initial, equilibrium (mixed) state. T_1 is always longer than T_2 , that is, slower spin-lattice

relaxation, for example because of smaller dipole-dipole interaction effects. In some embodiments, the T_2^* value can be used to monitor the PCR reaction. The T_2^* , which is the actually observed decay time of the observed NMR signal, or free induction decay and depends on the static magnetic field inhomogeneity, which is quite significant.

Methods of Purifying and Extracting Nucleic Acids

DNA-binding fluorochromes (DBFs) can be attached to magnetic particles to form a surface functionalized magnetic particle (SFMP) as a method of extracting and purifying DNA. The SFMPs can be used to bind and extract DNA to which DNA binding proteins are attached. This method permits the extraction of DNA without denaturing the DNA, which removes important proteins that regulate gene activity. The method can be carried out by providing a sample of nucleic acids, either in a solution or reaction mixture, adding the SFMPs and allowing sufficient time for the SFMPs to bind to the nucleic acids. A magnetic field can then be applied to the nucleic acid solution to “draw out” the nucleic acids that are bound to the SFMPs.

Separating the Magnetic Particle from the Nucleic Acid

A number of methods can be used to separate the SFMPs from the nucleic acids, including heating the sample to obtain dissociation, e.g., at a temperature of about 90 °C, the SFMP-nucleic acid complex can also be employed to separate the nucleic acids from the SFMPs. Another strategy is to employ a cleavable linker with a reactive group that can be cleaved. The cleavable linker is attached to the magnetic particle at a first end and the DNA-binding moiety at a second end. Exemplary groups include sulfhydryl, amino, and phosphate groups. They can be coupled to amino-functionalized magnetic particles, which are part of through the use of reagents such as N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and long chain SPDP (lc-SPDP) that produce a cleavable disulfide bond between the nanoparticle and the oligonucleotide. Another example is the use of a light-labile linker that can be cleaved using light, e.g., UV light. A number of such linkers are known in the art,

PCR-NMR Combination Devices

Also provided herein are devices that can be used to perform the present methods that include both a thermal cycler and a relaxometer. The devices include a housing, and within the housing are a thermal cycler module comprising (i) an automated thermal cycler module capable of alternately heating and cooling, and adapted to receive, at least one reaction vessel containing an amplification reaction mixture comprising a target nucleic acid, reagents for nucleic acid amplification, and a detectable nucleic acid binding agent; and (ii) a detector module operable to detect a proton relaxation signal without opening the at least one reaction vessel, which proton relaxation signal is related to the amount of amplified nucleic acid in the reaction vessel. Exemplary automated thermal cycler modules are known in the art, e.g., as described above and in US 6814934 and references cited therein, all of which are incorporated by reference herein. Exemplary detector modules are known in the art, e.g., as described above and in Koh et al., *Sensors* 9:8130-8145 (2009); Lee et al., *Nat Med.* 2008 August; 14(8): 869–874; Josephson et al., *Angew. Chem. Int. Ed.* 2001, 40, 3204–3208; Issadore et al., *Miniature magnetic resonance system for point-of-care diagnostics* Lab Chip. 2011 May 5; and Perez et al., *Nat. Biotechnol.* 2002, 20, 816–820; and references cited in all of the foregoing, all of which are incorporated by reference herein. In some embodiments, the device comprises a magnetic field sweep, constant radiofrequency generator, amplifier, spinning sample compartment, and a recorder for NMR spectrum generation.

Kits

The present disclosure further comprises kits, comprising the compositions and described herein, e.g., for use in the methods described herein for performing a PCR reaction with SFMPs or purifying and extracting nucleic acids using SFMPs. In some embodiments, the kit can include instructions for use of the kits in the methods described herein. “Instructions” can define a component of promotion, and typically involve written instructions on or associated with packaging of compositions of the invention. Instructions also can include any oral or electronic instructions provided in any manner. The “kit” typically defines a package including any one or a combination compositions described herein and the instructions, but can also include

the compositions described herein and instructions of any form that are provided in connection with the composition in a manner such that a person having ordinary skill in the art of PCR or nucleic acid purification and extraction will clearly recognize that the instructions are to be associated with the specific composition.

5 The kits described herein can also contain one or more containers, which can contain reagents, e.g., SFMPs, DNA template, deoxynucleotide triphosphates (dNTPs), DNA polymerase, and primers. The kits also can contain instructions for mixing, diluting, and/or preparing the reagents.

EXAMPLES

10 The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Example 1. Monitoring PCR Reactions Using DNA-Binding Magnetic Nanoparticles

15 This example describes how SFMPs were prepared and used to monitor DNA amplification. The following materials and methods were used in this example.

Conjugation of Fluorochromes to Magnetic Particles

20 A general and preferred method of synthesizing magnetic particle-fluorochromes is (i) the synthesis of the N-hydroxysuccinimide (NHS) ester of a fluorochrome, (ii) the reaction of the NHS-fluorochrome with an aminated magnetic particle. A second general and preferred method is the use of click chemistry to conjugate the fluorochrome to magnetic particle.

25 Syntheses of NHS-Fluorochromes are described in Examples 1-3 and 5-10 of WO 2010/141833, which is incorporated by reference in its entirety. The attachment to magnetic particles is described in Examples 6 and 7 of WO 2010/141833. Use of the click reaction is described in Example 5 of WO 2010/141833.

Characterization of Magnetic Particle-Fluorochromes

30 The number of fluorochromes attached to magnetic particles can be assessed from fluorochrome absorbance or fluorochrome fluorescence. The optimum number of fluorochromes per magnetic particle maximizes the multivalent effects, which

increase the affinity of DNA for the surface of the magnetic particle. However, the charge of the particles should be negative and exhibit a negative zeta potential, to prevent electrostatic binding between negatively charged DNA and a positively charged surface of the magnetic particle. A wide variety of magnetic particles with varying sizes and compositions can be used.

Materials

Ferumoxytol (FERAHEME®, FH) was obtained from AMAG Pharmaceuticals. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). PD-10 or NAP-5 columns were purchased from GE Healthcare, Piscataway, NJ. Size by light scattering and zeta potentials were measured using a Nano-ZS Zetasizer (Malvern, Medford, MA), with size expressed as the Z-average. T2 relaxation times were determined on a 20 MHz MiniSpec at 40°C (Bruker Systems, Billerica, MA). Absorbance was obtained with an Evolution 300 spectrophotometer (Thermo Scientific, Madison, WI), while fluorescence was measured with the GloMax®-Multi Detection System (Promega, Madison, WI). To obtain size and T2 as a function of PCR cycle number (CN), a BioRad MYCYCLER™ (Bio-Rad laboratories) was used. To obtain fluorescence as a function of CN with Sybr Green or FH-TO, an ABI Prism 7000 (Applied Biosystems, Carlsbad CA) was used.

Synthesis of the Nanoparticles

FERAHEME® (FH) (30 mg Fe/mL) was transferred to 0.1 M MES, pH 6 by gel filtration using a Sephadex G-25 PD-10 column. To 1 mL of diluted FH (6 mg Fe, 107 µmoles Fe) was added 25 mg of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (CDI, MW= 191.7, 130 µmoles) and 5 mg hydroxybenzotriazole (HOBT, MW= 153.1, 32.6 µmoles), and the mixture was incubated for 20 minutes at room temperature. To this mixture was added 40 µL of 1M ethylene diamine (40 µmoles EDA) in 0.1 M MES and the mixture incubated at 50 °C for 90 minutes. The mixture was concentrated by lyophilization and purified on a PD-10 column equilibrated with MES buffer. Some 50 µL of amino-FH (250 µg Fe) was added 3.12 µL (62.4 µg, 79.6 nmoles) of the NHS ester of TO in DMSO (20 mg/ml in DMSO) and allowed to react for 3 hours at 25°C. Purification was performed using a

Sephadex G-25 column using water as eluent. Unreacted TO adhered to the column, eliminating possible contamination of FH-TO with low molecular fluorochromes.

The TO/NP ratio was determined spectrophotometrically, using the absorbance at 300 nm and FH standards for iron concentrations, and the absorbance at 501 nm and an extinction coefficient of $63,000 \text{ (M}\cdot\text{cm)}^{-1}$ for TO-PRO 1 concentration. According to the AMAG Pharmaceutical package insert, there were 5874 Fe/NP.

DNA/TO-PRO 1 Model

A model for TO-PRO 1 bound to DNA was generated based on the experimentally determined NMR structures (Prodhomme, et al. J Photochem Photobiol B 53: 60-69, 1999; Spielmann, et al. Biochemistry 34: 8542-8553, 1995). Briefly, the MOE-2007.09 docking suite (Chemical Computing Group, Montreal) was used to build 10 base pairs of ideal B-form DNA and an energy minimized structure of TO-PRO 1 (MMF94X force field to a constant of 0.05 kcal/mol). An initial TO-PRO 1/DNA model was generated by superimposing the coordinates for B-form DNA and TO-PRO 1 onto the respective groups in 108D.pdb. Finally, the local interactions between TO-PRO 1 and the model DNA were optimized and minimized using the MOE LigX function.

Reactions of FH-TO and DNA

DNA (lambda phage, 48,502 base pairs, MW= 31.5 million Da) from NEBiolabs (N3013S) in 10mM Tris-HCl, 2.5mM MgCl₂, 0.5mM CaCl₂, pH7.6 was added (200 μL) to a 96 well microtiter plate (Thermo-Fisher, 07-200-565). Then 2.5 μL of FH-TO (0.42 mg Fe/mL, 1.30 μM NP) was added and the plate placed on an orbital shaker for 2 hours at room temperature. T₂ by relaxometry and fluorescence were determined on undiluted samples. For size determinations, duplicate wells were pooled and 300 μL water was added.

PCR Reactions

The DNA template was a 100 base pair synthetic dsDNA with a sequence of 5'-GCG-CCT-AGG-CAT-TTT-GCT-GCC-GGT-CAC-GGC-TAA-TGT-ATG-TCT-AA TGTC-TAA-TGT-CTA-ATG-TCT-AAT-GTG-ATC-GAT-CGC-TTT-GTC-GAT-ACT-

GGTACT-AAT-G--3, SEQ ID NO:1 (IDT, Coralville, IA, USA). Forward primer (5'-CGC-GGA-TCC-GTA-AAA-CGA-CGG-CCA-GTG-CC-3', SEQ ID NO:2, T_m=69.2°C, MW=8897.8) and reverse primers (5'-CTA-GCT-AGC-GAA-ACA-GCT-ATG-ACC-ATG-A TT-AC-3', SEQ ID NO:3, T_m=59.2°C, MW=9801.4) were from
5 IDT. The PCR reaction employed 5 µL of 10x Thermopol Reaction Buffer (NEBiolabs, #B9004S), 1 µL of each for forward and reverse primers (1 µg/µL), 1 µL VentR polymerase, (2000 units/mL NEBiolabs, #M0254L), 1 µL of template diluted to give a 20% of maximal response using Sybr Green at about 25 cycles using the ABI Prism 7000 (Applied Biosystems) and 1 µL dNTP nucleotide mix (Thermo-Fischer
10 (BP2565-1) Carlsbad, CA). Some 36.2 µL of RNase free water was then added followed by 3.8 µL of FH-TO (0.1 mg Fe/mL). For T₂ by relaxometry measurements were made in a 50 µL capillary tube. For light scattering, samples were diluted with PBS.

15 *MRI*

Measurements were made using a 21 cm horizontal-bore 9.4 Tesla (9.4 T) scanner from Bruker Biospec (Billerica, MA, USA). PCR tubes were immersed in water with 5mM Gd-DTPA (Gadopentetic acid) (Bayer Schering Pharma, Berlin, Germany). The imaging protocol included a triplane pilot scan for localization of the
20 samples. Multi-slice multiecho (MSME) T₂-weighted imaging was performed using the following parameters: flip angle = 90 degrees; matrix size (320 x 100); TR = 2500 ms; TE = 11 equally spaced echoes at 4.3 ms intervals ranging from 8.7 ms to 52 ms; field of view (FOV) = 8 x 3 cm; slice thickness = 1 mm; orientation = coronal. Region-of-interest analysis and T₂ quantification was performed using a mono-
25 exponential fitting algorithm for the multi-TE data with a DICOM viewer from OsiriX (v.3.8.1).

Data Analysis

A single site binding equation or four-parameter logit equation using Prism 4
30 software was employed (GraphPad Software, San Diego, CA).

Results: Synthesis and Testing of a DNA-binding Nanoparticle

To synthesize a DNA-binding NP, the DNA-binding fluorochrome TO-PRO 1 was attached to the FERAHEME (FH) NP approved for the treatment of iron anemia. A "TO-PRO 1 NHS ester" was synthesized according to Garanger, et al. *Chem Commun (Camb)*, 4444-4446, 2009 and reacted with FeraHEME-amine, a strategy that permits TO-PRO 1 attachment to any amine (FIG. 2). TO-PRO 1 fluoresces when bound to DNA by intercalation (FIG. 3). The FH nanoparticle (NP) was selected for several reasons. After observing that carboxymethyldextran (CMD) coated NP's were remarkably stable to heat stress (Chen, et al. *Journal of Materials Chemistry* 19: 6387-6392, 2009), it was noted that FH also possessed a CMD coating and exhibited high thermal stability. High thermal stability is required for using a DNA binding magnetic NP to monitor a cycling PCR reaction. In addition, FH possessed a strong negative surface charge. Because of its clinical use, the FH NP has a publically available formula, and consists of an iron oxide core ($\text{Fe}_{5874}\text{O}_{8752}$) and 414 carboxyl groups due to its CMD coating. Carboxyl groups on FH were partially converted to amines and the aminated FH reacted with the "TO-PRO-NHS ester." The resulting NP, termed FH-TO, had 9.9 ± 2.4 TO-PRO 1's per NP attached through a 6-carbon, flexible linker. (Values are the means $\pm$ 1 SE, n=4.)

FH-TO had r_1 and r_2 relaxivities of 23.3 ± 2.2 and 122 ± 12 (mM Fe sec^{-1}), respectively, and a size of 59.8 ± 3.4 nm. The parent FH nanoparticle had a zeta potential of -37.8 ± 3 mV (pH 6) that was largely preserved with the attachment of 9.9 ± 1.4 TO-PRO 1's per NP; FH-TO's zeta potential was still highly negative at -28.3 ± 0.8 mV. FH-TO's negative charge presumably resulted from a large number of still unmodified carboxyl groups after partial amination. Preservation of a negative NP surface prevented a likely electrostatic binding between a negatively charged DNA and positively charged NP, and insured that when FH-TO and DNA interact, it would be via an intercalation of the Band Q rings of TO-PRO 1 (see FIG. 3).

FH-TO was incubated with a wide range of DNA concentrations (0-10 $\mu\text{g/mL}$), and the reaction monitored by a fluorescence increase (FIG. 4A), by a decrease in the T2 relaxation time (FIG. 4B), and by an increase in aggregate size by light scattering (FIG. 4C). Results were plotted as deltas, e.g., ΔT_2 is the difference between $T_{2(\text{NO DNA})}$ and $T_{2(+\text{DNA})}$. FIGs. 4D and 4E show results for T2 and size at

low DNA concentrations (0-1.0 $\mu\text{g/mL}$) from FIG. 4B and FIG. 4C, respectively. The hyperbolic curves obtained with fluorescence (FIG. 4A) were fit to a single site-binding model, yielding apparent K_d 's of $5.86 \pm 1.20 \mu\text{g/mL}$ and of $3.62 \pm 0.25 \mu\text{g/mL}$ for TO-PRO 1 and FH-TO, respectively. Both K_d 's by fluorescence were far higher than the K_d from T2 ($0.0379 \pm 0.007 \mu\text{g/mL}$, FIG. 4D). Aggregate size exhibited a hook effect discussed herein, with a maximum size at $0.25 \mu\text{g/mL}$.

A comparison of fluorescence and size measurements was used to deduce features of the FH-TO/aggregates (FIGs. 4A-H). Of note, TO-PRO 1 bound to DNA as a monovalent intercalating fluorochrome (FIGs. 2, 4A and 4F, $K_d = 3.62 \mu\text{g/mL}$). Since the K_d for FH-TO fluorescence was similar to that of monovalent TO-PRO 1 ($K_d = 5.86 \mu\text{g/mL}$), the TO-PRO 1's on FH-TO bound to DNA as if they were independent fluorochromes, though they were in fact part of a surface ensemble. The large extended aggregate formed at $0.25 \mu\text{g/mL}$ resulted from a cooperative, avidity-driven interaction; here fluorescence was only 1.18% of the theoretical maximum fluorescence obtained from the hyperbolic curve shown in FIG. 4A. Hence the extended aggregate depicted in FIG. 4G involved the binding of only a small fraction of the TO-PRO 1's on the surface of the FH-TO NP. On the other hand, the small aggregate, present for example at $10 \mu\text{g/mL}$ and depicted in FIG. 4H, was a "wrapped" DNA/FH-TO configuration, where most of FH-TO's TO-PRO 1's were intercalated and fluorescence was high (67% of maximum). Supporting the large extended aggregate and small wrapped aggregate models, the concentration of FH-TO (16.1 nM in NP throughout FIGs. 4A-H) exceeded that of DNA, consistent with many FH-TO's binding to a given DNA molecule. (A concentration of $10 \mu\text{g/mL}$ was 319 pM DNA, since lambda DNA has a molecular weight of 31.5 million Da.) The "high dose hook effect" (FIGs. 4C and 4D) is characteristic of solution phase reactions between multivalent antibodies and multivalent antigens, a situation analogous to the reaction between multivalent FH-TO and polymeric DNA.

Either light scattering or relaxometry can be used to determine aggregate formation in the low DNA concentration range (0-1.0 $\mu\text{g/mL}$, peak aggregate size @ $0.25 \mu\text{g/mL}$, T2 K_d @ $0.0379 \mu\text{g/mL}$). However, relaxometry has two advantages: T2 is a hyperbolic function of DNA concentration (no hook effect) and T2 is a radiofrequency-based method (no light based interferences). With light scattering and

hook effects, some aggregate sizes (*e.g.*, 200 nm) can reflect low or high concentrations and additional measurements with diluted samples are required. To estimate the sensitivity of DNA detection by T2, mean and standard deviation (SD) of a FH-TO solution was determined without DNA (a blank) as in FIG. 5. Here T2 =
5 136.8 ± 1.3 msec (n=20, ± 1 SD). Using the equation $Y = B_{\max} * X / (K_d + X)$, with $K_d = 0.0379$ and $B_{\max} = 61.3$ msec, a T2 change of 2.6 msec corresponded to a DNA concentration of 27 fM DNA.

The sensitivity of DNA detection, the thermal stability of FH, and the broad high dose plateau seen with T2 measurements (no hook) suggested FH-TO could be
10 added to a PCR tube. FH-TO was added to a PCR reaction in a thermal cycler (Biorad MYCYLER™) and, after the indicated cycle number (CN), tubes were opened for light scattering and T2 relaxometer measurements (FIG. 5). Identical tubes were also placed in an ABI 7000 q-PCR instrument and FH-TO and Sybr Green fluorescence determined as a continuous function of CN. The cycle threshold (Ct) for
15 detection of the PCR reaction with the ABI system is taken to be 20% of the maximum response, which corresponded to Ct values by fluorescence of 26.5 for FH-TO and 28.2 with Sybr Green. Detection of PCR products by aggregate formation, by T2 or light scattering, was at Ct's of 9.5 and 16.4, respectively, both of which were far below the Ct's from fluorescence. Thus the detection of FH-TO/DNA aggregates was
20 more sensitive by T2 than by fluorescence, based on their relative Kd's, and based on the fewer PCR cycles needed to achieve a Ct for detection for the PCR product. (The Kd by T2 was 0.0379 µg/mL while a Kd by fluorescence was 3.62 µg/mL).

To demonstrate that radiofrequency-derived T2 values could be determined from within a sealed PCR tube, selected tubes from FIG. 5 were placed in the
25 phantom (FIG. 6), and the T2 relaxation time determined by MRI (FIG. 7). With a full complement of PCR reagents, T2 increased progressively with CN, a response not seen with the FH-TO in the absence of DNA template (FH-TOI-T), or with the full complement of PCR reagents but with a NP lacking TO-PRO 1 (FH/+ T).

When dispersed FH-TO reacted with DNA (FIG. 6), small aggregates formed,
30 and a T2 decrease resulted, termed a Type I relaxation switch. After a single PCR cycle aggregates of 402 nanometers were already present, and these were further aggregated to a size of 2600 nanometers with additional cycles. Here aggregation

produced a T2 increase, termed a Type II relaxation switch (FIGs. 5 and 7). The differing effects of magnetic particle aggregation have been observed before (Koh, et al. *Anal Chem* 81: 3618-3622, 2009; Hong, et al. *Magn Reson Med* 59: 515-520, 2008; Koh, et al. *Angew Chem Int Ed Eng.* 147: 4119-4121, 2008) and can be explained by magnetic sphere relaxation theory. When dispersed NP's aggregate, larger magnetic field inhomogeneities are produced; these are more efficient dephasers of the T2 relaxation process and T2 decreases with aggregation. When aggregate size increases still further, micron-sized aggregates are formed; here a diffusion-limited condition resulted and T2 increases with aggregation. The production of larger aggregates in the PCR reaction reflects the higher FH-TO concentration employed (32 nM FH-TO NP's/FIGs. 5-7 versus 16.1 nM/FIGs. 4A-H), and an increase in aggregate size by PCR temperatures.

Example 2. Separation and Purification of Nucleic Acids

DNA-binding magnetic particles, e.g., nanoparticle or microparticle, are mixed with a solution of nucleic acid and allowed to bind their target nucleic acid. They are then removed through the use of a magnetic separator which can be a permanent magnetic (Dexter Magnetics, InVitrogen) or grid type separator (Miltenyi Biotech). The magnetic particle-fluorochrome/nucleic complex is broken apart by heating the solution to about 80-90 °C and performing the magnetic separation on solution at an elevated temperature. Alternatively, dissociating/denaturing agents like guanidinium chloride, NaI, guanidium thiocyanide, urea are used. Organic solvents ethanol or phenol may also be employed.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

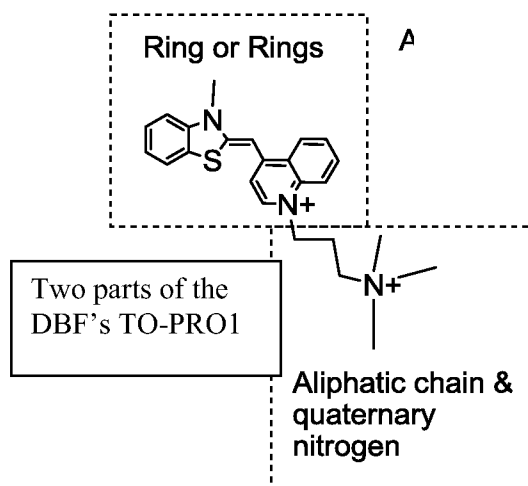
1. A method for monitoring amplification of DNA in a polymerase chain reaction (PCR) mixture, the method comprising:
 - providing a PCR reaction mixture comprising: DNA template, deoxynucleotide triphosphates (dNTPs), DNA polymerase, and primers;
 - contacting surface functionalized magnetic particles (SFMPs) comprising a DNA-binding moiety, a magnetic particle, and a linker with the PCR reaction mixture; and
 - determining progress of the PCR reaction by measuring proton relaxation time in the mixture.
2. A method for monitoring amplification of DNA in a polymerase chain reaction (PCR) mixture, the method comprising:
 - providing a PCR reaction mixture comprising: DNA template, deoxynucleotide triphosphates (dNTPs), DNA polymerase, and primers;
 - contacting surface functionalized magnetic particles (SFMPs) comprising a DNA-binding moiety, a magnetic particle, and a linker with the PCR reaction mixture; and
 - measuring a proton relaxation time in the mixture, thereby monitoring amplification of DNA.
3. The method of claim 1 or 2, comprising measuring a first proton relaxation time; performing at least one cycle of amplification; and measuring a second proton relaxation time, thereby monitoring the amplification of the DNA.
4. The method of any one of claims 1 to 3, wherein the DNA-binding moiety is a DNA-binding oligonucleotide.
5. The method of any one of claims 1 to 3, wherein the DNA-binding moiety is a DNA-binding fluorochrome.

6. The method of claim 5, wherein the DNA-binding fluorochrome is selected from the group consisting of: ethidium bromide, propidium iodide, TO-PRO 1, TO-PRO 3, YO-PRO, and 4',6-diamidino-2-phenylindole (DAPI), and bisbenzimidazole (Hoescht 33342).
7. The method of any of claims 1-6, wherein measuring a proton relaxation time comprises measuring one or more of T1, T2, T2*, or ratios of T1-T2, T2-T2*, T1-T2*.
8. The method of claim 7, wherein the measuring is performed using a relaxometer.
9. The method of claim 1 or 2, wherein the magnetic particles are microparticles or nanoparticles.
10. The method of claim 1 or 2, wherein the magnetic particles comprise ferumoxytol or a superparamagnetic cross-linked iron-oxide (CLIO) particle.
11. The method of claims 1 or 2, wherein the measuring is performed at intervals over time.
12. The method of claim 3, wherein the measuring of the T2 relaxation time is conducted with the PCR mixture in a closed tube.
13. A kit for use in the methods of any one of claims 1 to 12 comprising: deoxynucleotide triphosphates (dNTPs), DNA polymerase, and a surface functionalized magnetic particle (SFMP) comprising a DNA-binding moiety.
14. The kit of claim 13, wherein the DNA-binding functional group is a DNA-binding fluorochrome.

15. The kit of claim 14, wherein the DNA-binding fluorochrome is selected from the group consisting of ethidium bromide, propidium iodide, TO-PRO 1, TO-PRO 3, YO-PRO, 4',6-diamidino-2-phenylindole (DAPI) and bisbenzimidazole (Hoescht 33342).
16. The kit of claim 13, wherein the SFMP comprises a magnetic microparticle or nanoparticle.
17. The kit of claim 13, wherein the SFMP comprises ferumoxytol or a superparamagnetic cross-linked iron-oxide (CLIO) particle.
18. A device for monitoring PCR amplification comprising (i) an automated thermal cycler module capable of alternately heating and cooling, and adapted to receive, at least one reaction vessel containing an amplification reaction mixture comprising a target nucleic acid, reagents for nucleic acid amplification, and a SFMP comprising a DNA-binding functional group; and (ii) a detector module operable to detect a proton relaxation signal without opening the at least one reaction vessel, which proton relaxation signal is related to the amount of amplified nucleic acid in the reaction vessel.

1 / 6

FIG. 1



2 / 6

FIG. 2

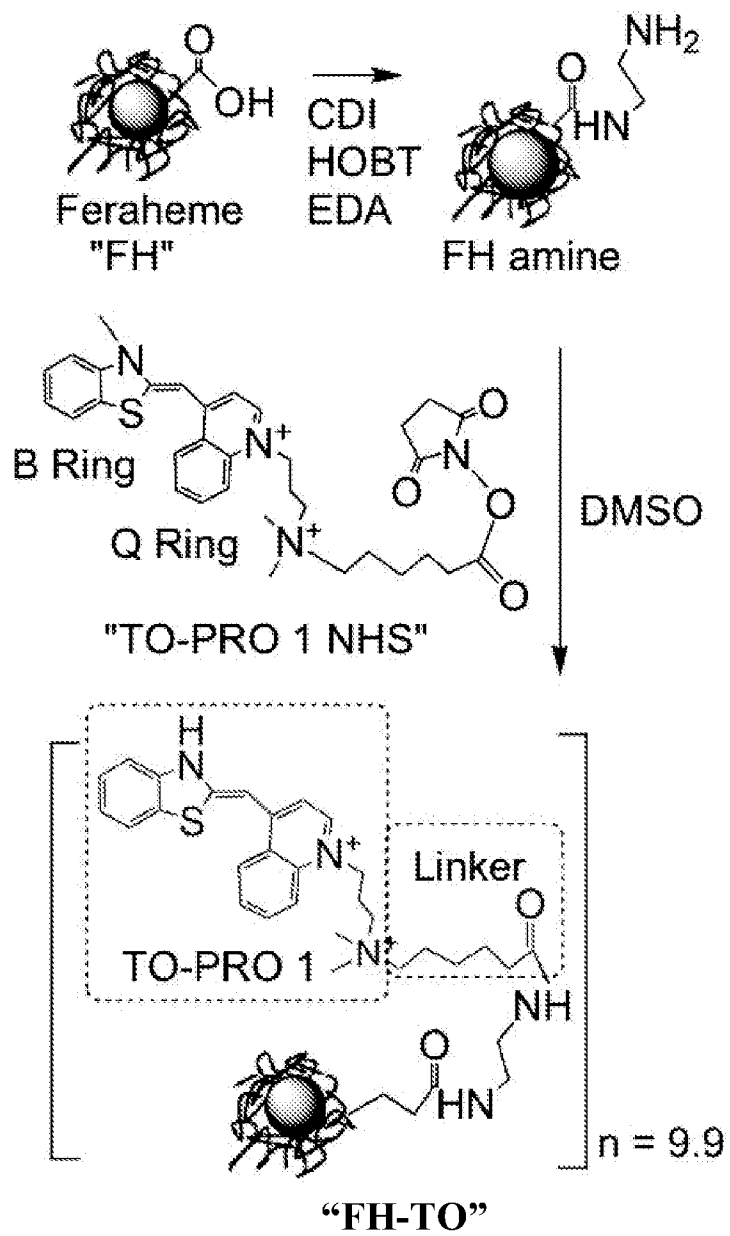
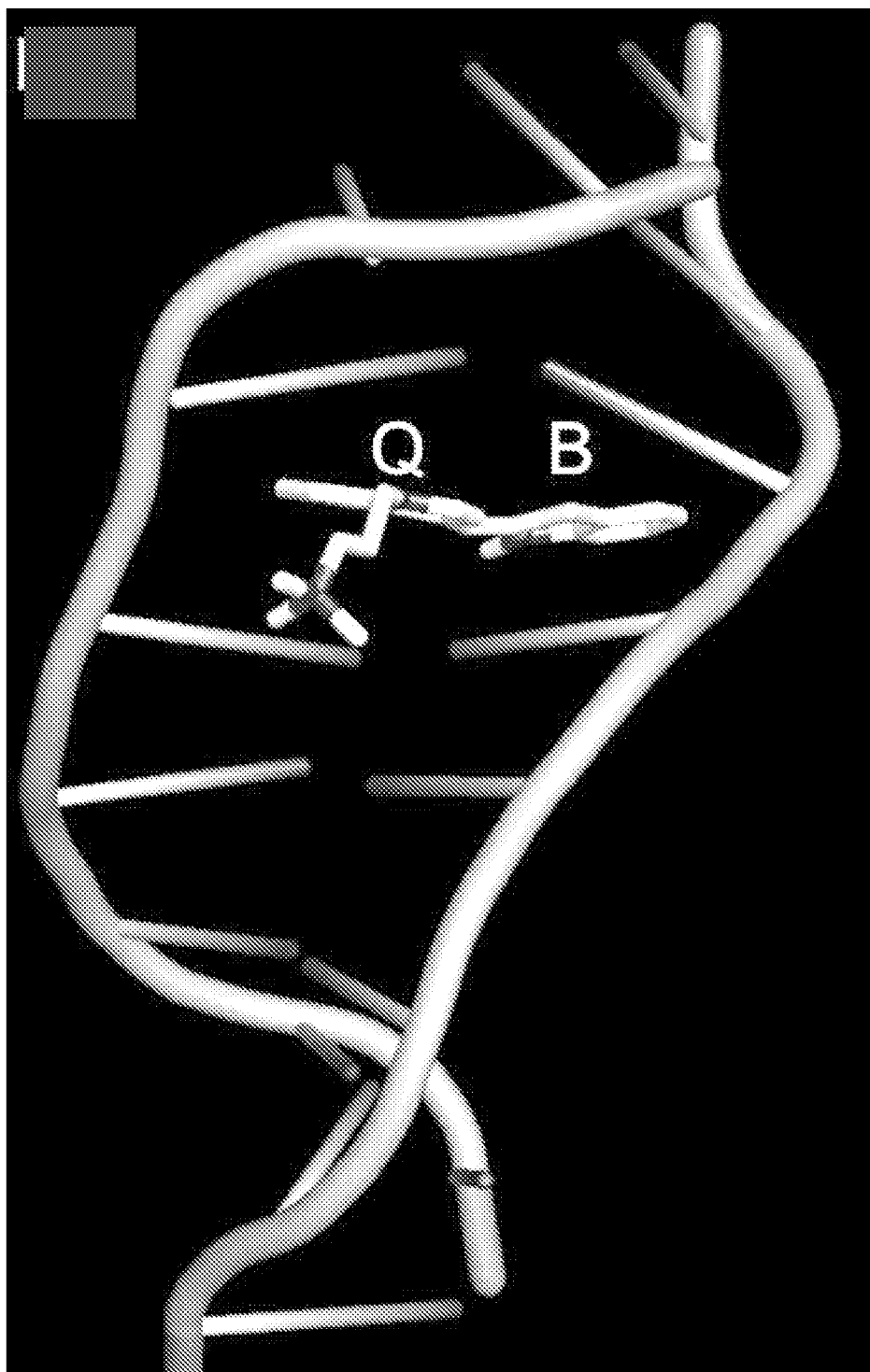
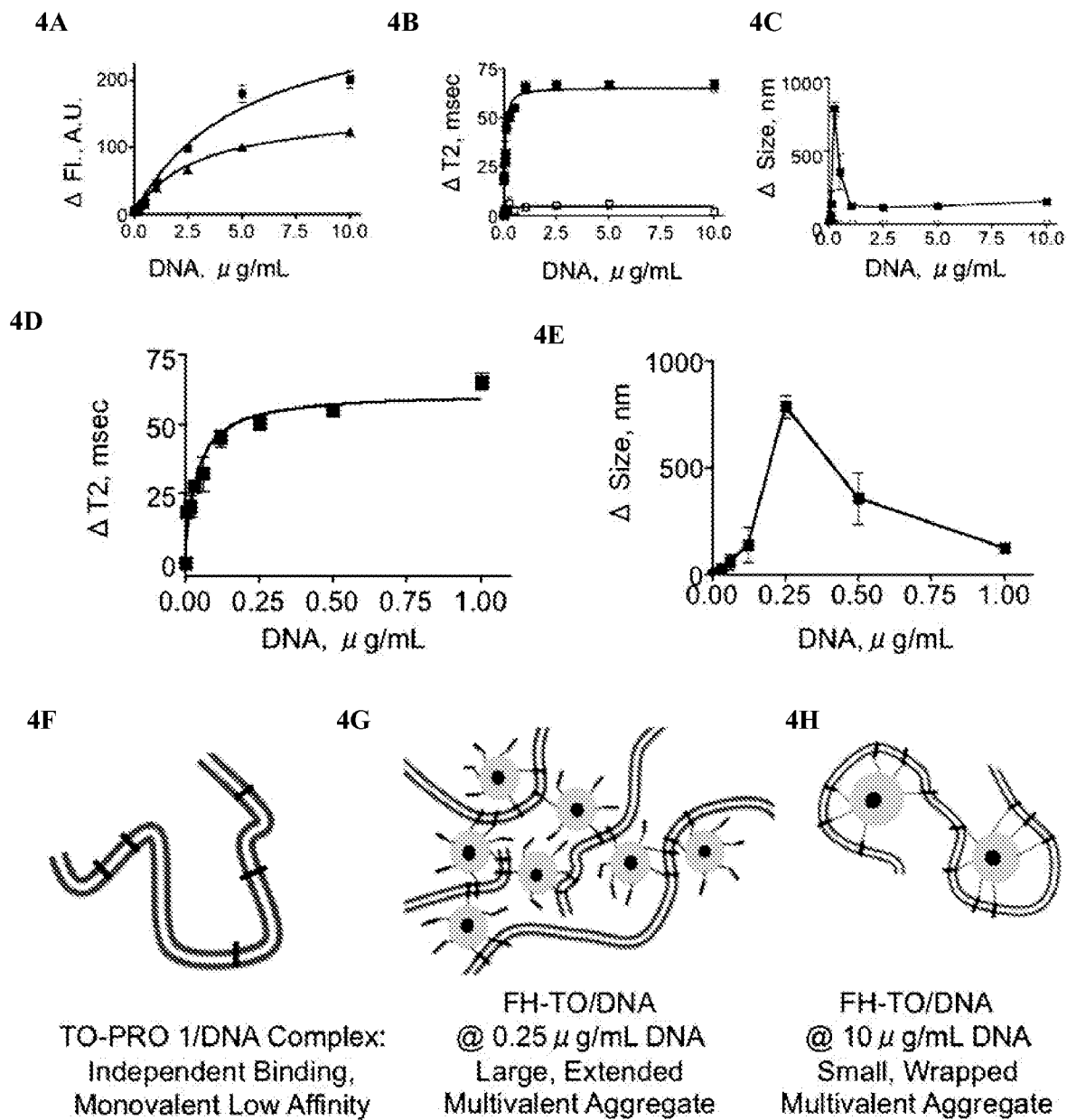


FIG. 3



FIGs. 4A-H



5 / 6

FIG. 5

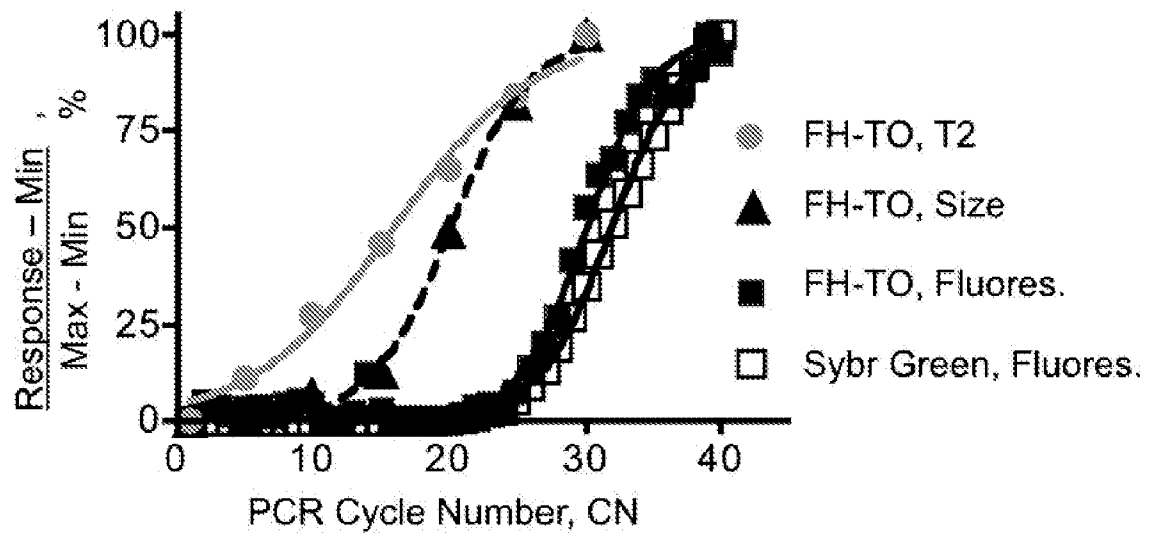
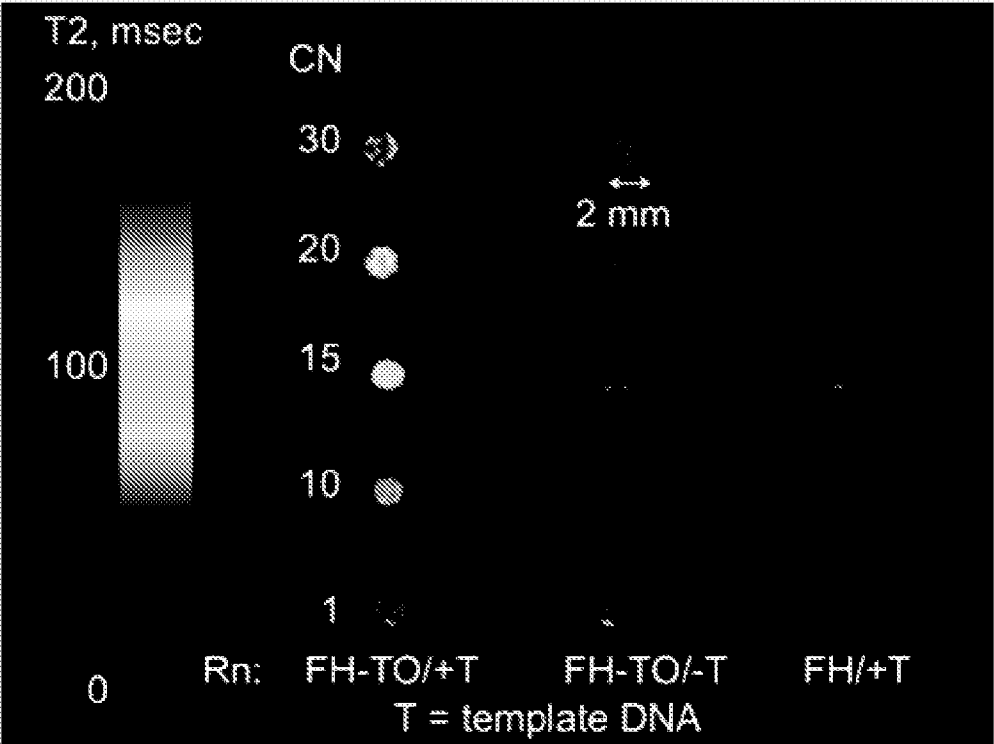


FIG. 6



FIG. 7



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2012/036505

A. CLASSIFICATION OF SUBJECT MATTER		<i>C12Q 1/68 (2006.01)</i> <i>C12P 19/30 (2006.01)</i> <i>G01N 24/00 (2006.01)</i> <i>G01N 27/72 (2006.01)</i> <i>G01N 33/553 (2006.01)</i> <i>A61B 5/055 (2006.01)</i>
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
C12Q 1/68, C12P 19/30, G01N 24/00, 27/72, 33/553, A61B 5/055		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
PatSearch (RUPTO internal), Esp@cenet, PAJ, USPTO		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2009/0068664 A1 (THIRD WAVE TECHNOLOGIES, INC.) 12.03.2009, ex.	1-17
Y	US 20060140871 A1 (LAUREL SILLERUD O. et al.) 29.06.2006, claims	1-18
Y	EA 200870288 A1 (USTAV MAKROMOLEKULARNI KHEMIE AKADEMIE VEDCHESKE RESPUBLIKI, V.V.I. et al.) 30.06.2009, claims	10, 17
Y	US 2011/0046004 A1 (THE GENERAL HOSPITAL CORPORATION) 24.02.2011, [0129]-[0148], ex., claims	4
Y	Elisabeth Garanger et al. A DNA-binding Gd chelate for the detection of cell death by MRI. Chem Commun (Camb). 2009 August 7; (29): 4444-4446	5-6, 14-15
☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
02 August 2012 (02.08.2012)		20 September 2012 (20.09.2012)
Name and mailing address of the ISA/ FIPS Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1		Authorized officer O. Fedonova
Facsimile No. +7 (499) 243-33-37		Telephone No. 499-240-25-91

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2012/036505

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Saher Afshan Shaikh et al. A molecular thermodynamic view of DNA-drug interactions: a case study of 25 minor-groove binders. Archives of Biochemistry and Biophysics 429 (2004) 81-99	5-6, 15
Y	US 6814934 B1 (RUSSELL GENE HIGUCHI) 09.11.2004, claims	18
Y	US 20110018538 A1 (T2 BIOSYSTEMS, INC.) 27.01.2011, claims	8, 18