# Direct Visual Detection of Nucleic Acid using Gold Nanoparticles

## Field

The invention relates to gold nanoparticles and their use in nucleic acid detection by a visual change.

## Background

DNA assays have traditionally been lab-based, primarily because of our reliance on PCR as the basis for target enrichment and DNA detection. Systems that have been developed for point-of-care DNA detection still leverage on PCR, and focus on finding ways to make the PCR portable. This has been achieved through the adoption of sophisticated optics[1], fluidics[2], electrochemical methods[3] and thermocycling[4], which require the consumption of portable power and reagents that pose issues with thermostability. Nevertheless systems like the Cepheid and R.A.P.I.D. have been developed for on-site molecular diagnostics of bio-terror agents.[5] There is a need for a method and system that can be easily used in any field conditions that is simple enough for almost anyone to use.

Some species of bacteria are pathogenic and can affect the safety of food and water. Such bacteria include *Vibrio cholerae*, *Salmonella*, and *Escherichia coli*. *V. cholerae* causes cholera which often causes epidemics in area struck by disasters such as floods, tsunamis, earthquake or area where water sanitation is difficult. In disaster relief situations it is often not possible to move equipment or have access to electricity to conduct tests to determine water or food safety. Salmonella has been responsible for many severe food poisoning epidemics. Within the US alone it accounted for 129 outbreaks and 3290 human infections annually from the period from 2003 to 2007.[6] Detection at the point of care and the capability to monitor the quality control of food-stocks is thus a major concern in food security; especially in an environment where emergent threats can cause serious concerns, for example the recent shiga-toxin producing *E.coli* O104:H4 outbreak in Europe.[7] There is a need for an

easy to use, nucleic acid assay to potentially remove the need to send samples back to a laboratory.

Earlier studies on visual assays reported the use of various techniques such as G-quadruplex[8-10], molecular beacons[11, 12], quantum dots[13,14] and gold nanoparticles[15-18] to detect the presence of a target nucleotide sequence. Although these methods are effective in detecting synthetic DNA sequences, all require a PCR or an enrichment step. This may be instrument dependent, time-consuming, labour-intensive and as a result delays the reporting of results. An ideal point-of-care nucleic acid assay should be robust and effective, easy to use, and have a small logistic footprint.

Gold nanoparticle (AuNPs) have for the last decade been applied highly effectively as a visual detection tool. These nanoparticles produce a visual readout based on varying inter-particle distance when target reagents are added, providing a capability for sensing. These particles can be coated with DNA or ligands, for target specific detection. When AuNPs are suspended in colloids, well dispersed AuNP appear red. As the inter-particle distance decreases, the plasmon resonance shifts, resulting in the AuNPs appearing purple, violet or blue. In addition, highly aggregated AuNPs precipitate out of the colloid to form black/grey sediments, hence providing a means for biological and chemical sensing.[19-22] Pioneering work by Mirkin's group has demonstrated that synthetic DNA sequences can be detected using AuNP decorated with thiolated probes.[23] A variety of assay formats have followed using AuNPs, nearly all of which have applied this technique to the use of synthetic or amplified genomic sequences. Rothberg's group established that single stranded DNA (ssDNA) could adsorb onto AuNP surface whereas, double stranded DNA (dsDNA) are too stable to dissociate and do the same.[24]

There is a need for a point-of-care nucleic acid assay that is robust and effective, easy to use, with a small logistic footprint.

The present invention seeks to ameliorate at least one of the problems mentioned above.

## Summary

Accordingly a first aspect of the invention includes a method of detecting double stranded nucleic acid comprising the steps of:

a. Making a thiolated probe with complimentary nucleic acid base pairs to a target nucleic acid;

b. Adding the thiolated probe with a sample suspected of containing the target nucleic acid and a gold nanoparticle wherein a colour change is observed if the sample contains the target nucleic acid.

This method may have the advantage of detecting double stranded nucleic acid visually, by the eye, without the need for a power source or sophisticated instrumentation. This method thus opens in-roads to direct visual detection, bringing sophisticated DNA analysis capabilities to the point of need.

Another aspect of the invention includes a nucleic acid detection system for visually detecting double stranded nucleic acid comprising a thiolated probe having complimentary nucleic acid base pairs to a target nucleic acid capable of hybridising to a target nucleic acid in a sample suspected of containing the target nucleic acid; and a gold nanoparticle capable of a colour change if the sample contains the target nucleic acid.

Another aspect of the invention includes a device for visually detecting double stranded nucleic acid comprising a first compartment containing a thiolated probe having complimentary nucleic acid base pairs to a target nucleic acid; and a second compartment containing a gold nanoparticle.

Other aspects and advantages of the invention will become apparent to those skilled in the art from a review of the ensuing description, which proceeds with reference to the following illustrative drawings of preferred embodiments.

## Brief Description of the Figures

**Figure 1.** Visual assay for whole genome DNA sensing. The addition of thiolated probes specific to the target gene aggregates AuNPs resulting in a colorimetric change to violet. In the presence of a non-target genomic DNA extract, no visual colour change occurs.

**Figure 2.** Visual readouts for L+R thiolated invA probes incubated with genomic DNA and AuNPs. A visual colorimetric change from red to violet was observed only in the tube with Salmonella target DNA. The tube containing human DNA and a negative control, with no DNA added, remained red even after prolonged incubation. Absorbance readings at 620nm and 520nm were obtained using a nanodrop spectrophotometer. Error bars refer to standard deviations from 3 independent sets of experiments.

**Figure 3.** Effect of the order of reagent addition on visual output. There is a wide range of responses between the different orders of addition. Best sensitivity was achieved when target DNA was pre-incubated with the probes for 5 minutes before the addition of AuNPs. **A** Order of addition: 1.Target DNA, 2. Probes then 3. Gold Nanoparticles. **B.** Order of addition: 1. Gold Nanoparticles, 2. Probes, then 3. Target DNA. **C.** Order of addition: 1. Gold Nanoparticles, 2. Target DNA, then 3. Probes

**Figure 4.** Varying NaCl amounts and its effects on visual output. Effect of increasing salt concentrations. a) Right Probe only. b) Left Probe only. c) No target. c) Profile in a double probe system lacking the target exhibited aggregation of AuNP at final buffer concentration of 423mM of NaCl, after a 1 hour incubation. To be safe, any signals observed over 256mM of NaCl may be considered false positives, under this set of assay conditions. Signals observed in a) and b) at 560mM of NaCl, may be attributed to be false positive results.

**Figure 5.** Effect of mutations in probes on visual output. Effect of having mutations in the probes reduced the signal. As long as there is a fully

complementary probe (LP or RP) aggregation seems to occur spontaneously although the visual readout is compromised due to AuNP precipitation as illustrated in the picture taken beyond 5 hours later. Graph depicts $A_{620}/A_{520}$ readings for the final time-point.

**Figure 6.** Effect of using a single probe on visual output. Single probe system is used to compare its effectiveness against the double probe system. The single probe system assays were prepared by incubating the single probe solution with the target genomic DNA extract and adding 0.3M NaCl, 1× PBS, 0.01%SDS to the mixture to make up for the buffer concentration. Despite the absence of another probe, the end-point for aggregation was similar to that of the positive control. Graph depicts $A_{620}/A_{520}$ readings for the final time-point.

**Figure 7.** Effect of having non-target genomic DNA on visual output. Different non-target samples tested under the same set of conditions and Salmonella probes. This demonstrated the specificity and robustness of the probes in being able detect Salmonella amongst *E.coli* and human genomic DNA extracts (human cell lines obtained from Corriell Laboratories). The final assay concentrations of Salmonella and *E. coli* DNA used were approximately 1ng/µl and human DNA was used at approximately 3 ng/µl.

**Figure 8.** TEM Images of gold nanoparticles. The visual assay was performed for a subset of the available targets and probes for Salmonella detection, with the intent to assess the organization patterns of the AuNP clusters using electron microscopy. It was observed that the size of aggregates in TEM varied in a sequence specific manner. Where aggregation did not take place (in the absence of target or in the presence of non-target DNA), the particles were highly diffuse, with no clear aggregation. Aggressive aggregation was observed in the positive control. Where mismatched probes were used, small clusters of particles were observed. Line in the inset SEM images depicts 50nm, 100nm, 50nm, 50nm and 50nm lengths from left to right, respectively.

**Figure 9.** The visual assay represents the colorimetric change in each assay with the above combinations of target and probe DNA sequences. The bar chart represents the absorbance ratio: A620/A520 which captures the changes in the UV-Vis spectrum. Error bars refers to standard deviations from 3 independent sets of experiments.

**Figure 10.** A device for visual detection of a target nucleic acid from double stranded nucleic acid showing the step wise use of the device: **A** Opening the device; **B** adding the sample suspected of containing the target nucleic acid to the thiolated probes; **C** removing the nanoparticles from the second compartment; **D** mixing the nanoparticles with the thiolated probes and the sample; and **E** detection of a colorimetric change to violet where the sample contains the target nucleic acid.

**Detailed description of preferred embodiments**

In attempting to move beyond PCR as the method for target specific double stranded nucleic acid detection, we have herein developed a visual genomic nucleic acid detection system using gold nanoparticles (AuNPs,) and thiolated probes. We modelled this system with a common food-borne pathogen *Salmonella enterica*, by designing probes specific to invasion A gene (invA). A visual, and easy to use, nucleic acid assay can thus contribute greatly to far-forward detection capabilities, potentially by removing the need to send samples back to a laboratory. We have expanded this to include E. Coli and V. *cholerae* and it appears that the method and system may be suitable for a wide range of bacteria or any double stranded nucleic acid (dsDNA) such as from other prokaryotic cells, eukaryotic cells, viruses and any other nucleic acid sequences that may need detection.

We hypothesized that dsDNA genomic extracts could be detected sequence specifically by using AuNPs, and designed a series of experiments to optimize conditions that could achieve this outcome (Fig. 1). One would not expect that

dsDNA could be detected in this manner, simply because of the need for genomic nucleic acid to be denatured for optimal hybridization with detection probes. We nevertheless wanted to overcome this, and attempted the assay under ambient room temperatures.

In summary, the assay is simple and moderately rapid. It allows both the preparation and detection step to occur at room temperature and does not necessarily need a PCR step. With these advantages, the AuNP-based detection has desirable qualities that can be employed at the point of care, potentially in areas of environmental surveillance and food security.

Even though the sensitivity is at the low femtomolar range, which is nowhere near the single molecule sensitivity of PCR, the assay works with simple and readily available agents such as thiolated probes which may be readily designed and synthesized, and AuNP which are all stable and can be stored conveniently. Since power is not required to perform the visual assay, this platform could also be applied broadly during humanitarian assistance and disaster relief operations.

Referring to Figure 1 the method of detecting double stranded nucleic acid comprising the steps of; (a) making a thiolated probe 4 with a complimentary nucleic acid base pairs to a target nucleic acid; and (b) adding the thiolated probe 4 with a sample suspected of containing the target nucleic acid 2 and a gold nanoparticle 6 wherein a colour change is the sample contains the target nucleic acid 8. Similarly where the thiolated probe 4 is added to a non-target nucleic acid 10 and a gold nanoparticle 6 and no colour change is observed 12 the sample does not contain the target nucleic acid.

Preferably the method is conducted without amplification of the nucleic acid; hence the nucleic acid is not amplified or has not been amplified by PCR or other techniques known in the art.

Preferably, the incubation is conducted in an ionic solution having an ionic concentration of 400mM or less. More preferably, the incubation is conducted in an ionic solution having an ionic concentration of 250mM or less. In one embodiment the ionic buffer is Sodium Chloride NaCl. The incubation solution may include other buffering or chemical solutions that are known in the art to support a biological system.

Preferably, the gold nonoparticle 6 is added after the thiolated probe 4 has been incubated with a cell suspected of containing the target nucleic acid 2.

Preferably, the sample suspected of containing the target nucleic acid 2 is a bacterium, such as *Salmonella; Escherichia Coli*; *Bacillus anthracis; Vibrio cholera;* or any other bacteria containing double stranded nucleic acid known to be pathogenic in mammals including but not limited to humans, horses, cows, dogs or cats.

Preferably, the target nucleic acid 2 is any double stranded nucleic acid sequence unique to a single species or type. Preferably, the species is a bacterium, such as *Salmonella; Escherichia Coli*; *Bacillus anthracis; Vibrio cholera;* or any other bacteria known to be pathogenic in mammals including but not limited to humans, horses, cows, dogs or cats.

Preferably, the thiolated probe 4 comprises a complimentary nucleic acid base pair with at least 90% contiguous to a target nucleic acid. In a preferred embodiment the thiolated probe 4 comprises any one of SEQ ID NOS. 1, 2, 3, 4, 9, 14, 15, 12, 13, 16 or 17. The probe can be selected from any one of SEQ ID NOS. 1, 2, 3, 4, 9, 14, 15, 12, 13, 16 and 17.

In one embodiment the method further comprises the step of lysing the cell suspected of containing the target nucleic acid 2 prior to the incubation. . This can be achieved by any method known in the art suitable for lysing cells Such as; Tris-EDTA buffer, chelex-100, 2% SDS and 10% Triton-100; Urea-SDS-

proteinase; 10% SDS, heating at 65°C; Lysozyme (with and without freezing); or Grinding/bead beating or any other established method.

The nucleic acid detection system for visually detecting double stranded nucleic acid comprises a thiolated probe 4 having complimentary nucleic acid base pairs to a target nucleic acid 2 capable of hybridising to a target nucleic acid in a sample suspected of containing the target nucleic acid; and a gold nanoparticle 6 capable of a colour change if the sample contains the target nucleic acid. Has the advantage of being able to detect the presence or absence of pathogenic bacteria anywhere just visually by eye without requiring a power source.

The system includes a kit for visually detecting double stranded nucleic acid comprises a thiolated probe 4 having complimentary nucleic acid base pairs to a target nucleic acid 2 capable of hybridising to a target nucleic acid in a sample suspected of containing the target nucleic acid; and a gold nanoparticle 6 capable of a colour change if the sample contains the target nucleic acid.

Preferably the thiolated probe of the system is at least 90% contiguous complimentary nucleic acid base pairs to the target nucleic acid. In one embodiment the thiolated probe 4 of the system comprises any one of SEQ ID NOS. 1, 2, 3, 4, 9, 14, 15, 12, 13, 16 or 17. In another embodiment the thiolated probe can be selected from any one of SEQ ID NOS. 1, 2, 3, 4, 9, 14, 15, 12, 13, 16 and 17.

Preferably the gold nanoparticle ranges in size from 1nm to 100nm. In a preferred embodiment the gold nanoparticle is about 15 nm. This means that when there is a suspension formed of gold nanoparticles the average size of the gold nanoparticle is 15 nm with the possibility that the suspension includes nanoparticle that range from 10 to 20 nm.

In a preferred embodiment the system may further comprise an ionic solution having an ionic concentration of 400mM or less. In one embodiment the ionic solution of the system has an ionic concentration of 250mM or less.

In one embodiment the system further comprising a means of cell lysing a cell such as a cell lysing buffer or a mechanical lysing device. Any means known in the art suitable for lysing cells Such as; Tris-EDTA buffer, chelex-100, 2% SDS and 10% Triton-100; Urea-SDS-proteinase; 10% SDS, heating at 65°C; Lysozyme (with and without freezing); or Grinding/bead beating or any other established means of lysing a cell.

The method may be conducted according to any technique or using any device known in the art.

In one embodiment the method may be conducted using the device depicted in Figure 10. Whereby the device is a modified Ependorf tube comprising a compartment containing a thiolated probe 4 having complimentary nucleic acid base pairs to a target nucleic acid hermetically sealed 16 within the compartment; and a second compartment containing a gold nanoparticle 6. The gold nanoparticle 6 is contained in a compartment within the lid of the ependorf tube. The compartments may be sealed off with parafilm, a suitable polymer or any other material that is suitable to protect the probe 4 and the nanoparticle from perishing or denaturing but is able to be perforated or removed quickly when testing is required.

Preferably the device for visually detecting double stranded nucleic acid comprises a first compartment containing a thiolated probe having complimentary nucleic acid base pairs to a target nucleic acid; and a second compartment containing a gold nanoparticle.

Preferably, the thiolated probe 4 of the device comprises a complimentary nucleic acid base pair with at least 90% contiguous to a target nucleic acid.In a preferred embodiment the thiolated probe 4 comprises any one of SEQ ID

NOS. 1, 2, 3, 4, 9, 14, 15, 12, 13, 16 or 17. The probe can be selected from any one of SEQ ID NOS. 1, 2, 3, 4, 9, 14, 15, 12, 13, 16 and 17.

Preferably, the device further comprises a delivery tube 14 suitable for piercing the seal 16; delivering a sample suspected of containing a target nucleic acid 2; delivering an ionic solution having an ionic concentration of 400mM or less; and piercing the second compartment containing the gold nanoparticle 6 such that the gold nanoparticle is able to fall into the compartment containing a thiolated probe 4 and the gold nanopartical 6 is capable of having a visual colour change in the presence of the target nucleic acid.

The device could easily be adapted to work in a 96 well plate or snap ampoules which allow components to be mixed with the sample suspected of containing a target nucleic acid 2 or any other device which is known in the art that will allow the thiolated probe 4, the sample suspected of containing a target nucleic acid 2 and the gold nanoparticle 6 to be added according the the method described herein.

The delivery tube could be any device able to deliver fluid to the system such as a syringe, a pipette or any other suitable means known in the art. Similarly the delivery tube does not need to be capable of piecing a seal as is shown in this embodiment. It would be understood that there could be many other means of opening the seal such as a separate implement for piecing the seal. Alternatively, the seal could be designed for perforation or a peel away detachment or could be a cap.

**Nucleic acids**

According to the invention there is provided a method system and device for visually detecting a nucleic acid molecule which molecule typically encodes a polypeptide. Specifically the nucleic acid may include DNA molecules; a polynucleotide; a cDNA; an RNA, DNA or a mixed polymer or others

molecules listed below or known in the art. The term embraces a nucleic acid sequence that includes recombinant or cloned DNA isolates and chemically synthesized analogs or analogs biologically synthesized by heterologous systems.

"gene sequence," " gene," " nucleic acids" or "polynucleotide" each refer to polynucleotides that are likely to be expressed in a cell suspected of containing the target nucleic acid. The gene sequence is intended to include coding sequences, intervening sequences and regulatory elements controlling transcription and/or translation. The gene sequence is intended to include all allelic variations of the DNA sequence.

A nucleic acid or fragment thereof is "substantially homologous" ("or substantially similar") to another if, when optimally aligned (with appropriate nucleotide insertions or deletions) with the other nucleic acid (or its complementary strand), there is nucleotide sequence identity in at least about 90%, and more preferably at least about 95-98% of the nucleotide bases.

Alternatively, substantial homology or (identity) exists when a nucleic acid or fragment thereof will hybridise to another nucleic acid (or a complementary strand thereof) under selective hybridisation conditions, to a strand, or to its complement. Selectivity of hybridisation exists when hybridisation that is substantially more selective than total lack of specificity occurs. Typically, selective hybridisation will occur when there is at least about 87% identity over a stretch of at least about 15 nucleotides, preferably at least about 90%. The length of homology comparison, as described, may be over longer stretches, and in certain embodiments will often be over a stretch of at least about nine nucleotides, usually at least about 15 nucleotides, at least about 20 nucleotides, at least about 30 or more nucleotides.

Thus, polynucleotides of the invention preferably have at least 87%, more preferably at least 90% homology to the sequences shown in the sequence listings herein. More preferably there is at least 95%, more preferably at least 98%, homology. Nucleotide homology comparisons may be conducted as

known in the art. A preferred sequence comparison program is the GCG Wisconsin Bestfit program.

Nucleotide sequences are preferably at least 15 nucleotides in length, more preferably at least 20, 30, 40, 50, 100 or 200 nucleotides in length.

Generally, the shorter the length of the polynucleotide, the greater the homology required to obtain selective hybridization. Consequently, where a polynucleotide of the invention consists of less than about 30 nucleotides, it is preferred that the % identity is greater than 90% or 95% compared with the nucleotide sequences for detection.

Nucleic acid hybridisation will be affected by such conditions as salt concentration, temperature, or organic solvents, in addition to the base composition, length of the complementary strands, and the number of nucleotide base mismatches between the hybridizing nucleic acids, as will be readily appreciated by those skilled in the art.

The "polynucleotide" compositions of this invention include RNA, cDNA, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those skilled in the art. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.), charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), pendent moieties (e.g., polypeptides), intercalators (e.g., acridine, psoralen, etc.), chelators, alkylators, and modified linkages (e.g., alpha anomeric nucleic acids, etc.). Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen bonding and other chemical interactions. Such molecules are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of the molecule.

cDNA or genomic libraries of various types may be screened as natural sources of the nucleic acids of the present invention.

The nucleic acid sequences used in this invention will usually comprise at least about five codons (15 nucleotides), more usually at least about 7-15 codons, and most preferably, at least about 35 codons. One or more introns may also be present. This number of nucleotides is usually about the minimal length required for a successful probe that would hybridize specifically with a target nucleic acid sequence.

As used herein, the terms " gene sequence," and " allele" refer to the double-stranded DNA comprising the gene sequence, allele, or region, comprising the gene sequence, allele or region (i.e. either of the coding and non-coding strands).

As used herein, a "portion" of the gene sequence or region or allele is defined as having a minimal size of at least about eight nucleotides, or preferably about 15 nucleotides, or more preferably at least about 25 nucleotides, and may have a minimal size of at least about 40 nucleotides.

Probe sequences may also hybridize specifically to duplex DNA under certain conditions to form triplex or other higher order DNA complexes. The preparation of such probes and suitable hybridisation conditions are well known in the art.

Detectably labeled nucleic acid molecules hybridisable to a DNA molecule of the invention are also provided and include nucleic acid molecules hybridisable to a non-coding region of a nucleic acid, which non-coding region is selected from the group consisting of an intron, a 5′ non-coding region, and a 3′ non-coding region.

"Probes". Polynucleotide polymorphisms associated with target nucleic acid sequences are detected by hybridisation with a polynucleotide probe which forms a stable hybrid with that of the target sequence, under moderately stringent hybridisation and wash conditions. If it is expected that the probes will be perfectly complementary to the target sequence, stringent conditions

will be used or at least 90% complimentary to the target sequence. Hybridisation stringency may be lessened if some mismatching is expected, for example, if variants are expected with the result that the probe will not be completely complementary.

Probes for double stranded nucleic acid may be derived from the sequences of the target nucleic acid region or its cDNAs. The probes may be of any suitable length, which span all or a portion of the region and which allow specific hybridisation to the region. If the target sequence contains a sequence identical or 90% similar to that of the probe, the probes may be short, e.g., in the range of about 8-30 base pairs, since the hybrid will be relatively stable under even stringent conditions. If some degree of mismatch is expected with the probe, i.e., if it is suspected that the probe will hybridize to a variant region, a longer probe may be employed which hybridises to the target sequence with the requisite specificity. The probes are thiolated.

Probes comprising synthetic oligonucleotides or other polynucleotides of the present invention may be derived from naturally occurring or recombinant single- or double-stranded polynucleotides, or be chemically synthesized.

**A Gold nanoparticle**

A Gold nanoparticle includes a nanoparticle that can reflect colours in a detectable manner. Detection may be via spectrophotometry or by eye. Hence a gold nanoparticle may comprise a plurality of gold nanoparticles at a concentration that can be detected for example seen with the eye. Preferably the gold nanoparticle comprises a nanoparticle suspension.

'Gold nanoparticles', are sub-micrometer-sized particles typically having dimensions ranging from 1-100 nm. Small nanoparticles absorb light in the blue-green portion of the spectrum (~400-500 nm) while red light (~700 nm) is reflected, yielding a deep red colour. As particle size increases, the wavelength of surface plasmon resonance related absorption shifts to longer, redder wavelengths. This means that red light is now adsorbed, and bluer light is reflected, yielding particles with a pale blue or purple colour.

Generally, gold nanoparticles are produced in a liquid ("liquid chemical methods") by reduction of choroauric acid ($H[AuCl_4]$), although more advanced and precise methods do exist. After dissolving $H[AuCl_4]$, the solution is rapidly stirred while a reducing agent is added. This causes $Au^{3+}$ ions to be reduced to neutral gold atoms. As more and more of these gold atoms form, the solution becomes saturated, and gold gradually starts to precipitate in the form of sub-nanometer particles. The rest of the gold atoms that form stick to the existing particles, and, if the solution is stirred vigorously enough, the particles will be fairly uniform in size.

To prevent the particles from aggregating, some sort of stabilizing agent that sticks to the nanoparticle surface is usually added. They can be functionalized with various organic ligands to create organic-inorganic hybrids with advanced functionality. It can also be synthesised by laser ablation. Other synthesis methods are known in the art. Any gold nanoparticle of less than 100nm would be suitable for use in the method system or device.

Preferably the gold nanoparticle ranges in size from 1nm to 100nm. In a preferred embodiment the gold nanoparticle is about 15 nm. This means that when there is a suspension formed of gold nanoparticles the average size of the gold nanoparticle is 15 nm with the possibility that the suspension includes nanoparticle that range from 10 to 20 nm.

**Sample**

A "sample", as used herein, refers to an sample that may contain double stranded nucleic acid. Such a sample may include a water sample, a food sample or a biological sample obtained from plants or animals, or from body fluid of an animal including a mammal such as a human. The sample may be a "clinical sample," which is a sample derived from a patient such as a fine needle biopsy sample. A "sample" may also include cells isolated from fluids such as blood, serum, saliva, urine, faeces and the like. Samples can be obtained by methods known to those skilled in the art.

**Target nucleic acid**

A "target nucleic acid" is from any double stranded nucleic acid of interest such as from prokaryotic cells, eukaryotic cells, viruses and any other nucleic acid sequences that may need detection. Preferably a double stranded nucleic acid that would be of interest to detect would come from a pathogen that may cause infection in a host but it is possible to detect any double stranded nucleic acid, hence, any double stranded nucleic acid could be the target nucleic acid.

Preferably, the target nucleic acid is from a bacterium, such as *Salmonella; Escherichia Coli*; *Bacillus anthracis; Vibrio cholera;* or any other bacteria containing double stranded nucleic acid known to be pathogenic in mammals including but not limited to humans, horses, cows, dogs or cats. Preferably the target nucleic acid is unique to the genetic code or sequence of a species or a strain or a type or a nucleic acid that is of interest for detection. A probe as described herein is then designed to match a conserved and or unique section of the target nucleic acid.

**Examples.**

Initially, we started out by attempting to detect double stranded PCR products (data not shown). A region within the invasion (invA) gene of *Salmonella enterica* was amplified alongside a region of non-target sequence and a negative control. The products were left to stand with 2 thiolated probes that bound to two contiguous 15bp stretches within the invA gene. After the addition of AuNP colloids with average diameters of 15nm, we were able to observe visual readouts for Salmonella, through a colorimetric change from red to violet. The negatives and non-target human genomic sequences remained red, as should be expected.

Since a visual differentiation was attainable within a post-PCR format, we wanted to extend this result with genomic DNA detection. Genomic DNA

extracts were left to stand with thiolated probes specific to Salmonella. After the addition of AuNPs to each of the mixtures, we were able to observe a visual change with the Salmonella genomic DNA (Fig. 2). The negatives which comprised of non-target human sequences and no target did not exhibit a colorimetric change and remained red thus, indicating a possibility of detecting DNA sequences within genomic targets.

We hypothesized that the hybridisation with two probes covering the full 30 bp stretch would produce significantly enhanced signals compared to using each thiolated left (L) and right (R) probe individually that target 15 bp each (invA) gene. Hence, we sought to examine how much the signals were compromised by the addition of just single probes. Assays, containing a single probe specific to 30 bp of (invA) gene of *Salmonella enterica*, were compared against the positive control which had two probes specific to the target gene. The visual readouts in both one probe and two probes containing assays were rather similar to our surprise, producing sufficiently discernible signals for detection visually, by eye. In the other test conditions, which comprised of non-specific probes, non-targets and no DNA, no colorimetric change was observed. (Fig. 6)

Increasing ionic concentration, for example with the addition of salts such as NaCl, can mask the electrostatic forces between the citrate stabilised AuNPs and mediate aggregation. In order to establish the robustness of the assay, we tested the assay across varying salt concentrations. This was to ensure that truly negative samples did not exhibit any colorimetric change under higher NaCl concentrations in the buffer and thus, result in false positives. Hence, we performed the visual assay under varying concentrations of NaCl, and with different combinations of probes and targets. (Fig. 4) The assays were pictured at three time points to reflect the stability of AuNPs over at least an hour as compared to the positive control in which the AuNPs aggregated within 15 minutes. Interestingly, we found that the negative controls did not aggregate until at least about three times (256 mM) the normal NaCl concentration than that used in our routine assay conditions. This

demonstrates that the assay well-tolerates ionic salt concentrations, and can potentially be deployed under varying concentrations of salt.

Since two out of the three components of the assay were allowed to stand before observing a visual readout, we were curious to find out if the order of addition would have an effect on the visual readout. We performed parallel Salmonella genomic DNA concentration dependent assays by pre-mixing two of the three main components at room temperatures, before adding the third reagent to trigger the colour change. This included premixing: 1. genomic DNA, probes; 2. AuNPs, probes; 3. AuNPs, genomic DNA (Fig. 3). In all cases, it was possible to elucidate positive signals, but at different detection thresholds. The best sensitivity was obtained when genomic DNA and probes were allowed to stand before the addition of AuNPs, the sensitivity threshold for the target was at 37fM (data not shown). A weaker sensitivity of 320 fM was observed when AuNPs were allowed to stand with genomic DNA. Another feature that both target and probe concentration dependant profiles revealed was that the increase in visual readout was not a gradual response. Instead, it resembled a sigmoid curve and hinted a transition concentration beyond which the assay would produce a distinct colour change.

Despite being able to detect the Salmonella genome in this visual manner, we wanted to ensure that this was no co-incidence and that binding was truly dependent on the designed sequence of our probes. In order to establish the specificity of the probes used so far in the visual Salmonella detection mechanism, we designed probes with inherent single and double point-mutations and tested them to pick out the invA gene of *Salmonella enterica*. We were expecting to see weaker or no visual readouts from using these probes, when applied in parallel experiments to detect the Salmonella genome. The mutant probes were tested in various combinations. After the addition of AuNPs, the visual readouts were compared against the positive control and the negative controls comprising non-targets, non-specific probes and no-target assays (Fig. 5). The presence of a single mis-matched position in the probe did marginally reduce the signal intensities obtained with the

salmonella target. In assays that contained probes with 2 mismatched sequences, there was a significantly reduced colour conversion. These tubes exhibited a negligible colour change, and remained red.

These mutated probes were also used in a single probe setting (Fig. 6), and produce similar results. In assays that had a probe specific to the invA gene, there was visual colorimetric change, whereas in the probes increasing numbers of point-mutations significantly changed the visual output.

Furthermore, in order to test the specificity of the probes to the genomes of choice, they we tested against various different genomic DNA samples (*Escherichia coli* and Human genome samples). Upon addition of AuNPs, only samples with Salmonella DNA present displayed a violet colouration, all other tubes containing non-target DNA sequences remained red (Fig. 7). In addition, we tested a separate set of thiolated probes designed to detect *Bacillus anthracis*. Under the same set of test conditions, no colour change was exhibited when they were tested with Salmonella DNA, demonstrating the requirement of probe specificity (Fig 5).

In addition, we performed TEM experiments to image the aggregation profiles of these AuNPs with varying probes and targets. Intense clustering of AuNPs were observed with the positive controls, with relatively smaller clusters observed with mis-matched probes (Fig 8). As expected no observable clusters were observed in the absence of target or with human genomic target, it was also determined that probe thiolation was critical to visual detection. Probes with the Salmonella targeting sequence but without the thiol group produced no visual readouts (data not shown). This indicated the importance of the probe interactions to the gold AuNP for production of the visual colour change.

To investigate the underlying mechanism further, we performed gel retardation experiments, to determine the interaction sequence and to further understand the need for ordered addition of the reagents, for optimal visual

output. Taken together, these results indicate that the salmonella probes independently complex with the double stranded target sequences. The thiol groups on the probes then mediate the interaction with the AuNPs, resulting in the observed aggregation and the colour change.

With these results, we have demonstrated the ability to detect Salmonella genomic DNA extracts visually using AuNPs. This may be the first report that establishes the utility of gold nanoparticles to detect genomic DNA sequence specifically, without the need for PCR. This in itself is a significant milestone that can be capitalized on in the field of visual DNA detection.

Next we extended the panel of genomic DNA targets to be assayed and integrate the DNA extraction step to this visual detection system with *E. coli*. This will significantly accelerate the capability to detect double-stranded DNA, sequence specifically, outside a laboratory environment.

For E. coli it was first necessary to lyse the target cell prior to incubation with the thiolated probe. This can be achieved by any method known in the art suitable for lysing cells. The following methods and reagents may be considered for bacterial genomic DNA extraction for the visual DNA assay with gold nanoparticles:

1) Tris-EDTA buffer, chelex-100, 2% SDS and 10% Triton-100
2) Urea-SDS-proteinase
3) 10% SDS, heating at 65°C
4) Lysozyme (with and without freezing)
5) Grinding/bead beating

**Materials and equipments.** Autoclaved MilliQ water was used for all buffers, stock solutions of oligonucleotides. Oligonucleotides were synthesised by local manufacturers (Suprenom Pte. Ltd.) with standard HPLC purification (Table 1). Thiolated probe sequences and *Bacillus anthracis* sequences were purchased from Suprenom Pte. Ltd. The Salmonella probe sequences were

purchased from 1st Base Primers Pte. Ltd. All genomic DNA samples were extracted using the Qiagen DNA extraction kit. Aqueous solutions of 15nm diameter gold nanoparticles were purchased from Ted Pella (mean diameter listed by the manufacturer). Absorbance readings were obtained from an ND-1000 spectrophotometer.

***Salmonella enterica* target amplification:** A 10$\mu$L PCR reaction was prepared using 9 ng of genomic DNA with 1.5mM $MgCl_2$, 1x PCR Buffer, 0.3µM Salmonella forward and reverse primers, with 0.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). Cycling conditions included an initial denaturation at 95°C for 3 mins, followed by 34 cycles of 95°C 1min, 53°C 1min, 72°C 1min.

**Non-target control amplification:** An 85bp Emilin1 gene segment from human genomic DNA was amplified. A 10 $\mu$L PCR reaction was prepared using 10 ng of HapMap Genomic DNA sample (extracted from immortalized cell lines, Cat. No: GM7034, Corriell Laboratories) with 1.5mM $MgCl_2$, 1x PCR buffer, 0.3$\mu$M forward and reverse primers and 0.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). Cycling conditions included an initial denaturation at 95°C for 3 mins, followed by 34 cycles of 95°C 1min, 63°C 1min, 72°C 1min.

**Probe preparation:** Protected 5' thiolated end of oligonucleotide probes (3nmoles) were deprotected in 0.1M DTT (final concentration) and purified on size exclusion NAP-5 sephadex spin columns (Amersham, GE Healthcare). The oligonucleotides were left to stand in 0.3M NaCl, 1× PBS. Then, aqueous 10% SDS, was added to the solution (final concentration: 0.01%). These oligonucleotides were used as probes in the visual assay.

**Genome copy number calculation:** The Salmonella enterica genome was approximated to be 5Mbp (UCSC Genome Browser). 3.28ng was added to a 27µl reaction, which corresponded to a concentration of 37fM. The average molecular weight of a base pair was assumed to be 650gmol$^{-1}$. Thus, the number of copies of genomic DNA for 3.28 ng of genomic DNA was

calculated to be $(3.28\times10^{-9}g\times6.022\times10^{23}mol^{-1})/(5\times10^{6}bps\times650gmol^{-1}bp^{-1})$=608 thousand copies.

**Genomic material:** Human genomic DNA was obtained from commercial immortalized human B-Lymphocyte cell lines (Corriell Laboratories), and cultured according to vendor's protocol. The genomic DNA was extracted from the cells using Puregene Cell and Tissue kit (Gentra Systems).

**TEM Imaging:** Drops of colloidal suspension were placed onto carbon coated copper grids and left to dry under a lamp. The copper grids were then analysed on a JEOL JEM 2010F HRTEM with an accelerating voltage of 200keV.

**Table 1.** Probes, primers and target sequences used in the study. The sizes of the resulting amplicons were 119bp for *invA* segment in salmonella DNA and 50bp for the *Emilin1* segment in human DNA.

| SEQ ID NO. | | SEQUENCE |
|---|---|---|
| 1 | Salmonella invA gene Left Probe sequence (L) | 5'HS-$(CH_2)_6$-$[T]_{20}$-GCC-AGT-ACG-ATA-TTC-3' |
| 2 | Salmonella invA gene Right Probe sequence (R) | 5'HS-$(CH_2)_6$-$[T]_{20}$-ATA-AAC-ACC-AAT-ATC-3' |
| 3 | Salmonella invA gene Left Mutated Probe sequence (LM1) (mutated base is underlined) | 5'HS-$(CH_2)_6$-$[T]_{20}$-GCC-AGT-A<u>T</u>G-ATA-TTC-3' |
| 4 | Salmonella invA gene Right Mutated Probe sequence (RM1) (mutated base is underlined) | 5'HS-$(CH_2)_6$-$[T]_{20}$-ATA-AAC-A<u>T</u>C-AAT-ATC-3' |
| 5 | Salmonella invA gene Left Double Mutated Probe sequence (LM2) (mutated bases is underlined) | 5'HS-$(CH_2)_6$-$[T]_{20}$-GC<u>T-G</u>GT-ACG-ATA-TTC-3' |
| 6 | Salmonella invA gene Right Double Mutated Probe sequence (RM2) (mutated bases is underlined) | 5'HS-$(CH_2)_6$-$[T]_{20}$-ATA-AAC-ACC-AA<u>C-G</u>TC-3' |
| 7 | Salmonella invA gene Forward Primer | 5'-CCTGATCGCACTGAATATCG-3' |
| 8 | Salmonella invA gene Reverse Primer | 5'-AACGACCCCATAAACACCAA-3' |

| | | |
|---|---|---|
| 9 | Salmonella target sequence within the InvA gene (Segments in underlined targeted by the L-probe, Segments in *italics* targeted by the R probe) | 5'-GAATATCGTACTGGC*GATATTGG TGTTTAT*-3' |
| 10 | Non-target *Emilin1* Forward Primer | 5'- TCTGCTGAGGCTCTCCTGTT-3' |
| 11 | Non-target *Emilin1* Reverse Primer | 5'-CTGCTTTGAAGTCCACGTAGC-3' |
| *12* | *Bacillus anthracis* Left Probe sequence | 5'HS-$(CH_2)_6$-$[T]_{20}$-TAA-CAA-TAA-TCC-CTC-3' |
| *13* | *Bacillus anthracis* Right Probe sequence | 5'HS-$(CH_2)_6$-$[T]_{20}$-ATC-CTT-ATC-AAT-ATT-3' |
| *14* | *Escherichia Coli* Left Probe sequence | 5'HS-$(CH_2)_6$-$[T]_{20}$-ATC-ACG-CAG-GTT-GGC-3' |
| *15* | *Escherichia Coli* Right Probe sequence | 5'HS-$(CH_2)_6$-$[T]_{20}$-AAC-CAG-ACG-ATA-GTT-3' |
| *16* | *Vibrio cholerae* Left Probe sequence | 5'- HS-$(CH_2)_6$-$[T]_{20}$-CCA-CCT-ACC-TTT-ATG-GTC-C-3' |
| *17* | *Vibrio cholerae* Right Probe sequence | 5'- HS-$(CH_2)_6$-$[T]_{20}$-GGT-TTG-TCG-AAT-TAG-CTT-CAC-C-3' |

**Visual Assay.** The final assay of 27$\mu$l contained a buffer comprising 90mM NaCl, 0.2X PBS, 0.002% SDS, pH 7.0, 320fM of Salmonella genomic DNA extract, 2.1nM each for Salmonella probe sequences and 1.6nM of 15nm AuNPs. 3$\mu$l of both probes with 20nM in buffer (0.3M NaCl, 1× PBS, pH 7.0 and 0.01% SDS) was left to stand with 1$\mu$l of genomic DNA extract (9pM) for 5 minutes. 20$\mu$l of AuNPs at a stock concentration of $2.8x10^{10}$ particles per ml (Ted Pella) was added to the mixture and allowed to stand for about 15 minutes. The absorbance readings were taken typically 15 minutes after adding the AuNPs. After prolonged standing, the AuNPs in the positive control precipitated and started to form black/grey sediments in the tubes. The tubes were photographed using a Samsung (ST50) digital camera 12.2 mega-pixel.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. The invention includes all such variation and modifications. The invention also includes all of the steps, features, formulations and compounds

referred to or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

Each document, reference, patent application or patent cited in this text is expressly incorporated herein in their entirety by reference, which means that it should be read and considered by the reader as part of this text. That the document, reference, patent application or patent cited in this text is not repeated in this text is merely for reasons of conciseness.

Any manufacturer's instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any document incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention.

The present invention is not to be limited in scope by any of the specific embodiments described herein. These embodiments are intended for the purpose of exemplification only. Functionally equivalent products, formulations and methods are clearly within the scope of the invention as described herein.

The invention described herein may include one or more range of values (e.g. size, concentration etc). A range of values will be understood to include all values within the range, including the values defining the range, and values adjacent to the range which lead to the same or substantially the same outcome as the values immediately adjacent to that value which defines the boundary to the range.

Throughout this specification, unless the context requires otherwise, the word "comprise" or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. It is also noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as "comprises", "comprised", "comprising" and the like can have the meaning

attributed to it in U.S. Patent law; e.g., they can mean "includes", "included", "including", and the like; and that terms such as "consisting essentially of" and "consists essentially of" have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention.

Other definitions for selected terms used herein may be found within the detailed description of the invention and apply throughout. Unless otherwise defined, all other scientific and technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

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**Claims:**

1. A method of detecting double stranded nucleic acid comprising the steps of;
    a. making a thiolated probe with complimentary nucleic acid base pairs to a target nucleic acid;
    b. adding the thiolated probe to a sample suspected of containing the target nucleic acid and a gold nanoparticle wherein a colour change is observed if the sample contains the target nucleic acid,

wherein the gold nanoparticle is added after the thiolated probe has been added to the sample suspected of containing the target nucleic acid.

2. The method of claim 1 wherein the double stranded nucleic acid is not amplified.
3. The method of claim 1 wherein the thiolated probe comprises at least 90% contiguous complimentary nucleic acid base pairs to the target nucleic acid
4. The method of claim 1 wherein the incubation is in an ionic solution having an ionic concentration of 400mM or less.
5. The method of claim 1 wherein the incubation is in an ionic solution having an ionic concentration of 250mM or less.
6. The method of claim 1 wherein the gold nanoparticle ranges in size from 1nm to 100 nm
7. The method of claim 1 wherein the gold nanoparticle is about 15 nm
8. The method of claim 1 wherein the sample suspected of containing the target nucleic acid is a bacterium.
9. The method of claim 8 wherein the bacterium is a *Salmonella*.
10. The method of claim 9 wherein the thiolated probe comprises SEQ ID NOS. 1, 2, 3, 4, or 9.
11. The method of claim 8wherein the bacterium is a *Escherichia Coli*.
12. The method of claim 11 wherein the thiolated probe comprises SEQ ID NOS. 14, or 15.
13. The method of claim 8 wherein the bacterium is a *Bacillus anthracis.*

14. The method of claim 13 wherein the thiolated probe comprises SEQ ID NOS. 12 or 13.
15. The method of claim 8 wherein the bacterium is a *Vibrio cholera.*
16. The method of claim 15 wherein the thiolated probe comprises SEQ ID NOS. 16 or 17.
17. The method of claim 1 further comprising the step of lysing a cell in the sample suspected of containing the target nucleic acid prior to adding the thiolated probe.
18. A nucleic acid detection system for visually detecting double stranded nucleic acid comprising a thiolated probe having complimentary nucleic acid base pairs to a target nucleic acid and which are capable of hybridising to said target nucleic acid in a sample suspected of containing the target nucleic acid; and a gold nanoparticle capable of a colour change if the sample contains the target nucleic acid, wherein said nucleic acid detection system does not require a power source so visual detection of DNA can be carried out at a point of need.
19. The system of claim 18 wherein the double stranded nucleic acid is not amplified.
20. The system of claim 18 wherein the thiolated probe comprises at least 90% contiguous complimentary nucleic acid base pairs to the target nucleic acid.
21. The system of claim 18 further comprising an ionic solution having an ionic concentration of 400mM or less.
22. The system of claim 18 further comprising an ionic solution having an ionic concentration of 250mM or less.
23. The system of claim 18 wherein the gold nanoparticle ranges in size from 1nm to 100 nm
24. The system of claim 19 wherein the gold nanoparticle is about 15 nm
25. The system of claim 18 wherein the double stranded nucleic acid to be detected is from a bacterium.
26. The system of claim 25 wherein the bacterium is a *Salmonella*.
27. The system of claim 25 wherein the bacterium is a *Escherichia Coli*.
28. The system of claim 25 wherein the bacterium is a *Bacillus anthracis.*

29. The system of claim 25 wherein the bacterium is a *Vibrio cholera.*
30. The system of claim 18 or 27wherein the thiolated probe comprises SEQ ID NOS. 14, or 15.
31. The system of claim 18 or 28 wherein the thiolated probe comprises SEQ ID NOS. 12 or 13.
32. The system of claim 18 or 26 wherein the thiolated probe comprises SEQ ID NOS. 1, 2, 3, 4, or 9.
33. The system of claim 18 or 29 wherein the thiolated probe comprises SEQ ID NOS. 16 or 17.
34. The system of claim 18 further comprising a means of lysing a cell.