Nanodiagnostics: fast colorimetric method for single nucleotide polymorphism/mutation detection

G. Doria, R. Franco and P. Baptista

Abstract: Advances in nanosciences are having a significant impact in many areas of research. The impact of new nanotechnologies has been particularly large in biodiagnostics, where a number of nanoparticle-based assays have been introduced for biomolecules detection. To date, applications of nanoparticles have largely focused on DNA-functionalised gold nanoparticles used as the target-specific probes. These gold nanoparticle-based systems can be used for the detection of specific sequences of DNA (pathogen detection, characterisation of mutation and/or single nucleotide polymorphisms) or RNA (without prior retro-transcription and amplification). Here a rapid and inexpensive nanoparticle-based method for single-base mismatch detection (single nucleotide polymorphism/mutation) in DNA samples is reported. Gold nanoparticles derivatised with thiol modified oligonucleotides complementary to DNA targets – Au-nanoprobes – are used to distinguish fully complementary from mismatched sequences, with a single-base mismatch. The authors have successfully applied this strategy to detect common mutations within the β-globin gene.

1 Introduction

Nanodiagnostics, defined as the use of nanotechnology (materials, devices or systems) for diagnostics purposes, has become a burgeoning field of interest as more and improved techniques are available to meet the demands of clinical diagnostics for increased sensitivity at lower costs [1]. Single analyte molecules can be assessed through one-to-one interaction between the target analyte and signal generating particles, such as noble metal nanoparticles, quantum dots and magnetic nanoparticles. These nanoparticle systems have been thoroughly applied in DNA/RNA characterisation, detection and separation of biomolecules. Among the best characterised technologies for DNA/RNA screening are noble metal nanoparticles functionalised with ssDNA capable of specifically hybridising to its complementary target.

Gold nanoparticles are associated colloids, which can be synthesised with dimensions ranging from 0.8 to 250 nm. Specific focus has been directed at colloidal gold nanoparticles with 3–100 nm size range, which are rather stable and whose properties can be easily tailored by chemically modifying their surfaces. These nanoparticles are normally charged and very sensitive to changes in the dielectric of the solution. For example, for citrate stabilised particles, the addition of NaCl shields the surface charge and leads to a concomitant decrease in the inter-particle distance and eventual particle aggregation [2]. In solution, monodisperse gold nanoparticles appear red and exhibit a relatively narrow surface plasmon absorption band centred at around 520 nm in the UV-visible spectrum. In contrast, a solution containing aggregated gold nanoparticles appears blue in colour, corresponding to a characteristic red shift in the surface plasmon resonance to higher wavelength. DNA or protein can be used as a linking molecule to aggregate the gold nanoparticles and thus taking advantage of the optical properties of disperse against aggregated gold particles for biodetection assays [3, 4].

The use of thiol-linked oligonucleotide modified gold nanoparticles (herein designated as 'Au-nanoprobes') for the colorimetric detection of DNA targets was first described by Mirkin *et al.* [5]. They used single-stranded oligonucleotide targets that could be detected using two species of Au-nanoprobes, each functionalised with a DNA-oligonucleotide complementary to one half of the given target. More recently, a non-cross-linking DNA hybridisation method was described, where aggregation of the oligonucleotide-functionalised gold nanoparticles is induced by an increasing salt concentration in the presence of complementary oligonucleotides [6]. As discussed earlier, a solution of non-functionalised gold nanoparticles aggregates instantaneously after NaCl addition, which is revealed by a colour change of the solution from red to blue. However, nucleic acid sequences protect gold nanoparticles against aggregation, possibly through electrostatic interactions between the negatively charged phosphate groups of the nucleic acid and the highly polarisable gold nanoparticles [7]. These properties were further exploited and applied in the detection and characterisation of gene expression (RNA target) [8, 9].

Since the human genome project was launched enormous amounts of data on genetic polymorphisms have been discovered [10]. One of the great challenges of modern

doi:10.1049/iet-nbt:20070001

Paper first received 29th January and in revised form 17th April 2007

G. Doria is with the CIGMH/SABT and REQUIMTE, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Campus da Caparica, Caparica 2829-516, Portugal

R. Franco is with the REQUIMTE, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Campus da Caparica, Caparica 2829-516, Portugal

P. Baptista is with the CIGMH/SABT, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Campus da Caparica, Caparica 2829-516, Portugal

E-mail: pmvb@fct.unl.pt

molecular detection strategies is the integration of new genetic information into procedures that can be implemented in rapid, cost-effective and reliable methods. There are several types of polymorphisms in the human genome (e.g. insertions and/or deletions of one or more bases, duplications), but single nucleotide polymorphisms (SNPs) are the more frequent (68%) type of polymorphism [11], with millions of different SNPs reported in several databases '*Celera's Human RefSNP*' (http://www.celera.com), '*NCBI dbSNP*' (http://www.ncbi.nlm.nih.gov/SNP/index.html), '*Human Genome Variation Database – HGVbase*' (http://hgvbase.cgb.ki.se), '*The Human Gene Mutation Database – HGMD*' (http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html) and the- '*Human Genic Bi-Allelic Sequences – HGBASE*' (http://hgbase.interactiva.de). SNPs are widespread in the human genome – located in coding region where they can modify the activity of a protein (non-synonymous SNP/missense SNP); located in the boundary exon/intron where they could modify the splicing process. However, other intronic SNPs and synonymous SNPs (base changes in the coding region that do not lead to an aminoacid change) could also change mRNA stability with potential implications for gene expression. This can also occur as a consequence of SNPs located in the promoter region or intragenic regions; in the latter case as a consequence of enhancing mechanisms [12–15]. Thus, the individual genetic variability as a consequence of SNPs has been associated with individual susceptibility to several multifactorial diseases such as cancer, diabetes and also with individual response to therapeutics [16, 17].

Our approach has been the development of a simple, easy-to-use and inexpensive assay for specific DNA/RNA sequence detection based on gold nanoprobes. The assay is based on a non-cross-linking hybridisation method, where aggregation of the Au-nanoprobes is induced by an increasing salt concentration – the presence of complementary target prevents aggregation and the solution remains red; non-complementary/mismatched targets do not prevent Au-nanoprobe aggregation resulting in a visible change of colour from red to blue. This method has been successfully applied to detect eukaryotic gene expression without retro-transcription or PCR amplification [8]; and in a fast and straightforward assay for *Mycobacterium tuberculosis* DNA detection in clinical samples [18]. Also, to attain better sensitivity and easily achieve quantification of target DNA/RNA samples, we have integrated this method in an amorphous/nanocrystalline silicon device, without the need to functionalise the glass surface with the sequence of interest [19].

Based on this approach we now present a method for the detection of SNP/single-base mutations in DNA samples. As a model for the proof-of-concept, we used common point-mutations in the β-globin gene responsible for β-thalassaemia. The target sequence harbours three of the most frequent mutations causing β-thalassaemia in the Mediterranean and Portuguese populations: ß^{0}IVS1, nt1 (G → A); ß$^{+}$IVS1, nt2 (T → C); and ß$^{+}$IVS1, nt6 (T → C) [20].

2 Materials and Methods

2.1 Materials

All primers and thiol-modified oligonucleotides were synthesised by MWG Biotech (Germany). All chemicals were from Sigma Aldrich and of the highest purity available.

2.2 Methods

2.2.1 Probe design: The probe oligonucleotide sequence 5′-thiol-AACCTTGATACCAAC-3′ was derived from the β-globin gene sequence (GenBank accession no. NG_000007) and designed to overlap a region harbouring three of the most frequent mutations in the Mediterranean and Portuguese populations causing β-thalassaemia [20].

2.2.2 Preparation of gold nanoparticles and Au-nanoprobe: The gold nanoparticles were prepared by the citrate reduction method described by Lee and Meisel [21]. Briefly, 250 ml of 1 mM $HAuCl_4$ were boiled while stirring in a 500 ml round-bottom flask. Twenty-five millilitres of 38.8 mM sodium citrate were quickly added and the mixture was refluxed for 15 min with continuous stirring. The flask was let to cool to room temperature and stored in the dark until use.

The Au-nanoprobe was prepared as described by Baptista *et al.* [8]. Briefly, an appropriate volume of 10 μM thiol-modified oligonucleotide was initially incubated with 6 ml of an aqueous solution of gold nanoparticles $\simeq$8.55 nM) for at least 16 h. The solution was then brought to 10 mM phosphate buffer (pH 7.0), 0.1 M NaCl and allowed to stand for 40 h. After centrifugation, the precipitate was washed with 5 ml of 10 mM phosphate buffer (pH 7.0), 0.1 M NaCl, recentrifuged and redispersed in 5 ml of the same buffer to a final concentration in gold nanoparticles of 8.5 nM. The resulting Au-nanoprobe was stored in the dark at 4°C.

2.2.3 DNA target samples: A 396 and 355 bp fragment of the β-globin and p53 genes, respectively, were PCR amplified from genomic DNA with the following primers: betaglob36 primer 5′-ACTCCCAGGAGCAGGGAGGG CAGG, betaglob103 primer 5′-CAG ATCCCCAAAGGA CTCAAAGAACC TGTG, Pex4-5′ primer 5′-CTGGTCCT CTGACTGCTC and Pex4-3′ primer 5′-CATTGAAGTCT CATGGAAGCC. PCR amplification of human genomic DNA (100 ng) samples was carried out in 50 μl volumes using 0.5 μM of primers and 0.2 mM deoxynucleotide triphosphates with Taq DNA polymerase (Amersham Biosciences) on a Tpersonal Thermocycler (Whatman Biometra, Germany). Following target denaturation at 94ºC for 5 min, PCR was performed for 34 cycles, each cycle consisting of 94ºC for 1 min, annealing at 69ºC for 1 min for the β-globin amplicon or 58ºC for 1 min for the p53 amplicon, extension at 72ºC for 1 min, with a final extension step at 72ºC for 7 min. All PCR amplicon fragments sizes were confirmed using a 1.0% Agarose Electrophoresis Grade gel (Invitrogen, UK) stained with ethidium bromide and visualised under UV light.

The genomic samples used for PCR amplification were screened for the presence of the mutations through amplification refractory mutation system (ARMS) and/or restriction enzyme digestion (RFLP analysis). Further confirmation of results was performed by direct sequencing using ABI Prism 3100 and ABI Prism Big Dye technology (Applied Biosystems).

2.2.4 Au-nanoprobe hybridisation and colour detection: Total volume of 60 μl assay solutions containing the Au-nanoprobe and target DNA were prepared by mixing the gold nanoprobe solution (final concentration 2.5 nM) with the appropriate amplicon (final concentration of 36 μg ml^{-1}) – complementary DNA ('Positive'); non-complementary DNA ('Negative'); 10 mM phosphate

buffer (pH 8) ('Blank'). After 10 min of denaturation at 95°C, the mixtures were allowed to stand for 30 min at room temperature and NaCl was added to a final NaCl concentration of 2 M. After 15 min at room temperature for colour development, photographs were taken and UV-visible spectroscopic measurements were carried out.

2.2.5 UV – visible spectroscopy and photography: Absorption spectra were performed in a UNICAM, model UV2, UV-visible spectrophotometer with Ultra-Micro quartz cells (Hellma, Germany). A 10 mM phosphate buffer with 0.1 M NaCl was used as reference. All photographs were recorded with a Coolpix E950 Digital Camera (Nikon Corp., Japan).

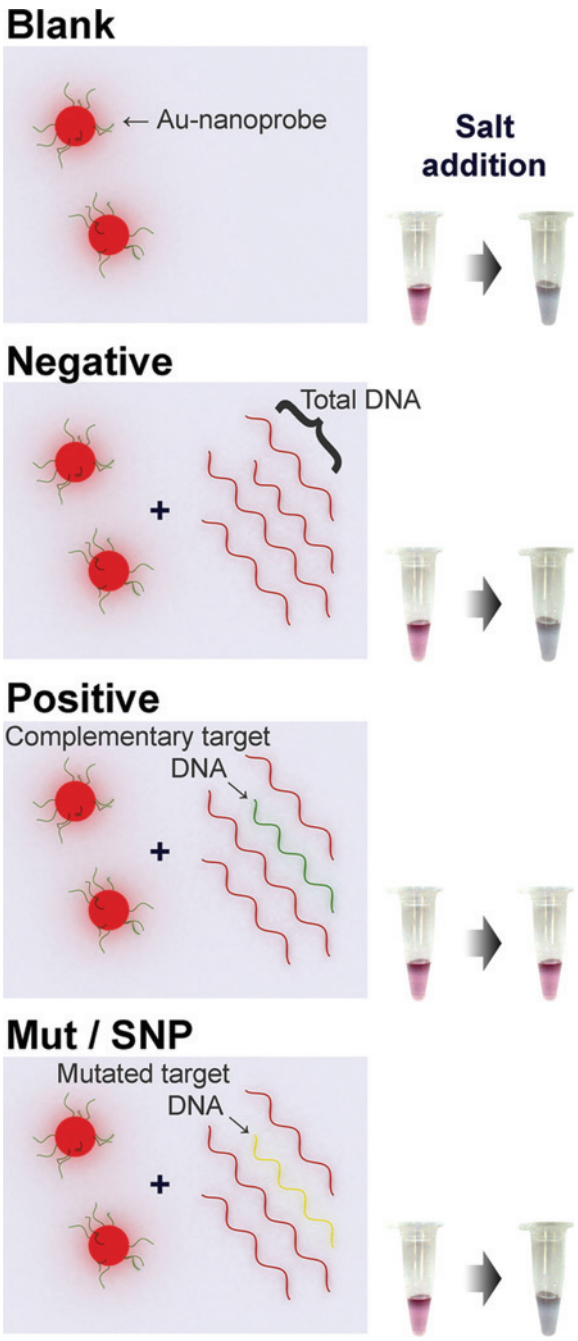

Fig. 1 *Schematic representation of the detection method*

The method consists on visual comparison of test solutions before and after salt induced Au-nanoprobe aggregation: (i) Au-nanoprobe alone – Blank; (ii) Au-nanoprobe in the presence of a non-related DNA sample – Negative; (iii) Au-nanoprobe in the presence of a wild-type DNA sequence – Positive; and (iv) Au-nanoprobe in the presence of DNA harbouring SNP/single point mutation – Mut/SNP

3 Results and discussion

We have been developing a simple colorimetric assay using Au-nanoprobes to detect specific DNA and RNA sequences [8, 18, 19]. The method is based on the colour change of a gold nanoprobe solution, upon increasing salt concentration, in presence of either a complementary or a non-complementary target sequence (see Fig. 1). As mentioned earlier, following an increase in salt concentration the Blank and Negative samples show extensive Au-nanoprobe aggregation, noticeable by the blue colour of the respective solutions; conversely, the Positive sample containing the complementary DNA does not show that effect and the solution retains the initial red colour. Full hybridisation of the DNA sequence on the Au-nanoprobe to its complementary DNA sequence (within the double-stranded DNA target), seems to stabilise the Au-nanoprobe in solution, avoiding aggregation. Colour change upon aggregation of Au-nanoprobes is corroborated by visible spectra, where an intense plasmon resonance band appears at 600–650 nm, with a concomitant decrease of the intensity of the original plasmon resonance at 526 nm (Fig. 2). The intensity of the plasmon resonance at 650 nm, is a convenient way to measure Au-nanoprobe aggregation.

This methodology was further applied for the detection of single-base mismatches with no loss of sensitivity. The probe overlaps a region of the β-globin gene harbouring three different single-point mutations causing β-thalassaemia, and very common in the Mediterranean and Portuguese populations [20]. The relative positions of each of the possible mutations within Au-nanoprobe sequence are presented in Fig. 3. Different amplicons harbouring single-point mutations at these three specific locations were used as target. The amplicons were attained by PCR amplification of genomic DNA samples previously screened for the presence of the mutations (see Section 2.2.1). After hybridisation and upon salt addition, a slight colour change from red to blue was observed. Nevertheless, after the complete revelation time elapsed, all assay solutions had changed colour to blue. UV–visible spectrophotometry was then used in order to obtain a more detailed picture of the amplitude of colour change observed by the naked eye. Fig. 3 shows the spectrophotometric change 15 min after increasing NaCl concentration to 2 M, in solutions containing Au-nanoprobe in

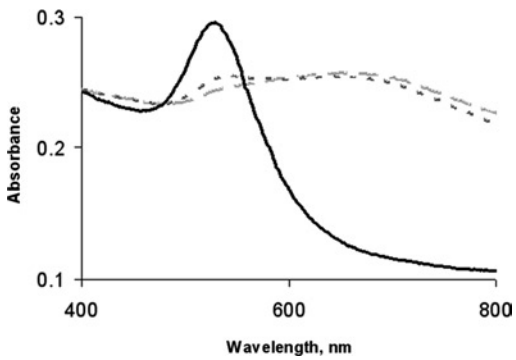

Fig. 2 *Au-nanoprobe aggregation profiles*

UV–vis spectroscopy data of samples after 15 min incubation with [NaCl] = 2 M. Blank (light-grey broken line), Positive (black solid line) and Negative (dark-grey dotted line)

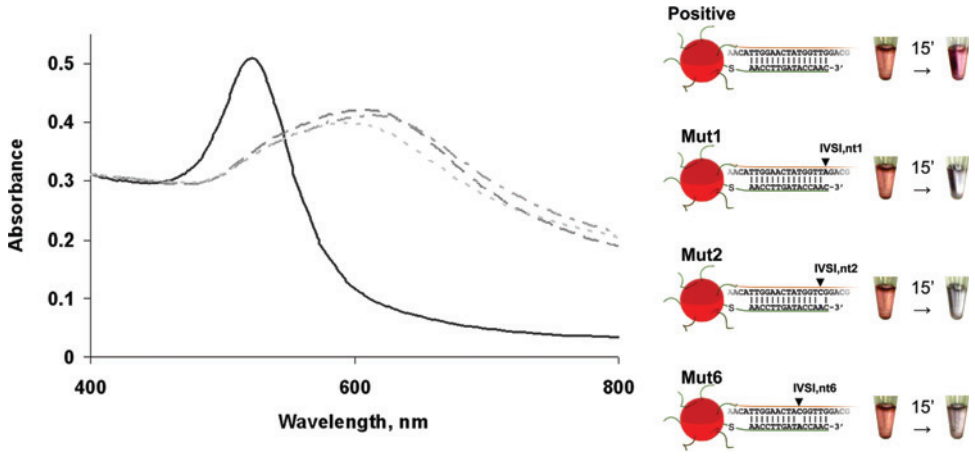

Fig. 3 *Mutation/SNP detection*

UV–vis spectroscopy data of samples after 15 min incubation with [NaCl] = 2 M. Samples are – DNA from normal β-globin gene (Positive – black solid line); DNA harbouring a single-point mutation IVS1, nt1 on the β-globin gene (Mut1 – dark-grey broken line); DNA harbouring a single-point mutation IVS1, nt2 on the β-globin gene (Mut2 – grey dash–dot–dash line); and DNA harbouring a single-point mutation IVS1, nt6 on the β-globin gene (Mut6 – light-grey dotted line). The inlet shows the relative position of the mutation within the Au-nanoprobe sequence, marked by an arrow – filled inverted triangle)

the presence of fully complementary target (Positive) or in presence of targets harbouring the mismatches at different positions within the region of overlap ('Mut1', 'Mut2' and 'Mut6'). From the observed results, one Au-nanoprobe design clearly distinguishes between fully complementary target sequences and the presence of any of the three single-base mutations. The detection assay was repeated at least four times and also using a serial dilution of target DNA. Even though most assays exhibited a clear colour distinction between positive and mismatched samples, best reproducibility of the assay was achieved with DNA concentrations between 18 and 36 μg ml^{-1}. Below that range, the difference in colour between positive and mismatched was not discriminating.

As mentioned earlier, nucleic acid sequences seem to have a protective effect on gold nanoparticles against aggregation, possibly through the electrostatic interactions between the negatively charged phosphate groups on the nucleic acid backbone and the charged gold nanoparticles in a salt solution [7]. This could explain the differences in aggregation observed for Positive against Negative and 'Mismatched'. The presence of nucleic acid in solution may act as a 'buffer' for increasing the ionic strength, thus stabilising the Au-nanoprobe in the non-aggregated form. The increased stability observed for the complementary target may be explained by this buffering effect added to the fact that the full length of the Au-nanoprobe has hybridised with the complementary target DNA sequence. The resulting duplex prevents the salt induced precipitation. The de-stabilisation of the duplex was derived from the mismatch in the sequence, and hence the level of Au-nanoprobe aggregation, might be dependant on the position of the point mutation. Several molecular methods make use of this de-stabilisation effect in order to achieve detection of single-base variation, for example ARMS, and so on [22, 23].

4 Conclusion

The work presented here describes an easy and inexpensive method for single-base mismatch detection (SNP/mutations) directly in DNA samples. Based on the colour changes of a specific Au-nanoprobe solution, fully complementary sequences can be easily distinguished from mismatched sequences, with single-base mismatch. Results can be assessed without the need of further signal enhancement or complicated technological solutions, which are common with other nanoparticle based methodologies. Our methodology allowed for the successful detection of β-globin gene mutations, with one Au-nanoprobe being able to detect three different individual mutations. Since this methodology is limited to a single colour change, two individual reactions are required for full SNP characterisation – distinction of the three possible genotypes (homozygous wild-type, homozygous mutant and heterozygous mutant). Other methods, such as fluorescence, offer single tube detection because they have more than one colour, but are more expensive and more complicated to implement.

Working at the nanoscale may decrease the cost associated with genetic tests and the amount of biological sample needed. The method presented here proved to be selective, sensitive and inexpensive, as the overall cost for one mismatch analysis is only €0.05/test. The inexpensive experimental set-up, the short developing time without the need of signal amplification or temperature control and the fact that colour change can be assessed visually are of great advantage and may prove to be valuable in SNP identification. The proposed colorimetric method is very easy to perform, taking less than 15 min, rendering it suitable for use at point-of-care diagnosis. The use of this methodology without the need for a previous PCR amplification is under investigation; future studies should clarify the influence of genomic DNA in background signal. Further developments include heterozygosity discrimination and other SNP targets.

5 Acknowledgments

This work was supported by FCT/MCES (CIGMH, and Grant POCTI/QUI/45141/2002), and FCGulbenkian (Ref 76436). GD was supported by FCT/MCTES (SFRH/BDE/15544/2005) and STAB Vida, Lda. FLAD (Luso-American Foundation) is acknowledged for financial support. We thank L. Osório-Almeida for genomic DNA samples and valuable discussions in the preparation of the manuscript.

6 References

1 Jain, K.K.: 'Nanodiagnostics: alication of nanotechnology in molecular diagnostics', *Expert Rev. Mol. Diagn.*, 2003, **3**, pp. 153–161

2 Storhoff, J.J., Lazarides, A.A., Mucic, R.C., Mirkin, C.A., Letsinger, R.L., and Schatz, G.C.: 'What controls the optical properties of DNA-linked gold nanoparticle assemblies?', *J. Am. Chem. Soc.*, 2000, **122**, pp. 4640–4650

3 Storhoff, J.J., Elghanian, R., Mirkin, C.A., and Letsinger, R.L.: 'Sequence-dependent stability of DNA-modified gold nanoparticles', *Langmuir*, 2002, **18**, pp. 6666–6670

4 Taton, T.A., Mirkin, C.A., and Letsinger, R.L.: 'Scanometric DNA array detection with nanoparticle probes', *Science*, 2000, **289**, pp. 1757–1760

5 Mirkin, C.A., Letsinger, R.L., Mucic, R.C., and Storhoff, J.J.: 'A DNA-based method for rationally assembling nanoparticles into macroscopic materials', *Nature*, 1996, **382**, pp. 607–609

6 Sato, K., Hosokawa, K., and Maeda, M.: 'Rapid aggregation of gold nanoparticles induced by non-cross-linking DNA hybridization', *J. Am. Chem. Soc*, 2003, **125**, pp. 8102–8103

7 Sandström, P., Boncheva, M., and Åkerman, B.: 'Nonspecific and thiol-specific binding of DNA to gold nanoparticles', *Langmuir*, 2003, **19**, pp. 7537–7543

8 Baptista, P., Doria, G., Henriques, D., Pereira, E., and Franco, R.: 'Colorimetric detection of eukariotic gene expression with DNA-derivatized gold nanoparticles', *J. Biotechnol.*, 2005, **119**, pp. 111–117

9 Huber, M., Wei, T., Müller, U.R., Lefebvre, P.A., Marla, S.S., and Bao, Y.P.: 'Gold nanoparticle probe-based gene expression analysis with unamplified total human RNA', *Nucl. Acids Res.*, 2004, **32**, e137

10 Goodstadt, L., and Ponting, C.P.: 'Sequence variation and disease in the wake of the draft human genome', *Hum. Mol. Genet.*, 2001, **10**, pp. 2209–2214

11 Weber, J.L., David, D., Heil, J., Fan, Y., Zhao, C., and Marth, G.: 'Human diallelic insertion/deletion polymorphisms', *Am. J. Hum. Genet.*, 2002, **71**, pp. 854–862

12 Tabor, H.K., Risch, N.J., and Myers, R.M.: 'Candidate-gene appproaches for studying complex genetic traits: practical considerations', *Nat. Rev. Genet.*, 2002, **3**, pp. 391–397

13 Zhao, H., Pfeiffer, R., and Gail, M.H.: 'Haplotype analysis in population genetics and association studies', *Pharmacogenomics*, 2003, **4**, pp. 171–178

14 Collins, F.S., Brooks, L.D., and Chakravarti, A.: 'A DNA polymorphism discovery resource for research on human genetic variation', *Genome Res.*, 1998, **8**, pp. 1229–1231

15 Wang, Z., and Moult, J.: 'SNPs, protein structure, and disease', *Hum. Mutation*, 2001, **17**, pp. 263–270

16 Hung, R.J., Hall, J., Brennan, P., and Boffetta, P.: 'Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review', *Am. J. Epidemiol.*, 2005, **162**, pp. 925–942

17 Shastry, B.S.: 'Pharmacogenetics and the concept of individualized medicine', *Pharmacogenomics J*, 2006, **6**, pp. 16–21

18 Baptista, P.V., Koziol-Montewka, M., Paluch-Oles, J., Doria, G., and Franco, R.: 'Gold nanoparticle probe-based diagnostic system for rapid and sensitive detection of Mycobacterium tuberculosis', *Clin. Chem.*, 2006, **52**, pp. 1433–1434

19 Martins, R., Baptista, P., Raniero, L., Doria, G., Silva, L., Franco, R., and Fortunato, E.: 'Amorphous/nanocrystalline silicon biosensor for the specific identification of unamplified nucleic acid sequences using gold nanoparticle probes', *Appl. Phys. Lett.*, 2007, **90**, p. 023903

20 Faustino, P., Osório-Almeida, L., Barbot, J., Espirito-Santo, D., Gonçalves, J., Romão, L., Martins, M.C., Marques, M.M., and Lavinha, J.: 'Novel promoter and splice junction defects add to the genetic, clinical or geographic heterogeneity of beta-thalassaemia in Portuguese population', *Hum. Genet.*, 1992, **89**, pp. 573–576

21 Lee, P.C., and Meisel, D.: 'Adsorption and surface-enhanced Raman of dyes on silver and gold sols', *J. Phys. Chem*, 1982, **86**, pp. 3391–3395

22 Newton, C.R., Graham, A., Heptinstall, L.E., Powell, S.J., Summers, C., Kalsheker, N., Smith, J.C., and Markham, A.F.: 'Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS)', *Nucl. Acids Res*, 1989, **17**, pp. 2503–2516

23 Nickerson, D., Kaiser, R., Lapppin, S., Stewart, J., Hood, L., and Landegren, U.: 'Automated DNA diagnostics using an ELISA-based oligonucleotide ligation assay', *Proc. Natl Acad. Sci*, 1990, **87**, pp. 8923–8927