

Ultraminiaturized assay for rapid, low cost detection and quantification of clinical and biochemical samples

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Abstract Herein, we report a simple, sensitive, rapid and low-cost ultraminiaturized assay technique for quantitative detection of 1 μ l of clinical or biochemical sample on a novel ultraminiaturized assay plate (UAP). UAP is prepared by making tiny cavities on a polypropylene sheet. As UAP cannot immobilize a biomolecule through absorption, we have activated the tiny cavities of UAP by 1-fluoro-2-nitro-4-azidobenzene in a photochemical reaction. Activated UAP (AUAP) can covalently immobilize any biomolecule having an active nucleophilic group such as amino group. Efficacy of AUAP is demonstrated by detecting human IgE, antibody of hepatitis C virus core antigen and oligonucleotides. Quantification is performed by capturing the image of the colored assay solution and digitally quantifying the image by color saturation without using costly NanoDrop spectrophotometer. Image - based detection of human IgE and an oligonucleotide shows an excellent correlation with absorbance - based assay (recorded in a NanoDrop spectrophotometer); it is validated by Pearson's product-moment correlation with correlation coefficient of $r=0.9545088$ and $r=0.9947444$ respectively. AUAP is further checked by detecting hepatitis C virus Ab where strong correlation of color saturation with absorbance with respect to concentration is observed. Ultraminiaturized assay successfully detects target oligonucleotides by perfectly hybridizing with their respective complementary oligonucleotide probes but not with a random

oligonucleotide. Ultraminiaturized assay technique has substantially reduced the requirement of reagents by 100 times and assay timing by 50 times making it a potential alternative to conventional method.

Keywords Ultra miniaturized assay · ELISA · Color saturation · Activated ultra miniaturized assay plate

1 Introduction

Conventional immunoassay is carried out in a well of a transparent polystyrene microtiter plate requiring about 50–100 μ l of sample and reagents for analysis (Hornbeck 2001). However, often samples are precious and not available in plenty; hence, it is necessary to develop the assay in miniaturized platform that can save precious sample and reduce subsequent reagents, thereby making it cost effective. The history of miniaturized immunoassays started in the late 1980s, when Ekins et al. created the first microspot multi-analyte immunoassay (Ekins 1989, 1998; Ekins et al. 1990). Miniaturization of assays can be carried out into: (i) low volume well plates, (ii) array platforms, and (iii) microfluidic platforms. Low volume plate is evolved from a gradual change from 96 -well plate to 384 and 1536 -well plates (Kricka 1998; Dunn et al. 2000; Roda et al. 2003). Such plates have reduced cost of assay due to reduction in sample and reagent volume; nevertheless the quantification method is conventional spectrophotometer or fluorescence spectrophotometer. Second miniaturized assay technique is microarray technology which is considered to be the most practically used miniaturization techniques (Rubenstein 1999) for DNA microarray (Hacia and Collins 1999) and protein microarray (Arenkov et al. 2000; Taylor and Giuliano 2005; Rivas et al. 2008; Vamum et al. 2004) etc. However, microarrays have been criticised as expensive

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for routine use. Also, low volume assay is carried out in microfluidic platform which is the most versatile for the miniaturization of heterogeneous assay (Kamholz 2004; Wu et al. 2009; Haeberle et al. 2007; Harrison et al. 1992). However, the main drawbacks of such assays are sensitivity and specificity which are prerequisites for assays involving diagnostic. Microfluidic technology is mainly qualitative and used for point of care (POC) testing thus restricting itself in providing quantitative result.

Recently, NanoDrop Spectrophotometer is becoming popular for quantification of small volume (0.5–2 μl) of a sample (Desjardins et al. 2009). However, here only one sample at a time can be quantified and after each reading sample-holder (pedestal) has to be cleaned causing the quantification procedure time consuming, cumbersome and costly. The advanced instrument such as NanoDrop 8000 Spectrophotometer can record the absorbance values for eight samples at a time but still it needs cleaning. Moreover, the instrument itself is costly. Besides, NanoDrop spectrophotometer is only for quantification purpose, and the main detection assay has to be done elsewhere such as on a microtiter or PCR plate requiring around 50 μl of sample and subsequent reagents. Also, NanoDrop spectrophotometer requires different filters for different colored solutions for quantification.

Miniaturized and automated immunoassays at nanoliter-scale can also be carried out by Gyrolab xPlore developed by Gyros. It uses microfluidic technology, optimizes immunoassay development, and enables faster assay development. (Andersson et al. 2007; Given et al. 2012). Gyrolab xPlore reduces manual labor, run time and material consumption, enhancing workflow efficiency. However, such system is costly (around 200000 USD) which is beyond the reach of many diagnostics and research laboratories in the developing countries.

Recently, image - based assay is emerging as a potentially useful solution for low cost assay system as it does not require costly spectrophotometer or ELISA reader (Rahman et al. 2004; Cheng et al. 2010; McGeough and O'Driscoll 2013). Several matrices are used for image- based assays such as transparency film (Tian et al. 2011), polypropylene (Parveen and Nahar 2013), polymethyl-methacrylate (PMMA) and polystyrene (Wang et al. 2011), thread (Reches et al. 2010) and paper (Martinez et al. 2007; Lee et al. 2011; Nash et al. 2010; Wang et al. 2010; Yager et al. 2006). Among them paper-based assays are getting attention in recent years as paper is comparatively cheap, absorbs analyte and reagents very fast, solution can be dried rapidly and scanning can be done easily. However, assay color on the paper disperse during drying resulting in non uniform color distribution; hence non uniform image. This may affect quantitative analysis. Moreover, as paper absorbs nonspecifically, sensitivity of assay on paper is much lower especially in case of diagnostics involving ELISA (Martinez et al. 2008; Cheng et al. 2010).

Therefore, there is a need to develop low cost assay platform that will detect as well as quantify a sample volume of 1 μl . The assay platform should be cheap, simple and can be used routinely for multipurpose analysis.

In this communication, we report an activated ultraminiaturized assay plate (AUAP) as a rapid, low cost assay platform. Efficacy of AUAP is checked by detecting human IgE, antibody of hepatitis C virus core antigen and three 20 mer oligonucleotides. The image of the plate having color assay solution is captured on a desktop scanner followed by quantifying the image by a color space. NanoDrop spectrophotometer is used for comparing the results obtained by AUAP.

2 Materials and methods

2.1 Materials

Hepatitis C virus core antigen, anti hepatitis C virus core antigen (produced in rabbit), bovine serum albumin (BSA), sodium dodecyl sulphate (SDS) and o-phenylenediamine dihydrochloride (OPD), horseradish peroxidase (HRP), anti - rabbit IgG conjugate and streptavidin-HRP conjugate were purchased from Sigma, USA. Human IgE ELISA quantitation set was purchased from Bethyl laboratories, USA. Synthetic oligonucleotides (Table 1) were purchased from Midland Certified Reagent Company, USA. Polypropylene sheets were purchased from Classic Enterprises Ltd., India. Activated ultra miniaturized assay plate (AUAP) was activated in an UV Stratalinker (Model 2400; Stratagene, USA) fitted with five 15-W tubes. 1-fluoro-2-nitro-4-azidobenzene (FNAB) was prepared according to published procedure (Naqvi and Nahar 2004). Biomolecules were immobilized by ultrasound waves on a sonicator bath (Elma, Germany). Colored assay solution in the ultraminiaturized assay plate were scanned on a desktop scanner, HP photo smart C6388, USA to get the image. Absorbance was recorded at 490 nm in a NanoDrop spectrophotometer (Nanodrop Spectrophotometer 2000c, Thermo fisher Scientific, USA)

All buffer solutions were freshly prepared in DW before use. For ELISA assay, Buffer A (0.01 M phosphate buffer, pH 7.2 and 0.85 % NaCl) was used. Washing buffer for these assays was made by adding 0.1 % Tween 20 to Buffer A. For oligonucleotide assay, Buffer B (0.1 M PBS, pH 7.2; 1 M NaCl) was used for reagent preparation and for washing 0.1 % SDS was added to Buffer B. Experiments were performed in triplicates.

2.2 Preparation of ultra miniaturized assay plate

Cavities in the UAP were made by gently pressing the locally purchased polypropylene sheet of 1 mm thickness with a fine steel rod. Cavity of the UAP has an average diameter of 1 mm

Table 1 Oligonucleotides used in the study (Mismatched base is shown in red color)

Oligonucleotide	Length (base)	Oligonucleotide sequence
Probe-1 (P1)	20	5'-NH ₂ -ACA AGA CGT TTT ACA GTT GC-3'
Probe-2 (P2)	20	5'-NH ₂ -ATG TGG AAA ATC TCT AGC AG-3'
Probe-3 (P3)	20	5'-NH ₂ -TCG GGG TTT TGG GTC TGA CG-3'
Target-1 (T1)	20	5'-Biotin-GCA ACT GTA AAA CGT CTT GT-3'
Target-2 (T2)	20	5'-Biotin-CTG CTA GAC ATT TTC CAC AT-3'
Target-3 (T3)	20	5'-Biotin-CGT CAG ACC CAA AAC CCC GA-3'
Random (R)	20	5'-Biotin-AGT TCG ATC ATT CAT CTA AG-3'
Mismatch -1 (M1)	20	5'-Biotin-GCA ACT GTA CAA CGT CTT GT-3'
Mismatch -2 (M2)	20	5'-Biotin-GCA GCT GTA CAA CGA CTT GT-3'

and a depth of around 0.5 mm measured by vernier caliper. Cavities can be made according to requirement. However, in a normal UAP we have made 9 rows each having 3 cavities. The plate should have supports at four corners of the plate which were made by punching the corners of reverse side by a paper punch. This was necessary to prevent contact of the assay solution with the scanner's glass top; thus preventing spillage.

2.3 Preparation of Activated ultra miniaturized assay plate (AUAP)

Methanolic solution of FNAB (0.0625 mg/1 µl of CH₃OH/cavity) was poured into the cavities (in triplicates) of first row of five UAP. The plates were then kept in a fume hood for complete evaporation of the solvent. FNAB coated plates were then exposed to UV light at a wavelength of 365 nm for 5, 10, 15, 20 and 25 min respectively in the UV Stratalinker. After the stipulated time the plates were washed with ethanol and dried. As a control, triplicate cavities (second row) of each plate were kept untreated. Efficacy of activation was checked by immobilizing HRP (10 ng/cavity) into the activated and untreated cavities. Immobilization was carried out by keeping the plates inside a plastic box surrounded by wet tissue paper (to maintain moisture level) and placing the box on a sonicator bath operating at a frequency of 40 kHz and output power of 120 W with a starting temperature of 37 °C for 2 min, modifying a procedure published earlier (Sharma and Nahar 2009). The plates were then washed with washing buffer and 0.8 µl of substrate - dye buffer (2.5 mg OPD, 2.5 ml H₂O₂ in 6 ml 0.1 M citrate buffer, pH 5.2) was added for color development. The color was stopped after 3 min by adding 0.2 µl of stop solution (5 % H₂SO₄). The plate with assay solution was then scanned on a desktop scanner and the scanned image was quantified as color saturation percentage. To do so, the scanned image was opened in Adobe photoshop to get the mean values of individual R, G and B from the histogram. This was then converted to HSB (Hue, Saturation, and Brightness) using freely available 'Macbeth color calculator' software. From this, only saturation (also

known as color saturation) is selected to plot the graph for quantification as it gives better correlation with absorbance than other color spaces (Parween and Nahar 2015).

2.4 Ultra miniaturized ELISA

2.4.1 Preparation of a standard curve of human IgE on an AUAP

Anti-human IgE (1 ng/µl/cavity) was immobilized onto the activated cavities of an AUAP by ultrasound waves in the sonicator bath in 6 min. Blocking solution (1 µl of 2 % BSA/cavity) was added and kept on the sonicator bath for 6 min. Human IgE (1 µl/cavity) in different concentrations (1000 ng- 31.25 ng/ml) was then added and incubated by ultrasound waves in 6 min. Anti - human IgE conjugate (1 µl/cavity of 1:2000 dilution) binding were carried out in 6 min by ultrasound waves. Washing by PBST (Buffer A with Tween 20) was carried out after each ELISA step. After color development, image of the assay plate was captured on a desktop scanner. From this plate color assay solution was taken for recording of absorbance at 490 nm in a NanoDrop spectrophotometer. From the image color saturation was calculated as mentioned in section 2.3 and a graph was plotted with color saturation percentage on primary y-axis and absorbance reading on secondary y-axis with respect to human IgE concentration on x-axis.

2.4.2 Quantification of hepatitis c virus Ab by Ultra miniaturized ELISA

Hepatitis C virus core antigen (1 ng/1 µl) was immobilized in the cavities of an AUAP by ultrasound waves. After blocking with 2 % BSA, anti hepatitis C virus Ab in different concentrations (1000 ng - 1 ng/ml) was immobilized. Next rabbit IgG conjugate (1: 2000) was bound to the plate. ELISA steps were carried out as in section 2.4.1. After color development image was captured and quantified as in section 2.3.

2.4.3 Interplate assay

Validation of AUAP was carried out by performing human IgE detection in three different plates at different times. It was carried out similarly as described in section 2.4.1. by binding of antihuman IgE (1 ng/ μ l/cavity), blocking, binding of human IgE in different concentrations (1000 ng–3.9 ng/ml) and finally binding anti - human IgE conjugate (1:2000 dilution) into the wells of 3 different AUAP. ELISA experiments on each plate was carried out at a gap of 3 h.

2.5 Oligonucleotide detection on an AUAP

2.5.1 Concentration dependent detection of Oligonucleotide

The cavities of an AUAP were loaded with probe 1 (1 μ l/cavity from a stock solution of 200 ng/ml) (Table 1) and immobilized by placing the plate on a sonicator bath for 6 min. After blocking with 2 % BSA, the immobilized probe was hybridized with 1 μ l of different concentrations (0.78, 1.513, 3.125, 6.25, 12.24, 25, 50, 100 and 200 ng/ml) of target oligonucleotide (Target 1) using ultrasound waves for 6 min. Finally, 1 μ l of streptavidin–HRP conjugate (1: 1000 dilutions) was loaded and incubated on a sonicator bath for 6 min. The plates were washed with washing buffer (Buffer B with 0.1 % SDS) after each step. Color was developed by adding 0.8 μ l of substrate - dye buffer and stopped after 3 min by adding 0.2 μ l of stop solution (5 % H_2SO_4). Image was scanned and quantified as color saturation. Absorbance was recorded at 490 nm in a NanoDrop spectrophotometer using the same color assay solution. As a control, the experiment was repeated on UAP (untreated plate).

2.5.2 Detection of different oligonucleotides on an AUAP

Three oligonucleotide probes (Probe-1, Probe-2 and Probe-3), were immobilized (1 μ l/cavity from the stock solution of 200 ng/ml) into the cavities of an AUAP in 6 min by ultrasound waves. After blocking, the probe-immobilized wells were loaded with (1 μ l/cavity of 200 ng/ml) respective biotin labelled oligonucleotide targets (Target-1, Target-2 and Target-3) and incubated by ultrasound waves for another 6 min. Binding of streptavidin-peroxidase conjugate and color development were carried out as described in section 2.5.1.

2.5.3 Mismatch hybridization

Stock solution of 100 ng/ml of probe and target oligonucleotides were made from which 1 μ l per cavity (test zone) was used. Covalent immobilization of probe 1 (rows 1, 4–6), probe 2 (2nd row) and probe 3 (3rd row) was carried out into the cavities of an AUAP in the sonicator bath for 6 min. Then target oligonucleotides (Target-1 perfect complementary; Mismatch-1: single-base mismatch; Mismatch-2: three-base

mismatches; Random: non-complementary) were added to probe 1 immobilized cavities. Target-2 (perfect complementary) and Target-3 (perfect complementary) were added into the probe 2 and probe 3 immobilized cavities. The plate was then incubated in sonicator bath for 6 min for hybridization. The hybridized sequences were then detected using streptavidin–HRP conjugate as in section 2.5.1.

3 Results and discussion

There are several advantages of miniaturized assay such as: i) it can be used for non invasive or minimal invasive diagnostics as blood samples can be taken even by pricking, ii) it can detect precious biomolecules such as protein which are not affordable in large quantity and iii) it is cost effective due to reduced requirements for sample, reagents and buffer. However, when image based quantification technique can be incorporated to such miniaturized assay system, it gives added advantage because there is no need of any costly spectrophotometer and image of assay color can be taken by a common desktop scanner or a smart phone.

Herein, we have developed a multipurpose assay system combining miniaturized assay platform with image based quantification. This assay technique is also rapid, sensitive and affordable (Fig. 1).

3.1 Preparation of an AUAP

UAP is made from inert polypropylene sheets and is used for assays where immobilization is not required. However, for assays where immobilization is required inert surface of UAP has to be activated so that it can covalently bind a biomolecule for assay. This was done by using a heterofunctional photolinker FNAB (Nahar et al. 2001; Naqvi and Nahar 2002). Azido group of FNAB on UV exposure leads to the

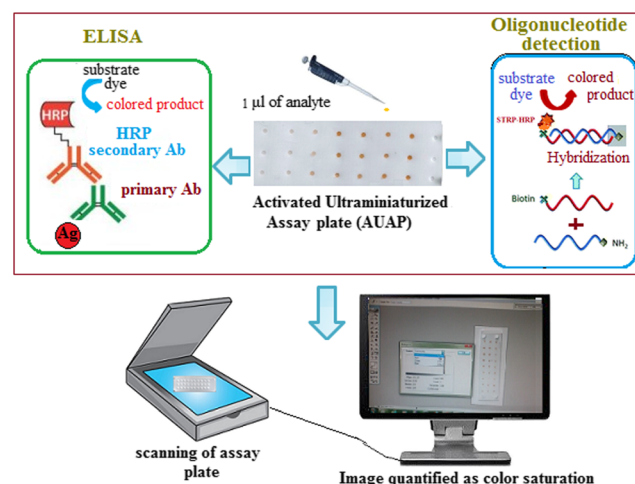


Fig. 1 Schematic representation of Ultraminiaturized Assay Technique on a ultraminiaturized assay plate

formation of highly reactive nitrene which inserts itself into the C-H bond of polypropylene surface by nitrene insertion mechanism. This simple, rapid and one step method of surface activation allows covalent binding of any biomolecule bearing a nucleophilic group such as amino group to AUAP. It was necessary to find the optimum UV irradiation time for activation of UAP. This was done by exposing the plates by UV light loaded with FNAB solution to 5, 10, 15, 20 and 25 min respectively. Efficacy of activation was checked by immobilizing HRP followed by its assay. Figure 2 (inset) shows the resultant color solutions in cavities of AUAP whereas no color is observed in the cavities of UAP indicating its inability to immobilize HRP. This was expected as untreated polypropylene could not bind a biomolecule through adsorption and thus preventing non-specific binding. The optimum time for UAP activation was 20 min of UV exposure beyond which no increase in activation was found.

3.2 Ultraminiaturized ELISA

ELISA has been the gold standard for clinical diagnostics, industrial analysis, environmental monitoring, food analysis and bioanalytical sciences. We have detected and quantified human IgE which is important for diagnosis of diseases caused by allergens. ELISA was performed in the cavities of an AUAP using human IgE ELISA quantitation set. After color assay the plate was scanned on a desktop scanner by placing the plate upside down (Fig. 1). Scanned image was then quantified as color saturation percentage. In the same time absorbance was recorded in a NanoDrop spectrophotometer at 490 nm. ELISA results by color saturation method shows excellent correlation with absorbance as seen from Pearson's product-moment correlation with correlation coefficient, $r=0.9545088$ (calculated by R software). The p value is 0.0008272 which indicates the significance of a relationship (Fig. 3a). This correlation shows that even 1 μ l of sample can

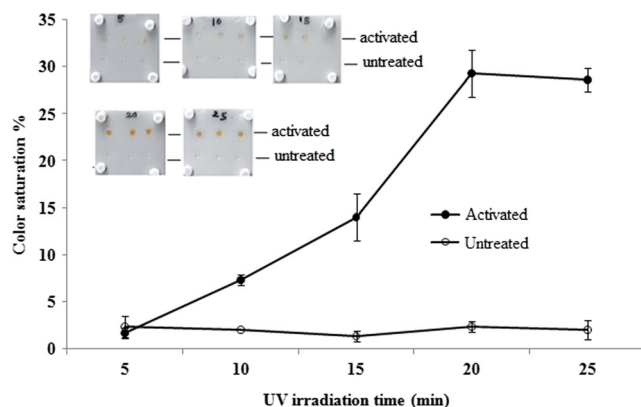


Fig. 2 Time dependent activation of UAP by UV irradiation. Scanned image of AUAP after assay (inset)

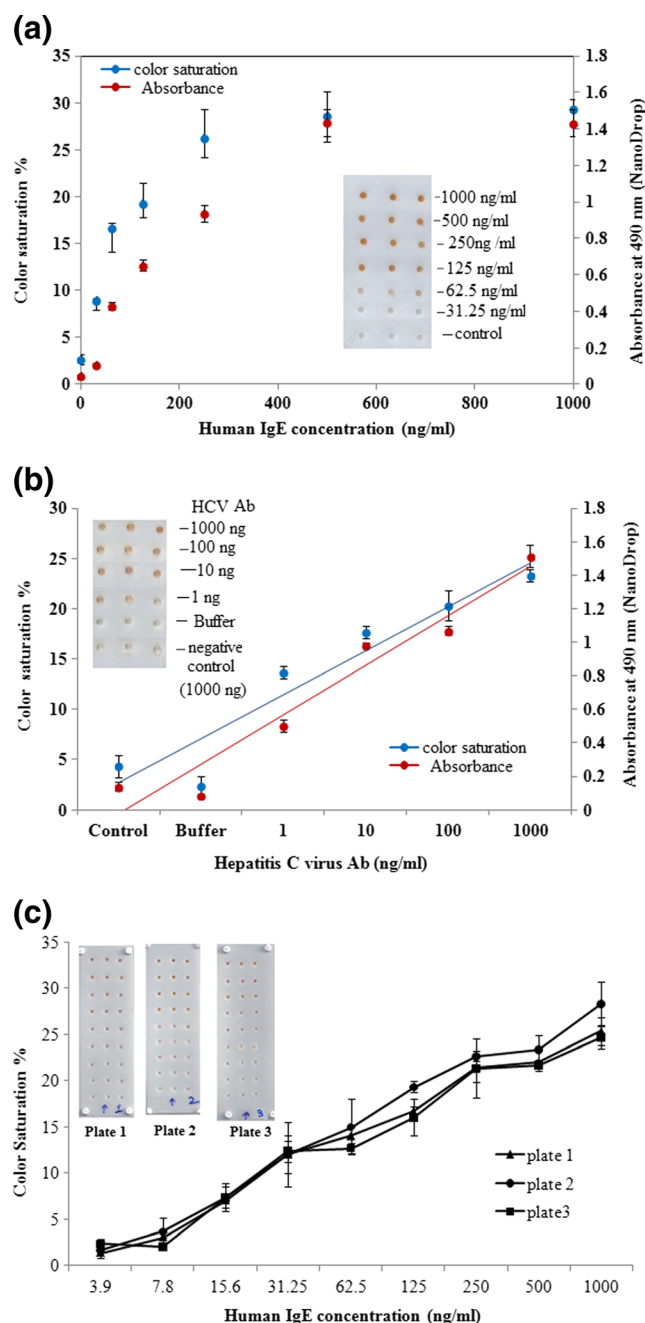


Fig. 3 Concentration dependent detection of (a) human IgE and (b) hepatitis C virus Ab on AUAP and their quantification by desktop scanner and NanoDrop spectrophotometer. c Reproducibility of immunoassay results on different AUAP

be quantified on an AUAP with high sensitivity even by a common desktop scanner and easily available software.

Further we have detected hepatitis C virus antibody on AUAP which is important because of increasing prevalence of disease caused by hepatitis C in recent time. Figure 3b shows strong correlation of color saturation with increasing concentration of Hepatitis C Virus Ab validating our novel assay system. Also, color saturation results strongly correlates

with conventional absorbance reading recorded from a NanoDrop spectrophotometer.

Plate to plate variations was also checked by performing an immunoassay on different plates carried out in different time. The inter-plate assay variability was calculated as percentage coefficient of variance from the three assay repeats on a single day and were in the range of 1–10 % (Fig. 3c).

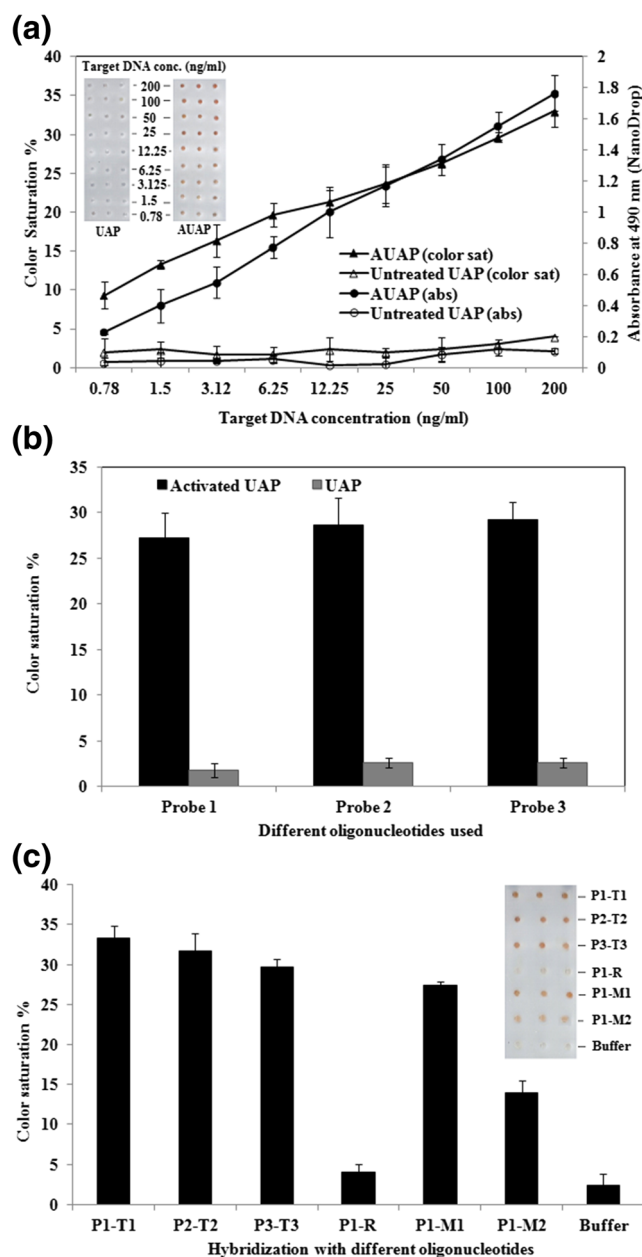


Fig. 4 **a** Concentration dependent oligonucleotide detection on an AUAP by color saturation and absorbance. **b** Detection of different oligonucleotide probes on an AUAP and a UAP. **c** Hybridization of complementary (P1-T1, P2-T2, P3-T3), single base mismatch (P1-M1), three base mismatch (P1-M2) and non complementary random oligonucleotide (P1 - R) with respective oligonucleotide probes immobilised on an AUAP

3.3 Oligonucleotide detection on AUAP

DNA-based assays are first becoming a widely used tool for the detection of clinical or bioanalytical samples (He et al. 2012; May et al. 2011; Li and Rothberg 2004; Doria et al. 2007; Scida et al. 2013). We have immobilized DNA probes of 20 nucleotides long having a NH_2 group on the 5-end on AUAP by ultrasound waves in 6 min. Subsequently, complementary sequence (target sequence) is detected by the hybridization process. Results show that intensity of the color increases as the concentration of the target molecule increases (Fig. 4a). Pearson's product-moment correlation was calculated between color saturation and absorbance by R software and correlation coefficient found was, $r=0.9947444$. The p value was $3.447\text{e-}08$ which is significant. Similar to protein, oligonucleotide does not show any binding on UAP.

The method was further validated by immobilizing three different oligonucleotide probes (Probe-1, Probe-2 and Probe-3) into both activated and untreated UAP and subsequently hybridizing with their respective complementary oligonucleotide targets (Target- 1, Target-2 and Target-3). As is shown in Fig. 4b, all the target oligonucleotides perfectly hybridize with their respective complementary oligonucleotide probes in AUAP whereas, in untreated UAP no color was seen indicating absence of any non-specific hybridization with the immobilized- probes.

This methodology was further applied for the detection of single-base mismatch with no loss of sensitivity. Figure 4c shows that target oligonucleotides (T1, T2, T3) perfectly hybridize with their respective complementary oligonucleotide probes (P1, P2, P3) whereas random target (R) fails to hybridise with probe (P1). As expected, decrease in color saturation is observed as the number of base mismatch is increased from one to three.

4 Conclusions

We have developed a simple, sensitive, cost-effective quantitative ultraminiaturized assay technique capable of detecting 1 μl of sample without compromising its specificity and sensitivity. Salient feature of this assay are: i) Biomolecules are immobilized through covalent binding in the test zones in 2–6 min, ii) Requirement of analyte or reagent is 100 fold less than used by conventional method thereby making it significantly cheaper, and iii) Ultraminiaturized assay technique can be used to quantify assays involving different colored solutions without the need of any filter as required in spectrophotometer, iii) ELISA performed in AUAP is rapid requiring only 24 min compared to 18 h conventional ELISA. Ultraminiaturized assay technique could be a useful tool for assays in different fields including diagnostics.

Acknowledgments The authors declare no competing interests. This work was funded by CARDIOMED project No. BSC 0122 of Council of Scientific and Industrial Research, Government of India, New Delhi, India. S.P. acknowledges CSIR CARDIOMED project No. BSC 0122 for the award of a senior project fellow.

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