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Naked-eye detection of rare point mutations in DNA

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TITLE:

Naked-Eye Detection of Rare Point Mutations in DNA

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KEYWORDS:

DNA, rare mutations, point mutations, gold nanoparticles, colorimetric test, naked-eye

SUMMARY:

This protocol permits the naked-eye identification of point mutated DNA in a 200-fold excess of wild type DNA molecules, by exploiting gold nanoparticles and paramagnetic microparticles.

ABSTRACT:

The protocol describes a naked-eye colorimetric test for the detection of somatic point mutations in an excess of wild type DNA. The final foreseen application of the method is the identification of rare mutations in circulating cell-free DNA from liquid biopsies, with a relevance in cancer diagnostics and stratification of oncological patients for personalized therapy. As a proof of concept, the test has been designed to detect the $BRAF^{V600E}$ mutation in the BRAF gene, which is important to identify the sub-group of melanoma patients that can benefit from targeted therapies with BRAF inhibitors. However, this colorimetric test can be easily generalized to other somatic mutations of clinical relevance due to the use of universal detection probes, thus providing strong potential in oncological diagnostics.

The test detects 0.5% of $BRAF^{V600E}$ in an excess of $BRAF^{WT}$ DNA, which matches the sensitivity of some commercial instrumental assays. Such sensitivity is clinically relevant for diagnostic purposes, allowing the early identification of drug-sensitive patients. In contrast to commercial assays based on real-time PCR, this test requires minimal instrumentation and processing, as it can be performed on DNA amplified with a standard PCR (or isothermal techniques) and provides a naked-eye readout with a one-tube reaction of a few steps in only one hour. At present, the

test has been used only on synthetic DNA samples. However, the latter have been designed to mimic a real sample amplified from circulating cell-free DNA, to favor the translation of the test to clinical diagnostics.

INTRODUCTION:

The purpose of the method is to detect underrepresented point mutations in a DNA sample with a minimally instrumented methodology and a naked-eye readout. The final aim is to have a proof-of-principle assay, suitable for applications in rapid tests for the detection of somatic mutations in circulating cell-free DNA (ccf-DNA) (e.g., from blood biopsy samples) for the early diagnostics and monitoring of cancer[1]. Cancer-related somatic mutations represent an important cancer biomarker[2] and are present in a minor (yet very variable)[3] fraction of ccf-DNA, making their identification challenging[4]. We chose, as a model target, the oncogenic mutation $BRAF^{V600E}$ that causes the constitutive activation of BRAF kinase. This mutation is present in 80% of all BRAF mutated cancers[5] and is generally represented in only <1% of circulating tumor DNA[6]. Identifying patients carrying this mutation is important as it is predictive of the therapeutic response to BRAF inhibitors. Therefore, several methods[7-10] to assess the BRAF mutation status have been developed, with sensitivities ranging from 0.01% to 2%.

The main advantage of this method over the state-of-the-art method is that its detection is instrument-free (naked-eye), as opposed to instrumental detection of fluorescent molecules by real-time PCR. Another advantage is its efficiency in discriminating one single mutated DNA molecule in an excess of 200 wild type DNA molecules. This discrimination factor of 0.5% is superior[11] or matches[12] that of some laboratory-based or commercially available kits, based on an instrumental detection and it is, thus, relevant for clinical diagnostic applications. On the other hand, as a laboratory prototype test, the method relies on the manual control of temperature-sensitive steps. However, the number of steps and the total duration of the assay is limited, making its future implementation in automated microfluidic systems conceivable.

This proof-of-concept method has been developed using synthetic DNA molecules. For its efficient translation to the clinics, it should be validated by using real-world samples amplified from patients' blood biopsies. We note that the future application field of the method is not intended to be the direct analysis of unprocessed complex biological matrices, such as bodily fluids. From the latter, DNA needs to be extracted with standard methodologies, and then amplified and purified. Consequently, the starting material for the analysis will always be purified and amplified DNA, which is reasonably comparable, in terms of possible interfering substances, to a synthetic DNA sample, such as that used for the development of this method.

PROTOCOL:

1. Synthesis of gold nanoparticle probes

1.1. Synthesize 40 nm citrate capped gold nanoparticles, using two-steps standard seeding growth method as detailed below.

1.1.1. Synthesize 15 nm citrate capped gold nanoparticles (AuNPs seeds) using the classical Turkevich–Frens method[13,14].

1.1.1.1. Wash all glassware with aqua regia ($HCl:HNO_3$ in a 3:1 v/v ratio).

1.1.1.2. Heat 250 mL of 0.25 mM $HAuCl_4$ to boil while uniformly stirring.

CAUTION: $HAuCl_4$ is corrosive and toxic.

1.1.1.3. Immediately add 25 mL of 38.8 mM Na_3·citrate.

1.1.1.4. Continue boiling and stirring for 30 min, while the solution turns to a red ruby color.

1.1.1.5. Remove the solution from the heat and let cool to room temperature while stirring.

1.1.1.6. Filter using 0.22 µm PTFE membrane syringe filters.

1.1.1.7. Store at 4 °C in a glass bottle.

NOTE: The experiment can be paused here.

1.1.2. Synthesize 40 nm citrate capped gold nanoparticles (40 nm AuNPs) by seeding growth[15].

1.1.2.1. Prepare solution A: 390 µL of freshly prepared 0.1 M hydroxylamine sulfate with 13 mL of AuNP seeds in a total volume of 120 mL in H_2O.

NOTE: The amount of AuNPs seeds needed to obtain the desired size can vary; it must be titrated for each new AuNPs seed stock.

CAUTION: Hydroxylamine sulfate is corrosive, toxic, mutagenic, and hazardous to the environment. For handling: Wash thoroughly after handling. Remove contaminated clothing and wash before reuse. Use with adequate ventilation. Minimize dust generation and accumulation. Avoid contact with eyes, skin, and clothing. Keep container tightly closed. Avoid breathing dust. Do not use with metal spatula or other metal items. For storage: Store in a tightly closed container. Store in a cool, dry, well-ventilated area away from incompatible substances. Keep away from metals.

1.1.2.2. Prepare solution B: 12.25 mL of H_2O + 0.25 mL of 0.1 M $HAuCl_4$.

1.1.2.3. Load solution B in a syringe and load the syringe in a syringe pump.

1.1.2.4. Set the syringe pump parameters: diameter of the syringe in mm; flow rate: 90 mL/h; total volume: 10.80 mL (of which, 0.8 mL are the excess needed for system equilibration, i.e., tube filling).

1.1.2.5. Start the syringe pump. After the initial 0.8 mL of solution B have filled the tubing and dropped out to a waste container, position the tube on top of the reaction flask containing solution A (kept under moderate and uniform stirring) and let solution B enter dropwise.

1.1.2.6. Cap by adding 2.65 mL of freshly prepared 0.1 M Na_3·citrate, and stir for 5 min.

1.1.2.7. Concentrate the obtained 40 nm AuNPs by centrifuging at 2,460 x *g* for 18 min at 10 °C.

1.1.2.8. Remove the supernatant by gently aspirating. Resuspend gently in the residual volume.

1.1.2.9. Store at 4 °C.

NOTE: The experiment can be paused here.

1.2. Functionalize 40 nm citrate capped gold nanoparticles by standard thiol chemistry[16].

1.2.1. Digest disulfide bond by incubating thiolated probe oligonucleotides (5' T(30)–(O–CH_2–CH_2)$_3$–SH 3') with 10 mM Tris(2-carboxyethil)phosphine (TCEP) at room temperature for 3 h under mild shaking (400 rpm).

1.2.2. Incubate a large excess of the digested oligonucleotides overnight with 10 nM AuNPs, at room temperature under mild shaking (400 rpm).

1.2.3. Bring the AuNPs-DNA mixture to 0.3 M NaCl in 10 mM phosphate buffer, pH 7.4, 0.01% SDS, by adding salt stepwise over a 10 h timespan, under mild shaking (400 rpm).

1.2.4. Incubate overnight at room temperature under mild shaking (400 rpm).

1.2.5. Wash the DNA-conjugated AuNPs 3 times with 0.25 M NaCl in 10 mM phosphate buffer plus 0.01% SDS, to remove the excess unbound oligonucleotides.

1.2.6. Store the obtained universal probes-AuNPs at 4 °C until use.

NOTE: The experiment can be paused here.

1.2.7. Determine the density of DNA probes on the AuNPs using a commercial fluorescence-based assay

1.2.8. Measure the concentration of AuNPs by UV-vis spectroscopy. In order to derive AuNPs concentration from the absorbance data, the Lambert & Beer's Law ($A=\varepsilon bc$) and published extinction coefficients for 40 nm AuNPs[16] can be used.

1.2.9. Characterize AuNPs probes by dynamic light scattering (DLS), and transmission electron

177 microscopy (TEM).
178
179 NOTE: The experiment can be paused here.
180
181 **2. Colorimetric discrimination of BRAF^{V600E} rare mutation**
182
183 2.1. Prepare target samples for analysis: mixtures of synthetic BRAF^{V600E} DNA and BRAF^{WT} DNA
184 at different ratios (BRAF^{V600E}:BRAF^{WT} 1:10, 1:100, 1:200, BRAF^{V600E} 100%, BRAF^{wt} 100%).
185
186 2.2. Equilibrate paramagnetic microparticles by washing them twice with hybridization buffer
187 (HB) (1x PBS pH 7.4, 5% w/v PEG 600) and resuspend them in a volume of HB equal to the starting
188 volume. For washing, put an aliquot of paramagnetic microparticles in a 1.5 mL tube (maximum
189 50 µL/tube), add 500 µL of HB and mix three times by gently pipetting, apply the magnet,
190 separate the clear supernatant, immediately add 500 µL of HB and repeat the procedure for a
191 second washing step. After removing the supernatant, immediately add the HB to avoid bead
192 drying, as advised by the manufacturer.
193
194 2.3. For each sample to be tested, prepare a tube containing 2.5 µL of washed paramagnetic
195 microparticles in HB.
196
197 2.4. Functionalize paramagnetic microparticles by incubating 2.5 µL of paramagnetic
198 microparticles with 10 µL of a 10 µM solution of a biotinylated first discriminating probe (DP1),
199 harboring BRAF^{V600E} mutation, for 5 min at room temperature.
200
201 2.5. Magnetically separate the microparticles from interfering unbound probes and resuspend
202 them in 12.5 µL of HB. To this aim, apply the magnet to the side of the tube, and wait until the
203 solution is transparent. Remove the solution by carefully pipetting, immediately add HB and
204 gently pipette until the beads are homogeneously resuspended.
205
206 2.6. Add to the suspension 10 µL of a 10 µM mixture of the DNA target samples described in step
207 2.1.
208
209 2.7. Incubate for 30 min at room temperature.
210
211 2.8. During the incubation, shake samples every 3 min to avoid sedimentation of the
212 paramagnetic microparticles.
213
214 2.9. Add to the suspension 10 µL of a 2 µM solution of a second discriminating probe (DP2),
215 designed to have a portion complementary to the target and a poly-A tail.
216
217 2.10. Incubate for 15 min at room temperature.
218
219 2.11. During the incubation, shake samples every 3 minutes to avoid sedimentation of the
220 paramagnetic microparticles.

2.12. Magnetically separate excess DP2 after incubation.

2.13. Add immediately 300 fmol of AuNPs conjugated with detection probes and incubate at room temperature for 5 min.

2.14. Magnetically separate supernatant containing excess AuNPs and perform the 1st washing step by adding 100 µL of HB at room temperature.

2.15. Magnetically separate the supernatant and perform a 2nd washing step by adding 100 µL of HB.

2.16. Incubate at 52 °C for 5 min.

2.17. Magnetically separate supernatant at 52 °C.

2.18. Resuspend immediately in 12.5 µL of HB for the readout of the colorimetric result.

2.19. Photograph results and store the samples at 4 °C.

REPRESENTATIVE RESULTS:

This method was used for the detection of $BRAF^{V600E}$ mutation in an excess of $BRAF^{wt}$ synthetic DNA. **Figure 1** shows the details of the detection strategy. The assay gives a colorimetric YES/NO result[17,18] where red corresponds to a positive result (YES) and yellow to a negative one (NO).

Briefly, streptavidinated paramagnetic microparticles were coated with biotinylated discriminating probes (DP1) harboring $BRAF^{V600E}$ mutation. The target samples to be analysed (a mixture of $BRAF^{wt}$ and $BRAF^{V600E}$ DNA) were added to microparticles. After a brief incubation, a second detection probe (DP2) was added to the tubes, followed by the addition of the colorimetric AuNP probe. $BRAF^{V600E}$ DNA in the sample binds DP1, then DP2 and the AuNP probe in turn binds, forming a sandwich that results in AuNPs decorating the surface of the paramagnetic microparticles (**Figure 2**) and conferring to the latter a red color (YES result). On the opposite, if the sample tested does not contain $BRAF^{V600E}$ DNA that binds DP1, the hybridization sandwich does not form and AuNP probes are washed away during the last washing step. The surface of the beads thus maintains its pristine yellow color (NO result). As a positive control, a sample containing only $BRAF^{V600E}$ DNA was used. This sample always returned a red (YES) result (**Figure 3**, tube A). Samples with different allelic fractions were tested to assess the limit of detection of the assay. As shown in **Figure 3**, the test could clearly discriminate the presence of $BRAF^{V600E}$ allele down to 1:200 dilution (**Figure 3D**), which corresponds to a fractional abundance of 0.5% ($BRAF^{V600E}/BRAF^{wt}$). In each test, a sample containing only the $BRAF^{wt}$ allele is always included as a negative control, and it returns a yellow (NO) result (**Figure 3E**).

These results demonstrate the detection of $BRAF^{V600E}$ as a proof-of-concept with relevance in the clinics. However, by changing the sequence of the oligonucleotide probes, the assay can be

adapted to the detection of any other point mutation of interest. Noticeably, the colorimetric AuNP probes are universal for any target, as they recognize and bind to a universal polyA portion of DP2. Another characteristic of the AuNP probes that is important for the sensitivity of the assay is their 40 nm size. This represent an optimal compromise between the colloidal stability of the nanoparticles and their high extinction coefficient, which enhances the sensitivity of the naked-eye detection. The characterization of AuNPs probes, including size, shape and monodispersity, is shown in **Figure 4**.

FIGURE AND TABLE LEGENDS:

Table 1. Sequences of synthetic DNA targets and probes
This table has been modified from Udayan et al.[1] with permission from The Royal Society of Chemistry.

Figure 1. Strategy for the colorimetric detection of $BRAF^{V600E}$ rare mutation. Samples containing mixtures of $BRAF^{V600E}$ and $BRAF^{WT}$ allele in different ratios were analyzed by the colorimetric test. The test employed a sandwich hybridization between the target and two probes, the first one linked to the microparticles surface and specific for the mutant allelic variant (DP1), and the second one complementary to a portion of the target (DP2). The latter also included a polyA tail, which is recognized by the polyT of a third probe, conjugated to AuNPs (AuNPs probe), which provides the colorimetric detection. Red result of the sample indicates the presence of the $BRAF^{V600E}$ allele in the sample. This figure has been republished from Udayan et al.[1] with permission from The Royal Society of Chemistry.

Figure 2. Characterization of paramagnetic microparticles complexed with AuNPs. (**A**) Transmission electron microscopy (TEM) images of microparticles complexed with AuNPs. AuNPs are visible as small black spheres on the microparticle's surface. (**B**) Scanning electron microscopy (SEM) images of microparticles complexed with AuNPs. AuNPs are visible as brilliant dots on the surface of the microparticles. This figure has been republished from Udayan et al.[1] with permission from The Royal Society of Chemistry.

Figure 3. Limit of detection of the colorimetric test. Samples containing different ratios of $BRAF^{V600E}$ and $BRAF^{WT}$ allele (**B, C, D**), positive control (**A,** containing only $BRAF^{V600E}$ allele) and negative control (**E,** containing only $BRAF^{WT}$ allele) were tested with the colorimetric assay. Red samples can be clearly discriminated by naked-eye until 1:200 dilution (**D**). Thus, the assay was sensitive down to a fractional abundance of $BRAF^{V600E}$ of 0.5%. This figure has been republished from Udayan et al.[1] with permission from The Royal Society of Chemistry.

Figure 4. AuNPs characterization. (**A**) UV-vis spectra of 40 nm AuNPs. (**B**) DLS characterization. (**C**) Transmission Electron Microscopy representative image of 40 nm AuNPs. (**D**) Statistics of size distribution of AuNPs as measured by TEM. This figure has been republished from Udayan et al.[1] with permission from The Royal Society of Chemistry.

DISCUSSION:

The core aspect of the method is the ability to discriminate a target DNA in the context of an excess of interfering non-target DNA, where target and non-target DNA only differ for one single nucleotide. Thus, the design of the probes and the hybridization conditions are critical to achieve a sensitive discrimination. The assay is designed to use universal colorimetric probes to be adapted to the detection of any point mutations of interest. However, it is possible that some minor optimization of the reaction conditions must be carried out each time a new probe pair is designed for a new mutation.

The only critical step in the method is step 2.17, where magnetic separation must be done at 52 °C. In this step, it is necessary to maintain the temperature of the previous washing step. This is needed because the assay is performed in very small volumes so, if magnetic separation is carried out without temperature control, the temperature will drop down very quickly, causing unspecific binding of non-target molecules to the beads. To ensure that this is not happening, check the color of the negative control, which has to be bright yellow at the end of the assay.

The assay is currently a lab prototype tested on synthetic DNA targets. The translation to the clinics requires the amplification of short portion of genomic DNA containing the target. As the assay needs single stranded DNA, which is readily hybridizable to the probes, it is advisable to amplify the target through a method that yields single strand amplicons. The latter can be obtained either via asymmetric PCR[19], or by different isothermal amplification techniques[20].

The sensitivity of the assay in detecting *BRAF*^{V600E} mutation is of ≥0.5%, which is one BRAF^{V600E} copy in 200 interfering BRAF^{wt} copies. This correspond to 500 fmol of the 100 pmol of sample needed for the test, and it is an amount of DNA that can be obtained with a standard PCR. This sensitivity, obtained with a naked-eye readout, is comparable to that obtained with a fluorescence readout by some commercially available rt-PCR assays[12], and is clinically relevant[6]. Moreover, this test does not need a step of allele-specific amplification[12].

Given the above, the test could reasonably find future applications in the detection of somatic point mutations in clinical diagnostics.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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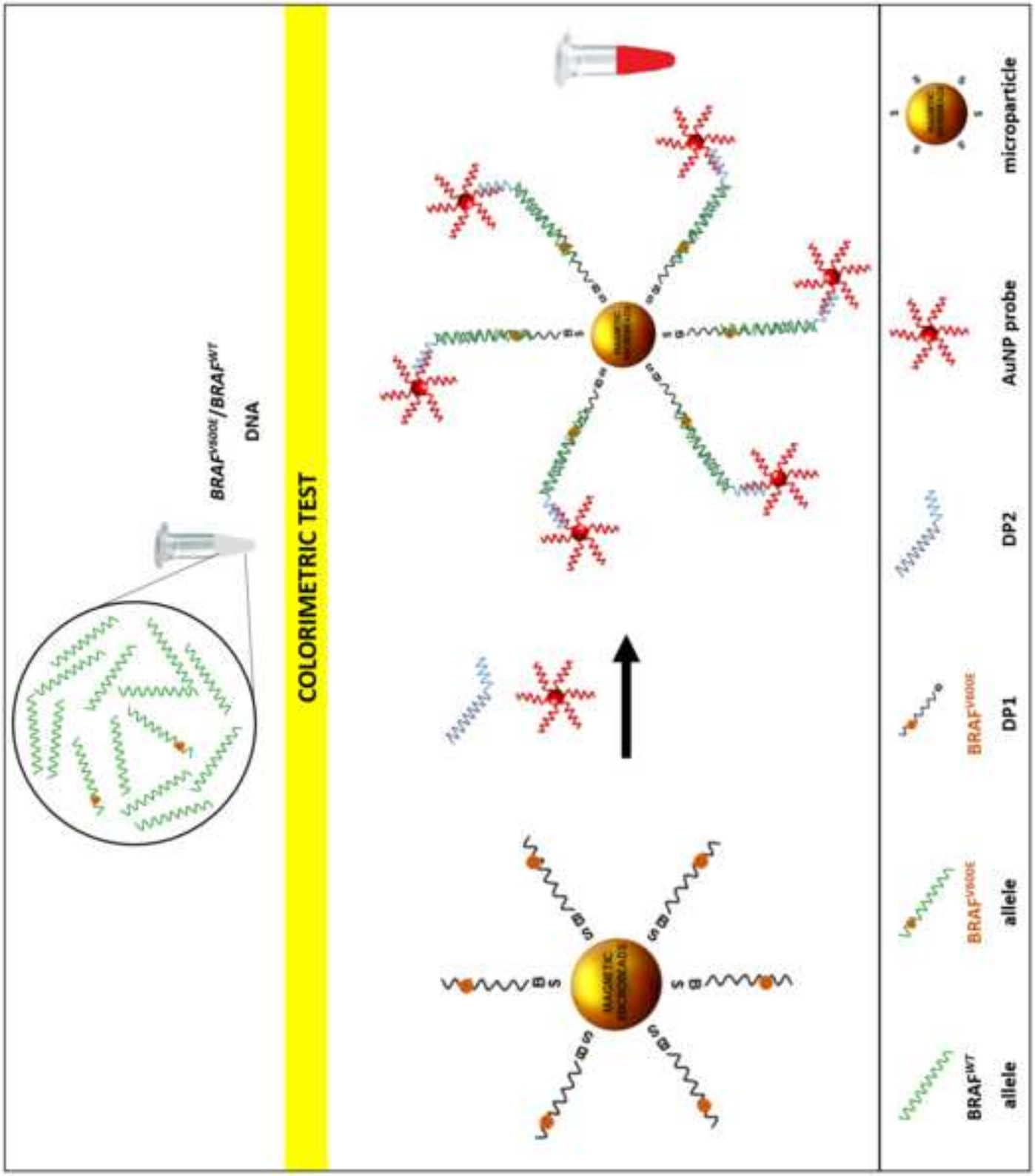

Figure 2

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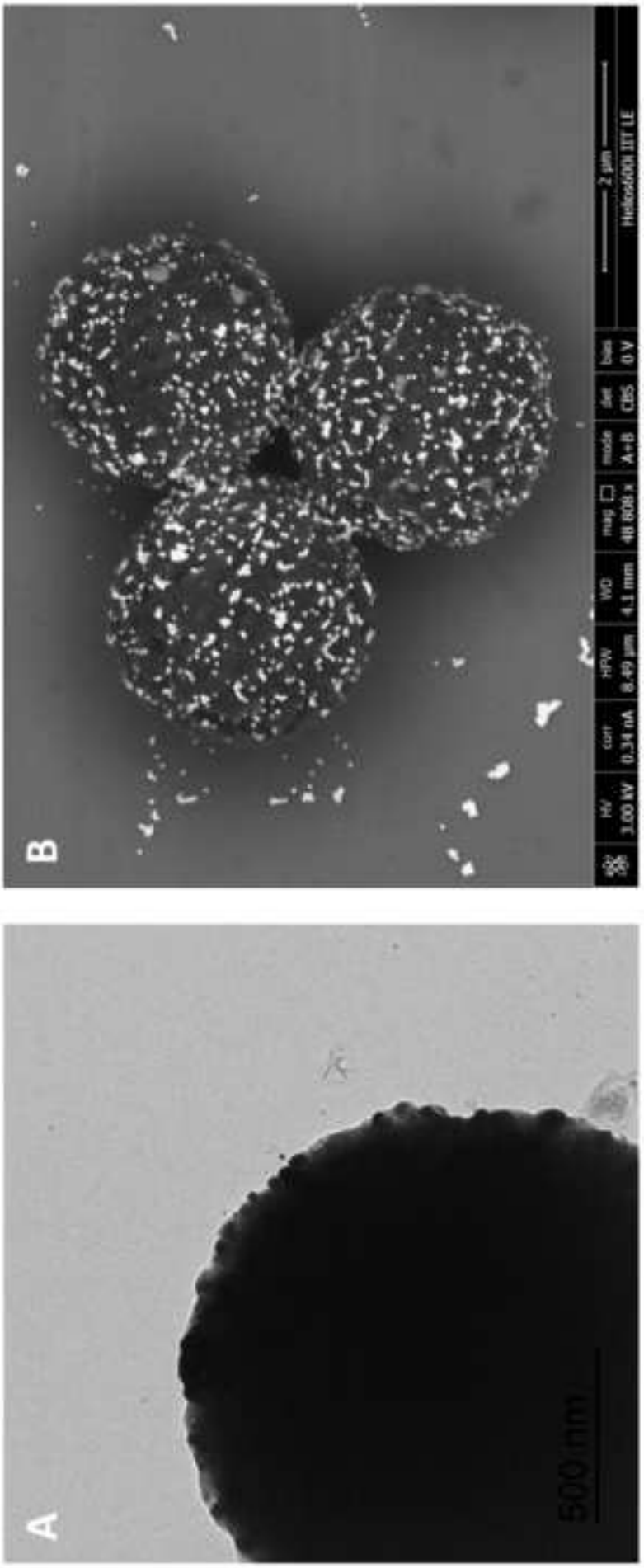

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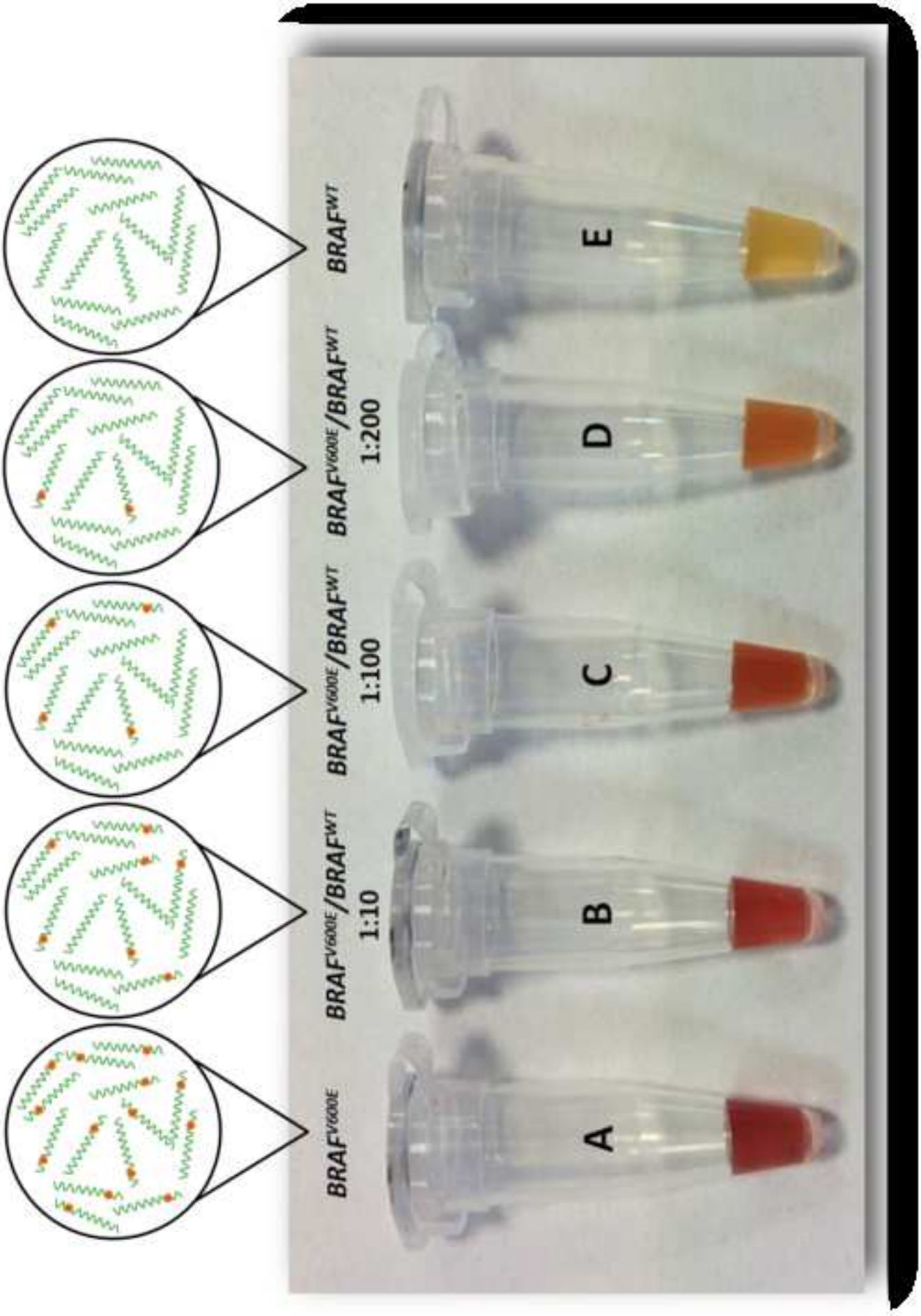

Figure 4

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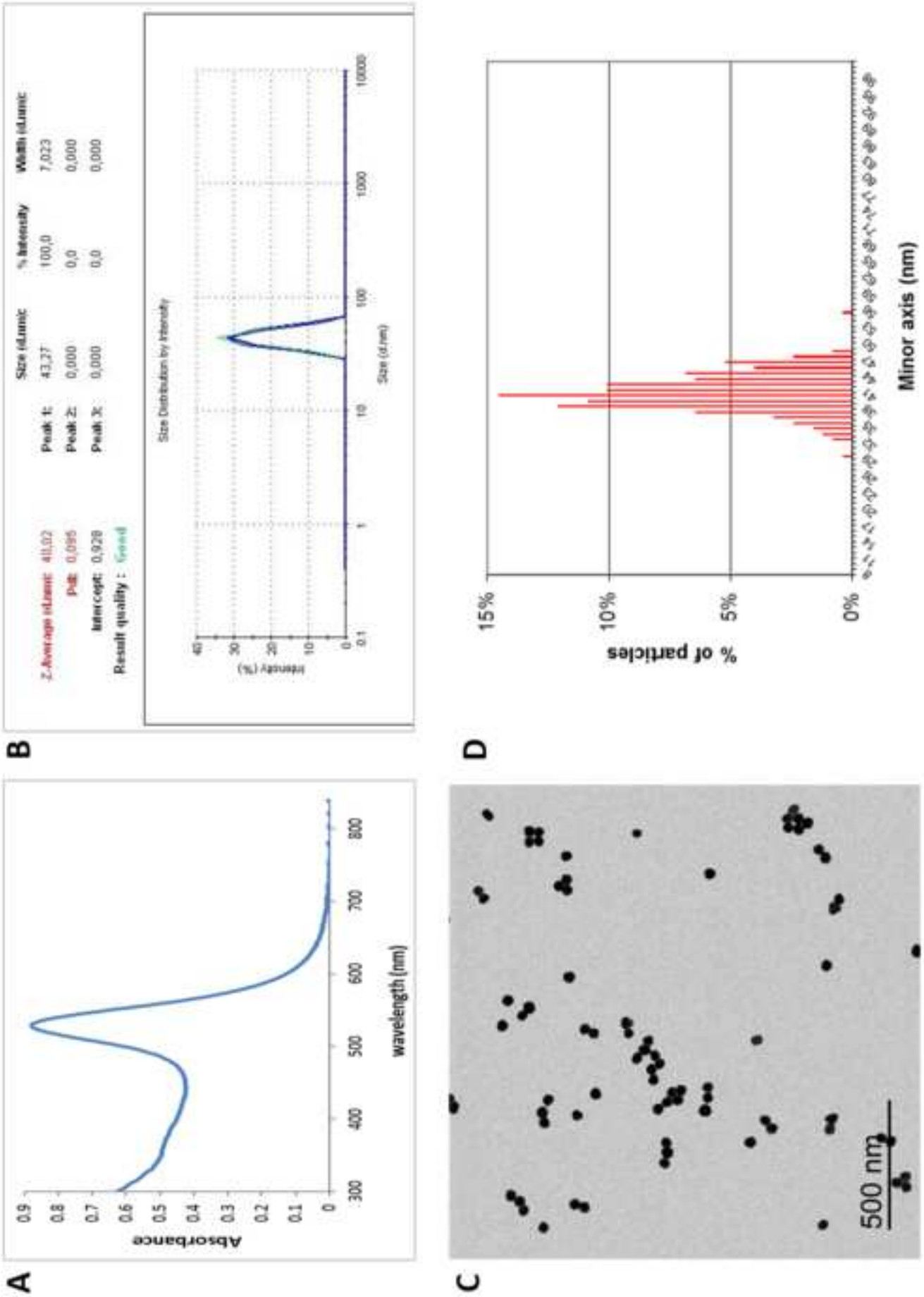

Table

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Synthetic DNA targets (ST)	
ST-BRAF ^{WT}	5′ ATA GGT GAT TTT GGT CTA GCT ACA G**T**G AA 3′
ST-BRAF ^{V600E}	5′ ATA GGT GAT TTT GGT CTA GCT ACA G**A**G AA 3′
Discriminating probes (DP)	
DP1- BRAF ^{V600E}	5′ /5BiotinTEG//iSp18/ TTC **T**CT GTA GC 3′
DP2	5′ TAG ACC AAA ATC ACC TAT AAA AAAAAAAAAAAAAAAAAAAAAAAAA 3′

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/ Equipment	Company
Bench Top Centrifuge- Allegra X 30	Beckman Coulter
DL-Dithiothreitol	Sigma-Aldrich/ Merck KGaA, Darmstadt, Germany
Dynabeads M-280 streptavidin paramagnetic microparticles	Invitrogen
Hydroxylamine sulfate	Sigma-Aldrich/ Merck KGaA, Darmstadt, Germany
KDS 100 Legacy Syringe Pump	kdScientific
NanoDrop OneC spectrophotometer	Thermo Fisher Scientific Inc.,Waltham, MA, USA)
Phosphate Buffered Saline	Sigma-Aldrich/ Merck KGaA, Darmstadt, Germany
Pierce™ TCEP-HCl, No-Weigh™ Format	Thermo Fisher Scientific Inc.,Waltham, MA, USA)
Polyethylene glycol 600	Sigma-Aldrich/ Merck KGaA, Darmstadt, Germany
PTFE 0,22 µm filters, Fluoropore	Millipore
Quant-iT™ OliGreen™ ssDNA Assay Kit	Thermo Fisher Scientific Inc.,Waltham, MA, USA)
Sodium citrate dihydrate	Sigma-Aldrich/ Merck KGaA, Darmstadt, Germany
Synthetic oligonucleotides	Integrated DNA Technologies, Inc. (IDT DNA)
Tetrachloroauric(III) acid	Sigma-Aldrich/ Merck KGaA, Darmstadt, Germany
Thiolated polyT DNA probes	Integrated DNA Technologies, Inc. (IDT DNA)
Transmission electron microscopy (TEM)	JEOL JEM 1011 microscope
Zetasizer Nano S - Dynamic Light Scattering System	Malvern Panalytical

Catalog Number	Comments/Description
A99473	
D0632-25G	
11205D	
379913-25G	
789100	
806552-500ML	
A35349	
202401	
FGLP04700	
O11492	
W302600	
520918	

Rebuttal Letter
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JoVE submission: JoVE61514 - [EMID:73d6ef3513d93a46]
Title: Naked-eye detection of rare point mutations in DNA
Authors: Gayatri Udayan‡, Alessandra Marsella‡, Paola Valentini*

Dear Dr. Alisha DSouza,

thank you very much for your e-mail.

We highly appreciated the Editorials and Reviewers' comments, which were valuable and helpful for revising and improving our manuscript. We have carefully replied to their comments in the following point-by-point scheme.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Response: Thank you for your comment; we performed a thorough proofreading of the manuscript and corrected some errors.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
1) 2.2: how is the washing performed?

Response: we included in the revised manuscript the following details regarding the washing: *"Washing procedure: put an aliquot of paramagnetic microparticles in a 1.5 ml Eppendorf tube (maximum 50 µl/tube), add 500 µl of HB and mix three times by gently pipetting, apply magnet, separate clear supernatant, immediately add 500 µl of HB and repeat the procedure for a second washing step. After removing the supernatant, immediately add the HB, in order to avoid beads drying, as advised by the manufacturer."*

2) 2.3: In HB?

Response: yes, we specified this in the revised manuscript.

3) 2.5: Describe magnetic separation in more detail.

Response: thank you for this comment. We added the following description in the revised manuscript: "*to this aim, apply magnet to the side of the tube, wait until the solution is transparent, remove the solution by carefully pipetting, immediately add HB and gently pipette until the beads are homogeneously resuspended."*

4) 2.6: 10 µM of DNA?

Response: yes, 10 µM of DNA. We specified this point in the revised manuscript.

• **Protocol Highlight:**

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.

Response: all the sub-steps are included in the highlighting.

2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

Response: thank you for the comment. We have read the manuscript once more and find the narrative of the highlighted steps cohesive.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs):

Response: thank you for the comment: We specify here below the paragraphs of the manuscript that address each of the highlighted points:

1) modifications and troubleshooting:

"The core aspect of the method is the ability to discriminate a target DNA in the context of an excess of interfering non-target DNA, where target and non-target DNA only differ for one single nucleotide. Thus, the design of the probes and the hybridization conditions are critical to achieve a sensitive discrimination. The assay is designed in order to use universal colorimetric probes, so to be adapted to the detection of any point mutations of interest. However, it is possible that some minor optimization of the reaction conditions has to be carried out each time a new probe pair is designed for a new mutation."

2) limitations of the technique

"The assay is currently a lab prototype tested on synthetic DNA targets. The translation to the clinics requires the amplification of short portion of genomic DNA containing the target. As the assay needs single stranded DNA, which is readily hybridizable to the probes, it is advisable to amplify the target through a method that yields single strand amplicons. The latter can be obtained either via asymmetric PCR19, or by different isothermal amplification techniques20."

3) significance with respect to existing methods

"The sensitivity of the assay in detecting BRAFV600E mutation is of ≥ 0.5%, that is one BRAFV600E copy in 200 interfering BRAFwt copies. This correspond to 500 fmoles of the 100 pmoles of sample needed for the test, and it is an amount of DNA that can be obtained with a standard PCR. This sensitivity, obtained with a naked-eye readout, is comparable to that obtained with a fluorescence readout by some commercially available rt-PCR assays11, and is clinically relevant5. Moreover, this test does not need a step of allele-specific amplification11."

4) future applications

"Given the above, the test could reasonably find future applications in the detection of somatic point mutations in clinical diagnostics."

5) critical steps within the protocol.

"The only critical step that we envision in the method is step 2.17, where magnetic separation has to be done at 52 °C. In this step it is necessary to maintain the temperature of the previous washing step. This is needed because the assay is performed in very small volumes so, if magnetic separation is carried out without temperature control, the temperature will drop down very quickly, causing unspecific binding of non-target molecules to the beads. To ensure that this is not happening, check the color of the negative control, which has to be bright yellow at the end of the assay."

• **Figures:**
1) Fig 2A: Add a scale bar.

Response: a scale bar is present in the left down corner of the picture. It is black over a dark grey image and maybe poorly appreciable, but this is the original image from the microscope and it cannot be modified unless we use image-editing softwares. For the moment, I left it as it is, please let me know if you need me to modify it.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Response: All the figures have been republished from a previous publication from an RSC Journal. This publisher allows the authors to freely republish the figures, provided that the correct acknowledgement is given with the reproduced material.

To see the editorial policy, please go to:
https://pubs.rsc.org/en/content/articlelanding/2020/nr/c9nr10030j#!divAbstract and press "Request Permissions" :

"Authors contributing to RSC publications (journal articles, books or book chapters) do not need to formally request permission to reproduce material contained in this article provided that the correct acknowledgement is given with the reproduced material…. For reproduction of material from all other RSC journals and books: Reproduced from Ref. XX with permission from The Royal Society of Chemistry."

Accordingly, the sentence "*with permission from The Royal Society of Chemistry*" has been added to the previous description in the figure legend.

Comments from Peer-Reviewers:

Reviewer #1:
I found that the paper is well written and it can be accepted in its present form
I have one query that there is no colour differentiation upon change in concentration. Authors may provide highly resolved figure.

Response: We acknowledge the Reviewer for its positive evaluation and for the comment. Figure 3 uploaded in the system is 1476 X 1041 pixels, horizontal and vertical resolution is 150 dpi x150 dpi. The colour difference that can be appreciated in this picture corresponds to that observed by eye (from dark red to orange). We are very sorry but we cannot envision, at least at this moment, any way to improve it.

Reviewer #2:
Manuscript Summary:
The protocol by Udayan and co-workers describes a colorimetric method to detect the single point mutation BRAFV600E. The point mutation detection is based on a colorimetric change induced by binding of functionalised gold nanoparticles with magnetic beads. BRAFV600E DNA target capture is achieved by the conjugation of complementary DNA probe to the magnetic bead to form a DNA-bead complex. A second DNA probe with a poly A tail is then incubated with the DNA-bead complex. Finally, functionalised gold nanoparticles that recognise the poly A tail of the second DNA probe are incubated and nanoparticle binding results in a colour change from yellow to red.

This work was previously reported by the authors for the detection of BRAFV600E (Nanoscale, 2020, 12, 2973) and KRAS (ACS Nano 2013, 7, 6, 5530-5538). The detection of BRAFV600E mutation is clinically significant, yet difficult. The simplicity of proposed method for BRAFV600E detection is appealing and could potentially be used as a simpler alternative to amplification-based approaches. The protocol is clearly structured and provides enough details to repeat the work. This reviewer recommends the publication of the protocol after addressing some comments listed below.

Major Concerns:
Although this work is a proof-of-concept demonstration, the assay is proposed for mutant DNA detection in complex samples (e.g., blood, liquid biopsy). The detection of target DNA in a complex samples is more difficult and can significantly impede the assay performance. It is questionable if the proposed assay would still be able to detect as little as 0.5% target DNA in a complex sample. It is suggested to include and comment on the steps required (e.g., sample preparation) to perform target DNA detection in complex samples in this protocol.

Response: we thank very much the Reviewer for its positive evaluation and this constructive comment on a very crucial issue. This comment allowed us to add a very important explanation in order to specific this point in the end of the introduction paragraph, where limitations of the method are highlighted.

The added paragraph is the following: " *This proof-of-concept method has been developed using synthetic DNA molecules. For its efficient translation to the clinics, it should be validated by using real-world samples amplified from patients' blood biopsies. We wish to highlight that the future application field of the method is not intended to be the direct analysis of unprocessed complex biological matrices, such as bodily fluids. From the latter, DNA needs to be extracted with standard methodologies, and then amplified and purified. Consequently, the starting material for the analysis will always be purified and amplified DNA, which is reasonably comparable, in terms of possible interfering substances, to a synthetic DNA sample, such as that used for the development of this method."*

Minor Concerns:
-For clarity, it would help to cite the author's initial paper (ref. 18) at the start of the manuscript (e.g., introduction), so that the reader would be referred to this for more details.

Response: thank you for the comment, we added reference n°18 in the introduction (reference n°1 in the revised manuscript)

-In 1.2.8., the AuNPs concentration is measured by UV-vis spectroscopy. How is the concentration derived from an absorbance value? Is a calibration curve with known particle concentration required? Details about how to measure AuNP concentration should be provided.

Response: extinction coefficients of spherical AuNPs of various sizes have been published in a number of systematic studies. In order to determine AuNPs concentration from UV-vis data, we used published extinction coefficients. Furthermore, we spot verified the accuracy of such determination by independently measuring AuNPs concentration via inductively coupled plasma atomic emission spectroscopy (ICP-AES), and always found good agreement of results generated with the two techniques.
In order to better specify this point, we added in the revised manuscript the sentence *"In order to derive AuNPs concentration from the absorbance data, the Lambert & Beer's Law ($A=\varepsilon bc$) and published extinction coefficient for 40 nm AuNPs[16] can be used."*