Amplification-free liquid biopsy by fluorescence approach

Jesper Uhd*, Anders Okholm**, Jørgen Kjems**, and Kira Astakhova*

*University of Southern Denmark, Department of Physics, Chemistry & Pharmacy Nucleic Acid Center, Campusvej 55, 5230 Odense M, Denmark. Correspondence: jeuhd14@student.sdu.dk
**Aarhus University, Interdisciplinary Nanoscience Center, Gustav Wieds Vej 14, 8000 Aarhus C.

ABSTRACT

Liquid biopsy is an attractive new paradigm of modern cancer research and clinical oncology. Synergy of fluorescence microscopy with mutation specific molecular probes is a method that we developed for the detection of tumor related circulating DNA, ctDNA. The present detection methods of ctDNA samples include amplification-based techniques that have multiple challenges, are often time consuming and rather expensive. In this work, we successfully applied the hybridization assay and advanced microscopy for the reliable amplification-free detection and quantification of cancer associated mutations in ctDNA.

Keywords: Fluorescence microscopy, mutation detection, ctDNA, nucleic acid, bioconjugation.

1 INTRODUCRTION

For cancer, oncogenes and their mutated variants such as *KRAS* G12D[1], *KRAS* G13D[1], and *BRAF* V600E[2] are well-known biomarkers. These genes affect intracellular signaling in the MAPK pathway which is associated with cell growth.[3,4] In cancer pathogenesis the concentration of cell free DNA (cfDNA) are increased due to apoptosis.[5,6] Typically, cfDNA are 300-500 nt long and therefore are easy to distinguish from other genomic fragments. All together, this makes cfDNA a relevant biomarker for solid tumors.

Detection of cfDNA from cancer cells or circulating tumor DNA (ctDNA) has three major challenges: the low diversity between ctDNA and cfDNA, the low concentration of ctDNA, and accurate quantification of the number of mutant fragments in the sample [6–8]. The mutations in human oncogenes are typically single nucleotide polymorphisms (SNP) [7,9], and since ctDNA only are present at low abundance (100-300 molecules per 100 µL analyte) [5,8,10], it requires highly sensitive and specific nucleic acid detection.[11,12] The present detection methods include amplification-based assays such as polymerase chain reaction (PCR). [11] PCR has multiple challenges that include sometimes low yield of the amplified DNA and moderate SNP specificity.[13,14] Furthermore, the initial sample's stoichiometry is affected by enzymatic reactions,[15] and amplification methods are time-consuming and tend to be fairly expensive.[16,17]

Locked nucleic acids (LNA) are synthetic nucleotide analogues that have a higher binding affinity and specificity to DNA and especially RNA targets [16,18]. This effect is caused by the structural and thermodynamic parameters of LNA, which also improves the detection of SNP.[19,20] Remarkably, LNA/DNA oligonucleotides have strong positional preference for specific annealing. The highest specificity is achieved when the LNA is positioned opposite to the SNP.[21,22]

Modern fluorescence microscopy has a huge impact on the detection of biomolecules down to the single-molecule level.[8,16,23] Fluorescence imagining is a fast, sensitive, and specific detection method which meets growing demands of both clinical diagnostics and research.[17,24] Using fluorescence microscopy, DNA and mutations in the DNA can be detected at low femtomolar to attomolar concentrations.[8] Fluorophores play a crucial role in the microscopy, and its utility depends on the chemical structure and optical properties [18,25]. In this setup, we use the recently developed DNA major groove binder EvaGreen, which changes its fluorescent properties dramatically in the presence of exclusively dsDNA. EvaGreen is non-toxic and photostable for imaging applications.[18,26,27]

Herein, we aim to develop enzyme-free detection of *KRAS* G12D, *KRAS* G13D and *BRAF* V600E mutations by novel LNA/DNA probes. To address this, we apply solid-phase target enrichment followed by the use of a mutation specific probe and EvaGreen.

2 EXPERIMENTAL

Reagents obtained from commercial suppliers were used as received. 3′ amino modifier LNA/DNA custom oligos were purchased from Exiqon and used after HPLC purification. 5′ amino modifier C6 LNA/DNA custom oligos were purchased from Exiqon and used after HPLC purification. EvaGreen dye (20× stock in water) was obtained from Biotium. Biotinylated probes were purchased from Integrated DNA Technologies and used after HPLC purification. Streptavidin MagneSphere® Paramagnetic Particles(Promega) where purchased from Promega. ProMag™ HP 3 Series Streptavidin(ProMag) where purchased from Bangs Laboratories. Dynabead® M-270(Dynabead) where purchased from ThermoFisher Scientific, invitrogen.

Microscopy images was obtained using an LSM 700 confocal microscope (Zeiss).

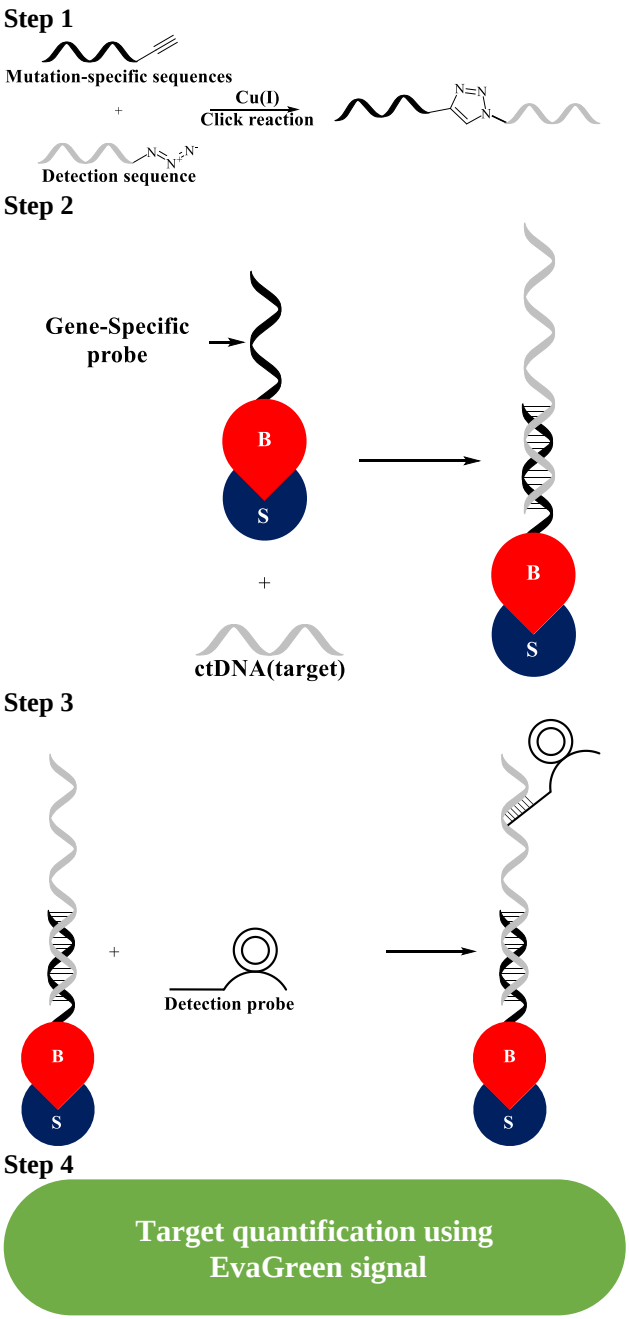

Figure 1 Main principle of ctDNA detection. Step 1: Coupling of the mutation-specific sequence and detection sequence by using click chemistry. Step 2: Catching gene specific ctDNA using magnetic streptavidin beads. Step 3: Transfer mutation specific probe with EvaGreen dye and washing. Step 4: Reannealing the ctDNA with the probe and make fluorescence detection. B is biotin, S is streptavidin.

2.1 Probe design

Three mutation specific probes were designed respectively for: *BRAF* V600E, *KRAS* G12D and *KRAS* G13D. The probes were a combination of two regions, tp aid the synthesis. The two regions where attract together with copper-catalysed azide-alkyne cycloadditions(CuAAC).[28] The *BRAF* V600E specific probe where designed with LNA on and beside the SNP, the LNA nucleotides are denoted with +nucleotide:

5′-/5AmMC6/TTTTTTTTTTGATTT+C+T+CTGTAG-3′

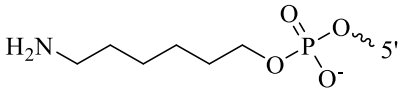

Figure 2 5AmMC6: Amino modifier C6.

The same design strategy where used for *KRAS* G12D specific probe:

5′-/5AmMC6/TTTTTTTTTTGAGCT+G+A+TGGCGT-3′

and *KRAS* G13D specific probe:

5′-/5AmMC6/TTTTTTTTTGAG+CTGGT+G+A+CGT-3′

The 5AmMC6 were further modified with pentynoic acid sulfotetrafluorophenyl ester (PNHS) using Lumiprobe NHS ester labeling of biomolecules amino protocol instructions. The detection sequence was:

5'-CTT+CG+ATTTAATT+CGTAAA +CAA-GCA+GTA+GTAATTC/3AmMO/-3′

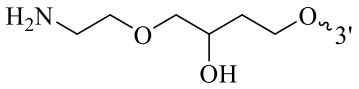

Figure 3 3AmMO: 3' Amino Modifier.

The 3AmMO were modified further with azidobutyric acid *N*-hydroxysuccinimide ester using Lumiprobe NHS ester labeling of biomolecules amino protocol instructions.

2.2 Click chemistry

Lumiprobe's protocol for click-chemistry labeling of oligonucleotides and DNA was used. The click chemistry was prepared in 1 mL reactor tube under argon using vigorous stirring in Emrys Creator (Personal Chemistry).

2.3 Test for non-fluorescent streptavidin

In our initial design, we planned to detect the ctDNA directly on the beads, given that the latter gives no background fluorescence signal. Promega, ProMag and Dynabead where tested for this purpose. 50 µL commercial streptavidin beads was placed in a 1.5 mL Eppendorft tube. The original buffer was easily removed from the solution using the beads' magnetic properties. Thereafter the beads were washed with 1×PBS(3 × 200 µL).

2.4 Genomic DNA test

To capture *BRAF* V600E ctDNA we used a previously developed target enrichment approach[8] and a 120mer biotinylated probe designed not to overlap with the SNP region of the ctDNA:

5′-AGCAAGCATTATGAAGAGTTTAGGTAAGAGATCTAATTTCTATAATTCTGTAATATATATTCTTTAAAACATAGTACTTCATCTTTCCTCTTAGAGTCAATAAGTATGTCTAAAACAAT/3Bio/-3′

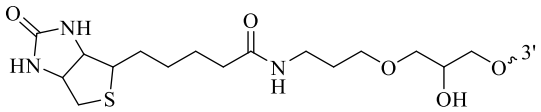

Figure 4 /3Bio/: biotin

To capture *KRAS* G12D and *KRAS* G13D ctDNA, another biotinylated 120mer was designed, also not overlapping with the SNP region of the ctDNA target:

5'-TGAAAGTTAAGTTATCTGAAATGTACCTTGGGTTTCA AGTTATATGTAACCATTAATATGGAACTTTACTTTCCTTG GGAGTATGTCAGGGTCCATGATGTTCACTCTCTGTGCATT TT/3Bio/-3′

2.4.1. Dynabead

100 µL Dynabeads was placed in a magnetic separation rack to remove and discard the clear supernatant and then washed with 1×PBS (3 × 100µL) and incubated with 20 µL capturing probe and 20 µL 1×PBS for 20 min at 21 ºC. Followed by a wash with 2×PBS (2 × 100µL) and annealed with 20 µL ctDNA and 20 µL 2×PBS for 40 min at 65 ºC followed by 30 min at 21 ºC. Hereafter, a wash with 2×PBS (2 × 100 µL) was performed, and the resulting complex was annealed with 15 µL detection probe, 5 µL DNA and 25 µL 2×PBS followed by 10 min at 92 ºC and a temperature gradient to 21 ºC for 20 min. Thereafter, the beads were washed with 2×PBS (2 × 100 µL) at 21 ºC. The resulting DNA complex was removed from the capturing probe at 92 ºC for 10 min, and placed in a solution with 3 µL EvaGreen and 10 µL 2×PBS, vortexed for 7 min, incubated at 92 ºC at 10 min followed by cooling to rt over 1 h.

2.4.1. Promega

100 µL Promega beads was placed in a magnetic separation rack to remove and discard the supernatant and then washed with 0.5×SSC (3 × 100µL), incubated with a solution with 5µL capturing probe for *BRAF* V600E, 2µL ctDNA, 2.5 µL milli-Q water and 9.5 µL 2×SSC at 92 ºC. The entire assay was prepared in a 0.2 mL low binder Eppendorf tube, heated 10 min at 92 ºC and thereafter 3 hours at 65 ºC. After washing with 0.5×SSC (3 × 100µL) the procedure followed by 20 µL *BRAF* V600E detection probe at 4 ºC at 24 hours and then heated to 42 ºC. Next, it was incubated with 3 µL EvaGreen stock at 92 ºC for 12 min followed by cooling to rt over 1h.

3. RESULTS AND DISCUSSION

Initially, the mutation specific probes were modified with an alkyne group. Next, the detection sequence was linked to them via an azido group at the 3' amino end. The aim of this was to be able to bioconjugate the two specific sequences of the detections probes using the gentle, effective and highly selective CuAAC method. Simultaneously, improved yields for long LNA/DNA sequences and versatile block-synthesis approach for expanding the potential targets were our goals.

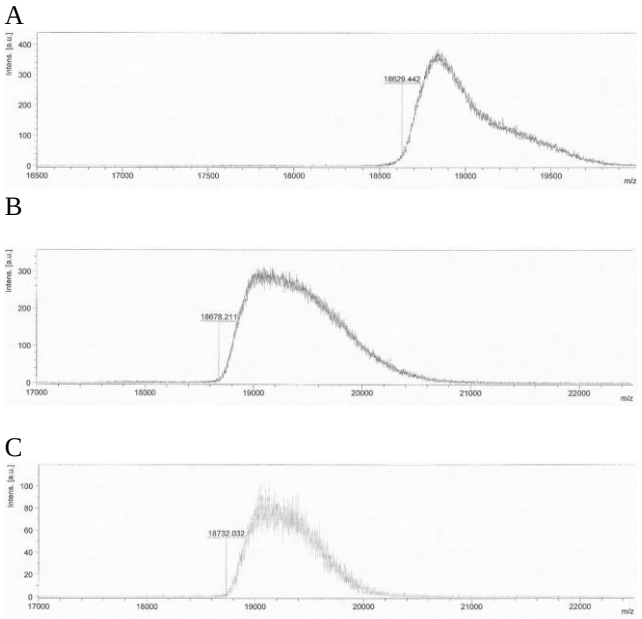

Figure 5 MS MALDI for the clicked detection probes, A *BRAS* V600E, B *KRAS* G12D, and C *KRAS* G13D respectely.

It is apparent from figure 5 that the desired molecular weights of the products were achieved, the calculated molecular weights were 18,629 g mol^{-1} for the *BRAF* V600E detection probe, 18,676 g mol^{-1} for the *KRAS* G12D detection probe, and 18,732 g mol^{-1} for the *KRAS* G13D detection probe respectely.

Next, several commercial streptavidin beads were tested for autofluorescence. Indeed, it turned out that all the commercial streptavidin beads gave background fluorescence. ProMag showed fluorescence under the UV light and Promega and Dynabead showed fluorescent in the microscopy setting as seen in figure 6.

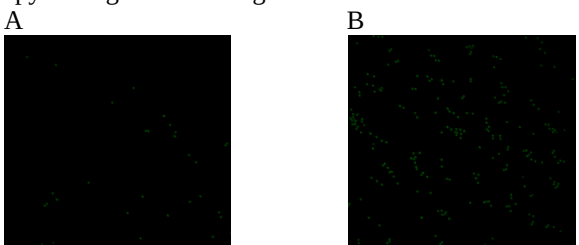

Figure 6 Fluorescent test for commercial streptavidin bead. A Dynabead and B Promega.

The purpose of the test was to check whether it was necessary to separate the detection probe from the biotinylated capture probe, and thereby making the detection method even more simple. However, due to the fluorescence background of the beads it was necessary to detach the detection probe from the capturing probe(figure 7).

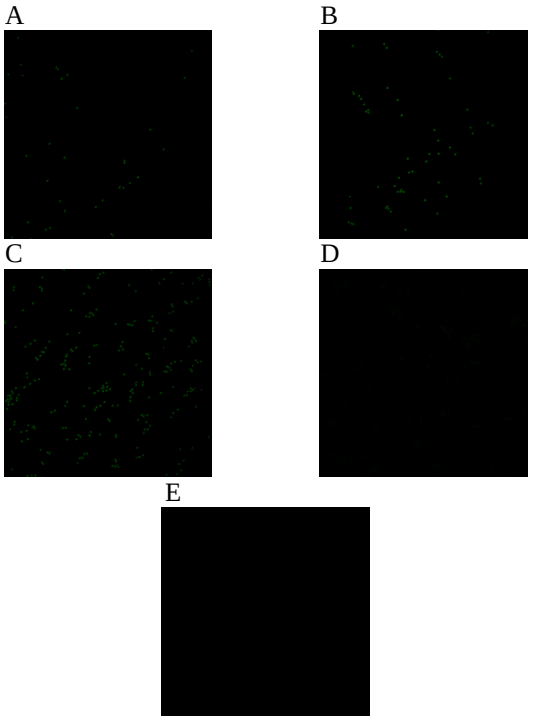

Figure 7 Imaging of genomic DNA test using Promega, with the use of *BRAF* V600E detection probe. A**,** HT29. B, human male control(HMC). C, patient 2 ctDNA tested *BRAF* V600E positive. D, Gp2d(cell line) 50% *KRAS* G12D. E, MQ water

It is apparent from figure 6 that imaging of MQ water and Gp2d 50 % *KRAS* G12D control showed almost no signal. Some artifact could be observed due to incomplete bead removal at the final stage.

The consequence of separating the detection probe from the capturing probe is a cumbersome maneuver, due to the detection and capturing probes great affinity for the specific ctDNA. However, the separation is necessary otherwise the detection method gives false positive signals.

We proceeded further with Dynabeads and Promega. We tested the assay using genomic DNA from human cell lines: HT29, HMC, Gp2d (obtained from ATCC). The genomic DNA was isolated as suggested by the supplier and restricted by EcoRI. The size analysis confirmed that genomic fragments of approx. 5000 base pairs were generated. Next, we applied these samples at concentrations 5-20 ng/µL and MQ control to the developed assay. The results are shown in Table 1. Thus, the DNA samplel from the *BRAF* V600E HT29 cell line (25% mutant) showed a low signal, which indicates the importance of the additional signal boosting. However, it was a positive finding that none of the negative controls showed any signal as well, also when the concentration was increased up to 100 ng/µL for HMC.

Assay	Signal
HT29	Low signal
HMC	No signal
Gp2d	No signal
Patient 2 *BRAF* V600E positive	No signal
MQ water	No signal

Table 1 Genomic DNA test using Dynabead.

4. CONCLUSION

In summary, an assay is described which has a potential for the fast analysis of cancer related mutations. The assay combines serveral principles: using a long (120mer) gene-specific capture probe, high specificty and binding affinity of LNA/DNA oligo nucleotide detection probes, and DNA detection by high resolution fluorescence microscopy. As a consequence of the low signal from HT29, the assay in its present form lacks sensitivity. However, since no signal was observed for any control samples, also at high concentrations, it suggests that the necessary level of specificity has been achieved.

It is noteworthy that the developed method can be applied to detect other nucleic acids of scientific and clinical importance such as microRNA and viral DNA/RNA.

REFERENCES

[1] S. Eser *et al.*, *Br J Cancer*, 2014, **111**, 817.
[2] M. Kobayashi, A. Tojo, *Blood*, 2014, **124**, 2610.
[3] W. Zhang, H.T. Liu, *Cell Research*, 2002, **12**, 9.
[4] D. Ahmed *et al.*, *Oncogenesis*, 2013, **2**, e71.
[5] L.A. Diaz, A. Bardelli, *J Clin Oncol*, 2014, **32**, 579.
[6] E. Heitzer, *et al.*, *Clinical Chemistry*, 2015, **61**, 112.
[7] Z. Gui *et al.*, *Talanta*, 2016, **154**, 520.
[8] L. Miotke *et al.*, *Plos One*, 2015, **10**, e0136720.
[9] H. Takeshita *et al.*, *DNA Cell Biol.*, 2012, **31**, 36.
[10] M. Ma *et al.*, *Ann Transl Med*, 2015, **3**, 235.
[11] K. Nafa, M. Hameed, M.E. Arcila, *Methods in Molecular Biology*, 2016, **1392**, 71.
[12] F. Janku *et al.*, *Oncotarget*, 2014, **5**, 3607.
[13] Y. Han *et al.*, *Bioinform Biol Insights*, 2015, **9**, 29.
[14] K. Chen *et al.*, *Analytical and Bioanalytical Chemistry*, 2016, **408**, 4371.
[15] F. Sun *et al.*, *Virology Journal*, 2016, **13**, 202.
[16] K. Astakhova, *Chemosensors*, 2014, **2**, 193.
[17] A.P.F. Turner, *Chem. Soc. Rev.*, 2013, **42**, 3184.
[18] H. Ji, K. Astakhova, in *RNA and DNA diagnostics*, (Erdmann, V. A., Jurga, S. & Barciszewski, J.) Springer International Publishing, 2015, 161.
[19] L. Miotke, M. Barducci, K. Astakhova, *Chemosensors*, 2015, **3**, 224.
[20] S. Karmakar, P.J. Hrdlicka, *Chemical science (Royal Society of Chemistry : 2010)*, 2013, **4**, 3447.
[21] D.A. Di Giusto, G.C. King, *Nucleic Acids Research*, 2004, **32**, e32.
[22] A. Simeonov, T.T. Nikiforov, *Nucleic Acids Research*, 2002, **30**, e91.
[23] E.C. Arnspang, J.R. Brewer, B.C. Lagerholm, *Plos One*, 2012, **7**, e48521.
[24] S. Gao *et al.*, *Scientific reports*, 2014, **4**, 4384.
[25] K.M. Brummond, *L.S. Kocsis*, *Acc. Chem. Res.*, 2015, **48**, 2320.
[26] C.D. Nowadly *et al.*, *Mil. Med.*, 2014, **179**, 626.
[27] F. Mao, W.-Y. Leung, X. Xin, *BMC Biotechnol*, 2007, **7**, 76.
[28] E. Haldón, M.C. Nicasio, P.J. Pérez, *Org. Biomol. Chem.*, 2015, **13**, 9528.