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Bio-analytical applications of nicking endonucleases assisted signal-amplification strategies for detection of cancer biomarkers -DNA methyl transferase and microRNA

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ABSTRACT

The low concentrations of cancer biomarkers in the blood have limited the utility of quantitative bioassays developed for the purpose. The advent of nicking endonucleases (NEases) as signal amplification tools have greatly enhanced the detection efficiency and provided a multi-optional platform to design target specific detection methods. The present review focuses on the prominent features of NEases, modified DNA probes (such as hairpin (HP) probes, molecular beacons, and G- quadruplex) that mediate cyclic cascade and role of helper enzymes. Application of NEase assisted signal amplification (NESA) has been discussed for diagnosis of two prominent cancer biomarkers *viz*. DNA methyl transferase (Dam MTase) and microRNA (miRNA). NESA mediated techniques such as rolling circle amplification (RCA), strand displacement amplification (SDA) and isothermal exponential amplification (EXPAR), have been compared in light of their future applications in clinical diagnosis. Significance of nanomaterials to achieve further amplification and NESA assays for simultaneous detection of miRNAs has also been conversed. It is anticipated that the information gained from the analyses of the prospects and limitations of NESA-based assays will be useful towards understanding the applications, and improvement of efficient isothermal exponential amplification strategies for highly sensitive and selective detection of cancer biomarkers.

1. Introduction

Cancer being the most dreadful disease necessitates early stage diagnosis to reduce mortality rate. A number of techniques are available for the purpose, but suffer from limitations like invasive procedures, low sensitivity, specificity, high cost and time consumption (Mittal et al., 2017). Considering that the concentration of biomarkers is ultralow in early stages of cancer, it is imperative to develop fast and sensitive methods for detection. Biosensors are alternative technique for the purpose and have created a distinct niche due to attributes of sensitivity, specificity, quick response, on-site monitoring and cost benefits. The initial era of bio-detection involved direct assessment of signals produced by interaction between target and bioreceptor in 1: 1 stoichiometric ratio and detection of signals produced by low concentration of target was not possible. But, now the biosensor technology has advanced to a stage where the signals produced by targets present in low concentration can be amplified through series of amplification steps and thus, the current scientific strategy of biosensors seems like an attractive alternative. Enormous literature is available regarding signal amplification using nanomaterials (Devi et al., 2015; Mittal et al., 2017; Vigneshvar et al., 2016; Yang et al., 2015), but only limited literature is available about role of specific enzymes like NEases as signal amplification tools.

NESA is a suitable strategy because it mediates amplification of single target DNA into cyclic generation of multiple triggers that facilitates ultralow levels of detection. It also employs simple instrumentation and has the feasibility to be utilized for routine analysis (Connolly and Trau, 2010). Basic design of NESA involves hybridization of target DNA with the probe to generate a NEase-restriction site, which is recognized by sequence-specific NEase. NEase-mediated cleavage of probe releases the target DNA and further, initiates next cycle of hybridization, cleavage and dissociation, which subsequently results in signal amplification (Yin et al., 2012). NESA has gained attention because it has been reported to possess discrimination capability against

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single-base mismatch (Li et al., 2011). NEase-based techniques have also been demonstrated to have potential to be used for protein detection, however such methods are difficult to handle and are time-consuming (Hun et al., 2010; Xue et al., 2012).

Recent studies have demonstrated that NESA has played an imperative role in improvement of detection sensitivity of target from two to five folds (Dong et al., 2015; Zhang et al., 2016a, 2013). Therefore, in an effort to stimulate interest in advances in biosensor technology and its diagnostic applications for cancer detection, the current article provides an overview on the recent advances in NESA-based assays, their prospective applications, approaches, and challenges, with a focus on their role in effective estimation of two important cancer biomarkers - DNA methyl transferase (Dam MTase) and microRNAs (miRNAs). For readers convenience a glossary is provided in the **Supplementary data** to understand typical terms of molecular biology.

2. Nicking endonucleases (NEases)

NEases are mutated forms of restriction endonucleases that have lost the ability to dimerize and hence cleave only one strand in the DNA duplex or DNA-RNA hybrid at a specific recognition sequence. They generate a nicked site in the DNA with extendable 3^{l} recessive ends, which can be regenerated further for the next cycle of nicking extension. (Higgins et al., 2001). Most of the natural NEases have been isolated from the *Bacilli sps*. Approximately 200 NEases have been identified and studied for their biological functions (Li et al., 2011). The important NEases with their recognition sequences and characteristics are enlisted in the Table 1.

NEases have immense applications in all streams of life sciences ranging from DNA nanotechnology to biosensor technology (Miao et al., 2015a; Yan et al., 2016). These have been employed in various electrochemical biosensors and optical bioassays for diverse targets such as metal ions, miRNA, DNA and enzymes (Chen et al., 2015b; Cheng et al., 2016, 2015; Cui et al., 2016; Li et al., 2016a; Ma et al., 2013; Wang et al., 2016a, 2016c, 2016e). The advantage of using NEases is their bio-compatibility with other enzymes, which makes them suitable for carrying out complex procedures of rolling circle amplification (RCA) and isothermal exponential amplification (EXPAR) (Song et al., 2016; Wang et al., 2014). The ability of nicked strand to initiate strand extension reaction is a desired attribute in such amplification procedures. NEases may distinguish between methylated and unmethylated recognition sequences which is an important characteristic feature used to design bioassays for epigenetic studies, such as evaluation of methylated DNA and estimation of Dam MTase activity (Chen and Zhao, 2013; Cui et al., 2016; Li et al., 2016e; Zhang et al., 2016d; Zhao et al., 2014, 2013b; Zhu et al., 2013). They have specific recognition sequence requirement that should be fulfilled to obtain single stranded cleavage products and may perform catalytic function on immobilized targets as well. Due to this property, NEases are being used in diverse bio-analytical techniques such as photo-electrochemical immunoassays for cancer biomarkers (Lv et al., 2018; Qiu et al., 2017; Shu and Tang, 2017; Zhang et al., 2018a). The detection efficiency of such bioassays may further be improved with integration of microfluidic approach for disease diagnosis (Zhou et al., 2018).

Table 1
Characteristics of important NEases.

Enzyme	Recognition sequence	Isolation source
Nb. BbvCI	3′- GGAGT‸CG - 5′	*Bacillus brevis* strain C
Nb. BsmI	3′- CTTAC‸GN- 5′	*Bacillus stearothermophilus* NUB 36
Nt. BstNBI	5′- GAGTCNNNNˇN- 3′	*Bacilluc stearothermophilus*
Nt. BspQI	5′- GCTCTTCNˇN- 3′	*Bacillus* sp. strain Q
Nt. BspD6I	5′- GAGTCNNNNˇN- 3′	*Bacillus* sp. strain D6
Nt. Bst9I	5′- GAGTCNNNNˇN- 3′	*Bacilluc stearothermophilus* strain 9
Nt. BstSEI	5′- GAGTCNNNNˇN- 3′	*Bacilluc stearothermophilus*
Nt. *Bsm*AI	5´- GTCTCNˇN- 3´	*Bacillus stearothermophilus* A664
Nt. AlwI	5′- GGATCNNNNˇN- 3′	Engineered endonuclease
Nb. BsrDI	3′-CGTTAC‸NN- 5′	Bacillus stearothermophilius D70

In addition to this, there are certain other enzyme characteristics such as temperature, pH, concentration etc. which play important role in bioassays. As far as optimum temperature is concerned, 37–55 °C temperature has been applied for assays involving Nt.Alwl and N.BstNBl endonucleases (Bi et al., 2013; Liu et al., 2012; Ma et al., 2013; Niu et al., 2010; Wang et al., 2014, 2013, 2016e), whereas for Nt.BbvC1, Nt.BsmA1 and Nt.BspQ1 optimum temperature of 30–37 °C have been reported (Chen et al., 2015a; Hun et al., 2013; Song et al., 2014; Wang et al., 2016c, 2016d; Yu et al., 2015; Zhao et al., 2013b; Zhu et al., 2013). Exceptions of using higher temperatures (50 °C) have also been reported for Nt.BbvC1 (Dong et al., 2015). Optimum pH for enzyme activity has been reported to be pH 7.9 for almost all the endonucleases (Jia et al., 2010; Li et al., 2016b).

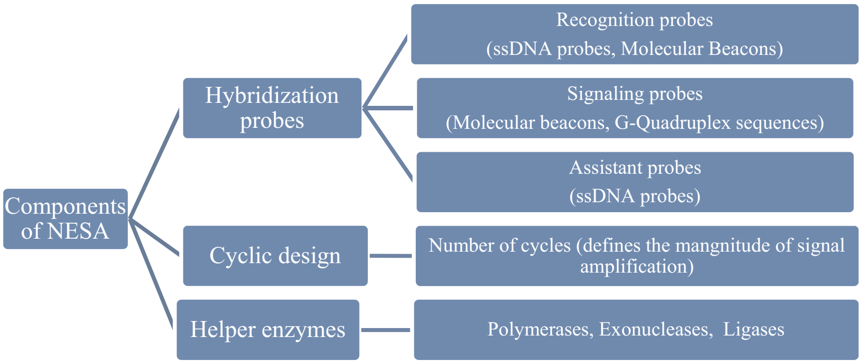
Most of the studies report that a minute amount (0.1–2.0 enzyme units) of NEases is sufficient to carry out the restriction process, however, some studies have also used 5–40 enzyme units for the process (Bi et al., 2013; Huang et al., 2017; Li et al., 2016b; Liu et al., 2013, 2017; Wang et al., 2014; Xue et al., 2015a; Yu et al., 2015). In rare investigations, concentration as high as 200 units ml^{-1} has also been optimized (Yin et al., 2017). To achieve maximum efficiency, appropriate reaction time is also required. Incubation period of 1–3 h have been reported to achieve complete restriction in most studies (Bi et al., 2013; Chen et al., 2015a; Dong et al., 2015; Wang et al., 2016c, 2013, 2016e). The optimum levels of all the enzyme parameters are highly needed for efficient working of NEases to achieve signal amplification.

3. Components of NESA based bioassays

Main components of NESA based bioassays have been illustrated in Scheme 1.

The working principle of a NESA based bioassays depend upon the target to be assessed. The most important criterion is the selection of appropriate bioreceptor. For Dam MTase and miRNA detection, a usual practise is the employment of linear or HP DNA probes as bioreceptors. The hybridization of target DNA with the probe is the basis of detection. Use of molecular beacons (MB) or HP probes as target capturing probes is very much preferred, as they remain in hair pin configuration without target and restrict the unnecessary progression of reactions further during the analysis. This reduces the background noise which is a desired attribute of any bioassay. Fluorophore at one end and quencher labelled at other end of MB enables development of fluorescence based bioassays (Chen et al., 2015b, 2013; Dong et al., 2015; Niu et al., 2010; Zhang et al., 2016b; Zhao et al., 2013b).

Another consideration for bioassay is whether a single probe is sufficient or a combination of linear DNA probes, HP probes or MBs are required to achieve the desired amplification. The inclusion of single or multiple amplification cycles in the assay is also an important deliberation. Since the bioassays designed for amplified signal involve a number of counterparts and numerous cycles of product generation, consumption and regeneration, association of final product with the signal producing moiety is the most critical parameter. This step needs proper functioning of all the previous stages to accomplish amplified signal. Generally amplification of target induced secondary signal is desirable rather than amplification of primary signal produced directly from the target-bioreceptor association (Liu et al., 2011). The further sections have been devoted to discuss in brief the different components of NESA based bioassays and considerations involved in selection of appropriate recognition probes and their assembly with other components of the system to produce an amplified signal. The role of NEase in the progression of recognition process has been focused.



Scheme 1. Components of NESA.

3.1. Hybridization probes

Hybridization Probes are the DNA or RNA probes complementary to the target sequences and are used to capture them through hybridization. These probes can be further divided as recognition probes, signalling probes and assistant probes depending on their function. The various hybridization probes are discussed as follows:

3.1.1. Recognition/capture and signalling/reporter probes

The recognition/capture probes have the responsibility of target recognition, whereas signalling/reporter probes are meant to produce a signal corresponding to the target concentration. Different domains with varied functions are incorporated in these probes, these necessarily includes a domain containing recognition site for NEase, one domain for target recognition and other for signal production and amplification (Cheng et al., 2016; Li et al., 2011; Mao et al., 2015; Wang et al., 2013; Zhu et al., 2013).

3.1.1.1. DNA probes. DNA probes are single stranded (ss) or double stranded (ds), sequence specific DNA oligonucleotides (20–30 bp) designed to capture target oligonucleotides and may contain NEases recognition sites at defined positions. Various types of probes such as dumbbell probes, locked nucleic acid (LNA) probes, assistant probes, etc. have been used as capture probes in various studies (Bi et al., 2013, 2016; Dong et al., 2015; Huang et al., 2017, 2018; Song et al., 2016). ssDNA probes are normally used to form a duplex with the target oligonucleotide/miRNA, followed by polymerase mediated extension which provides the recognition site for NEases. But, ssDNA probes have limitation over ds or HP probes as they can hybridize with newly generated sequences [produced through polymerase chain reaction (PCR) or RCA] leading to background signals (Liu et al., 2017). HP probes with long stem are preferred as capture probes as these provide the specificity and stability, but these also compromises with recognition affinity (Cui et al., 2017; Huang et al., 2018). As a consequence, in-stem-mismatched HP probe have been designed in a recent report for sensitive estimation of miRNA through NESA based bioassay (Huang et al., 2018).

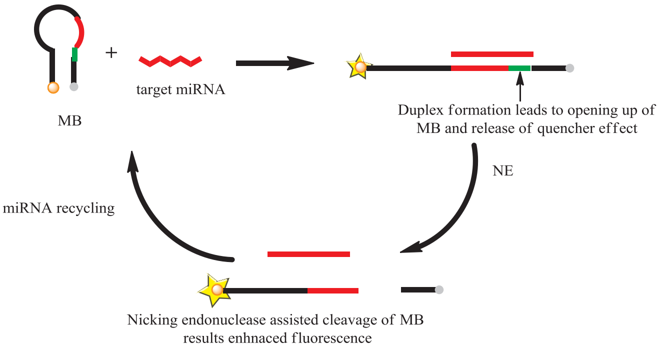


Fig. 1. MB based fluorescence response in the presence of target miRNA. Arrow defines the recognition site for NE.

3.1.1.2. Molecular beacons (MB). MBs are the oligonucleotide hybridization probes. As already mentioned, these are labelled with a fluorophore at one end, and a quencher at the other end. Their sequence promotes formation of HP shaped structures, which are disrupted to linear forms in the presence of complementary nucleic acid sequence (target). In this way, they are the non-radioactive hybridization precursors used to detect specific nucleic acid sequences. The versatile MBs with more than one domain work both as capture probes as well as reporter probes in NESA assays. The domains are specified for specific functions such as NEase recognition sequences and signal producing counterparts. Presence of target sequence or miRNAs opens up the stem of MB and restores fluorescence as illustrated in the Fig. 1 (Cui et al., 2016; Dong et al., 2015; Tan et al., 2014; Xu et al., 2016; Yin et al., 2013). The NEase assisted cleavage of MB further enhances fluorescence signal (Chen and Zhao, 2013; Chen et al., 2013; Cui et al., 2016; Dong et al., 2015; Wang et al., 2015; Zhang et al., 2016b; Zhao et al., 2013b). This also leads to release of target or target generated signal for another cycle of amplification. Certain studies have illustrated intelligent probe designing by employment of a HP probe along with a MB or two HP probes together, for target recognition and signal production simultaneously (Li et al., 2016c; Liu et al., 2017, 2014b; Xu et al., 2016; Yu et al., 2015; Zhang et al., 2014; Zhao et al., 2013b).

3.1.1.3. G-quadruplex. The most preferred signalling probes in NESA are the G-rich sequences that may fold into G-quadruplex, a highly stable DNA secondary structure capable of mimicking peroxidase activity (Ruttkay-Nedecky et al., 2013). These G-quadruplex structures have been exploited variably to produce output signal due to recognition specificity (Ma et al., 2016; Wang et al., 2016d). In most of the studies, G-quadruplexes formation has been related to the target concentration through H_2O_2 mediated oxidation of fluorescent or redox active compounds as depicted in the Fig. 2 (Bi et al., 2013; Cheng et al., 2015; Li et al., 2016a, 2016c; Liu et al., 2014b; Nie et al., 2014; Song et al., 2016; Wang et al., 2014, 2016d, 2013, 2016e; Xiang et al., 2014; Yu et al., 2015; Zhang et al., 2016d; Zhou et al., 2014, 2012). Target dependent formation of G-quadruplexes are also known to enhance the fluorescence intensity of some anionic porphyrins as N-methyl mesoporphyrin IX (NMM) [in the presence of potassium ions] and Zinc(II)-protoporphyrin IX (Bi et al., 2016; Cheng et al., 2015; Cui et al., 2017; Wang et al., 2016d; Xue et al., 2015a, 2015b; Zhang et al., 2016d). Thioflavin has also been employed as reporter probe as it specifically recognizes G-quadruplexes against ssDNA/dsDNA and has no effect on RCA and EXPAR. Hence, it provides the advantage of low background noise and real time monitoring (Huang et al., 2017; Jiang et al., 2016).

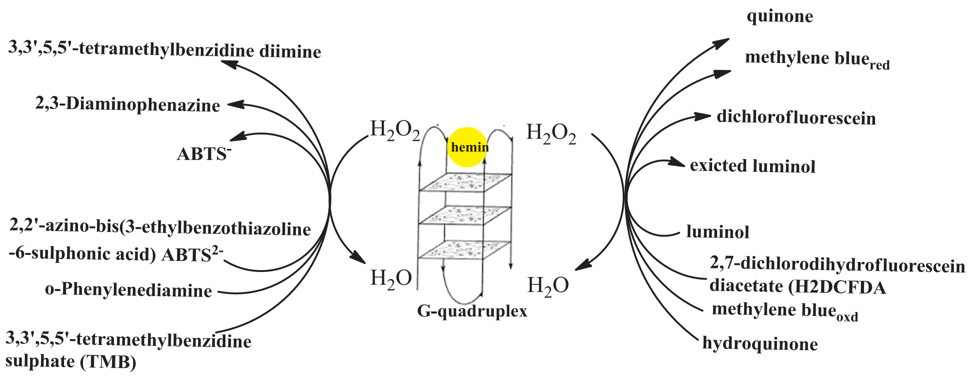


Fig. 2. G-quadruplex/hemin mediated oxidation of fluorescent or redox active compounds.

Association of oxidation products of G-quadruplex/hemin complex with distinct platforms have been used to produce amplified response against various targets. Wang et al. proposed a method that involved NEase assisted chemiluminescence resonance energy transfer (CRET) taking luminol as energy donor and polydopamine nanospheres (PDA) as quencher (Wang et al., 2016c). In this study, the sensing probe (extended to a G-quadruplex) was immobilized on PDA to produce chemiluminescence through G-quadruplex/hemin mediated oxidation of luminol. It was observed that in the absence of target miRNA, close proximity between the donor and the quencher resulted in quenched chemiluminescence, but, in the presence of target miRNA, a hybrid of sensing probe and miRNA was formed. Consequently, NEase assisted cleavage released G-quadruplex from the PDA surface in the solution leading to seized CRET and restoration of chemiluminescence (Wang et al., 2016c). In another report, NEase mediated formation of G-quadruplex/hemin complex was associated with graphene quantum dots (QD). In the study, NESA assisted formation of G-quadruplex/hemin complex mediated oxidation of o-phenylenediamine (OPD) to 2,3-diaminophenazine (DAP) which was able to quench the fluorescence of graphene-QD and hence produced target dependent response (Wang et al., 2016a).

3.1.1.4. Assistant/auxiliary probes. Assistant or auxiliary probes are the secondary probes employed in the bioassays to create duplexes for NEase catalytic action. These are designed as partially complementary to a capture probe and target (DNA or RNA) to capture the target in a Y-shaped junction (Chen et al., 2015a; Miao et al., 2015b). This creates a duplex situation in the Y-shaped assembly and generates recognition site for NEase that releases assistant probe and target for recycling (Dong et al., 2014; Song et al., 2016). The cleaved part of capture probe could be engaged in EXPAR reaction to finally generate G-quadruplex structures and an amplified signal through G- quadruplexes/hemin association as illustrated in the Fig. 3 (Song et al., 2016). G-quadruplexes in such assays function as the reporter probe that produces colorimetric or fluorescence signals as discussed in the previous section.

3.2. Cyclic design of amplification process

To achieve amplification, it is necessary to design the bioassay in a cascade manner that leads to regeneration of target or signal producing moieties repeatedly (Cheng et al., 2016; Cui et al., 2016; Miao et al., 2015b). The extent of signal amplification depends on the number of cyclic processes involved in the working principle. Usually bioassays with two or three cyclic processes are demonstrated to achieve higher signal magnification (Bi et al., 2016; Chen et al., 2013; Cheng et al., 2016; Hun et al., 2010; Li et al., 2016e; Liu et al., 2017; Song et al., 2016; Wang et al., 2016b; Yu et al., 2015). Cheng et al. reported a fine bioassay designed for detection of miRNA-21, with three cycles of amplification (Fig. 4). Cycle I was associated with generation of large number of replicas of target miRNA-21 (which were recycled again in the cycle) and DNAzymes which were used in cycle II for carrying out Zn^{2+} dependent cleavage of a substrate strand. The substrate strand was designed in a way that after cleavage it moulded into a G-quadruplex structure and carried out H_2O_2 aided oxidation of 3,3,5,5-tetramethylbenzidine sulphate (TMB) in cycle III (Cheng et al., 2016). The catalytic current signal of oxidized TMB assisted in determination of target concentration. These kinds of dual amplification strategies helped in the production of large number of signal sequences or G-quadruplexes. Similarly, three cascade amplification involving target recycling, nicking replication reaction and DNAzyme mediated fluorescence generation has also been reported for miRNA-27a and miRNA-21 detection (Yin et al., 2017; Zhou et al., 2016b). This strategy

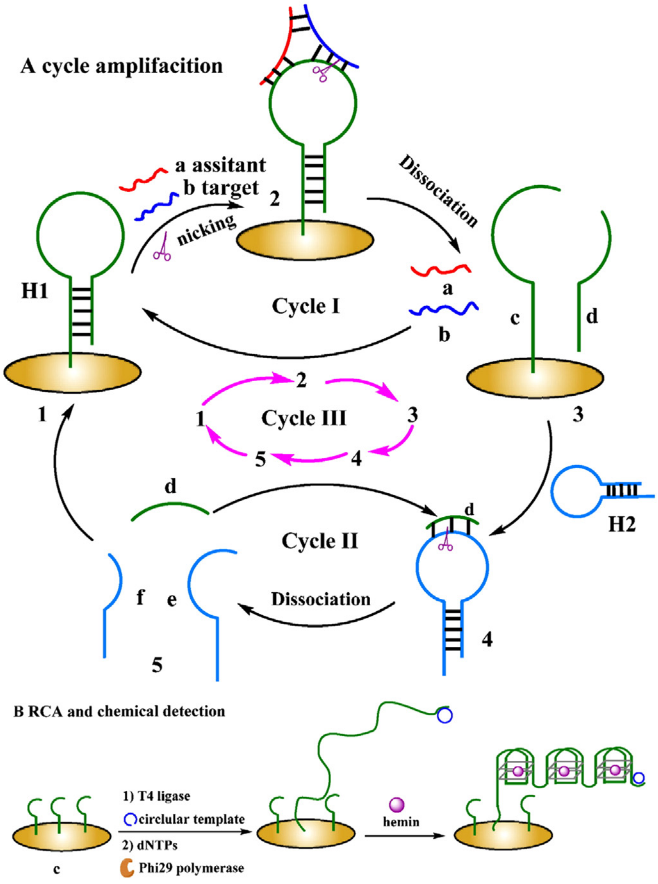


Fig. 3. Cyclic scheme of assistant probe mediated NESA assay for miRNA detection through the formation of Y-shaped junction and G-quadruplex/hemin complex (reprinted from Song et al., 2016 with permission).

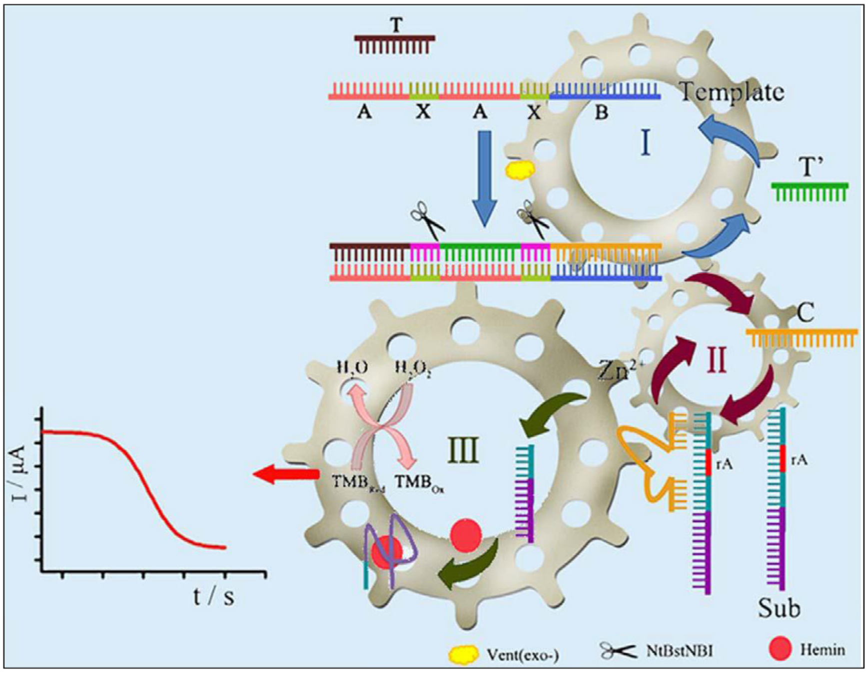


Fig. 4. Design of 3 cascade process for the detection of miRNA-21 (reprinted from Cheng et al., 2016 with permission).

provides the advantages as it do not require any sophisticated instruments, thermal cycling procedure or any washing and separation step and holds the potential for ultrasensitive miRNA detection. Further, the replacement of a probe in the unimolecular template could also extend the method to other targets and techniques, such as fluorescence, chemiluminescence, colorimetry etc.

Few studies have incorporated only a two cascade assay which have been reported equally efficient in signal amplification (Liu et al., 2017; Song et al., 2014; Xu et al., 2016; Yu et al., 2015; Zhang et al., 2014). It is a general observation that the nicking step acts as the linker between the two cycles and provides trigger for the last cycle to produce more and more reporter probes and thus amplify the signals. For example, Zhang et al. reported the formation of three-way junction (3 WJ) between the target miRNA, a 3WJ primer and a 3WJ probe to trigger a cyclic isothermal amplification cycle. In the first cycle these assemblies were cleaved by the NEase Nt.BbvCI to produce ssDNA products which were utilized in the second cycle to produce a MB mediated fluorescence response (Zhang et al., 2014). Such two cascade assays have gained detection limits upto attomolar levels of miRNA in very less time (30 min) as compared to duplex endonuclease mediated non-exponential signal amplification demonstrated by Tian & co-workers (Tian et al., 2013). NEases in association with some other enzymes such as polymerases and ligases enable the cyclic amplification of signals, and their specific roles are further discussed in the next section.

3.3. Helper enzymes

NEases are usually used in combination with other enzymes such as polymerases, ligases, exonucleases, restriction endonucleases etc., to carry out amplification. Such enzymes are regarded as helper enzymes. Polymerases are undoubtedly the most important enzymes in strand displacement amplification (SDA), RCA and EXPAR based bioassays and have been enormously used with NEases to design cyclic assays (Bi et al., 2013; Chen et al., 2015a; Cheng et al., 2016; Cui et al., 2016; Hun et al., 2010; Li et al., 2016b, 2016e; Liu et al., 2017; Mao et al., 2015; Song et al., 2016, 2014; Tan et al., 2014; Wang et al., 2016b, 2014, 2013; Yu et al., 2015; Zhang et al., 2016d; Zhao et al., 2013a, 2014; Zhu et al., 2013). Among these bioassays RCA is the most preferred method of amplification, due to its simplicity, versatility and ability to carry out reactions in biological environment, under mild reaction conditions with high specificity (Jiang et al., 2016; Wang et al., 2014). Additionally, RCA products may be modified in desired forms through designing circular probes (Zhang et al., 2018a). The use of more than one NEase along with exonuclease or endonucleases is also an option to enhance the efficiency of the bioassay. For example, Nb. BbvCI and Nb.BtsI have been used in a two stage isothermal assay to detect methylated DNA (Zhu et al., 2013). Exonuclease III and λ- exonuclease with NEases have been reported indispensable components in certain bioassays and their application has further helped in signal magnification (Cheng et al., 2015; Wang et al., 2016b; Xu et al., 2017; Zhang et al., 2018b; Zhou et al., 2012). Restriction endonucleases have also been incorporated in certain assays as helping enzymes to assist in cyclic regeneration of target and signal amplification (Zeng et al., 2013; Zhao et al., 2013b).

DNA Ligases are other important enzymes that mediate joining of two DNA strands via catalysing phosphodiester bond formation. Ligases have been used in NESA assays to regenerate recognition templates or MBs (Bi et al., 2013; Chen et al., 2013; Li et al., 2016a, 2016c; Song et al., 2016; Wang et al., 2015, 2014; Zou et al., 2015). The importance of ligase becomes more significant when padlock probes are used in the assay to amplify signal producing fragments through RCA (He et al., 2017; Jiang et al., 2016; Li et al., 2016c; Liu et al., 2013; Wang et al., 2014, 2016d). Padlock probes are specially designed probes with 5′ and 3′ ends complementary to the target sequence due to which these get circularized in hybridized form. The advantage attributed by such probes is that, they could be made completely circular with the use of ligase (as it has no gap in the target complementary region) and hence can trigger RCA for exponential signal amplification.

In bioassays designed for detection of Dam MTase, Dpn I and Hpa II are often used as auxiliary enzymes to carry out methylation sensitive restriction of target sequence/probe (Cui et al., 2017; Huang et al., 2017; Xue et al., 2015a, 2015b; Zeng et al., 2013; Zhao et al., 2013b). Fig. 5 illustrates the role of Dpn I in signal amplification for detection of Dam MTase.

4. Application of NEases in clinical diagnosis of cancer biomarkers

NEases have been involved in the diagnosis of diverse targets,

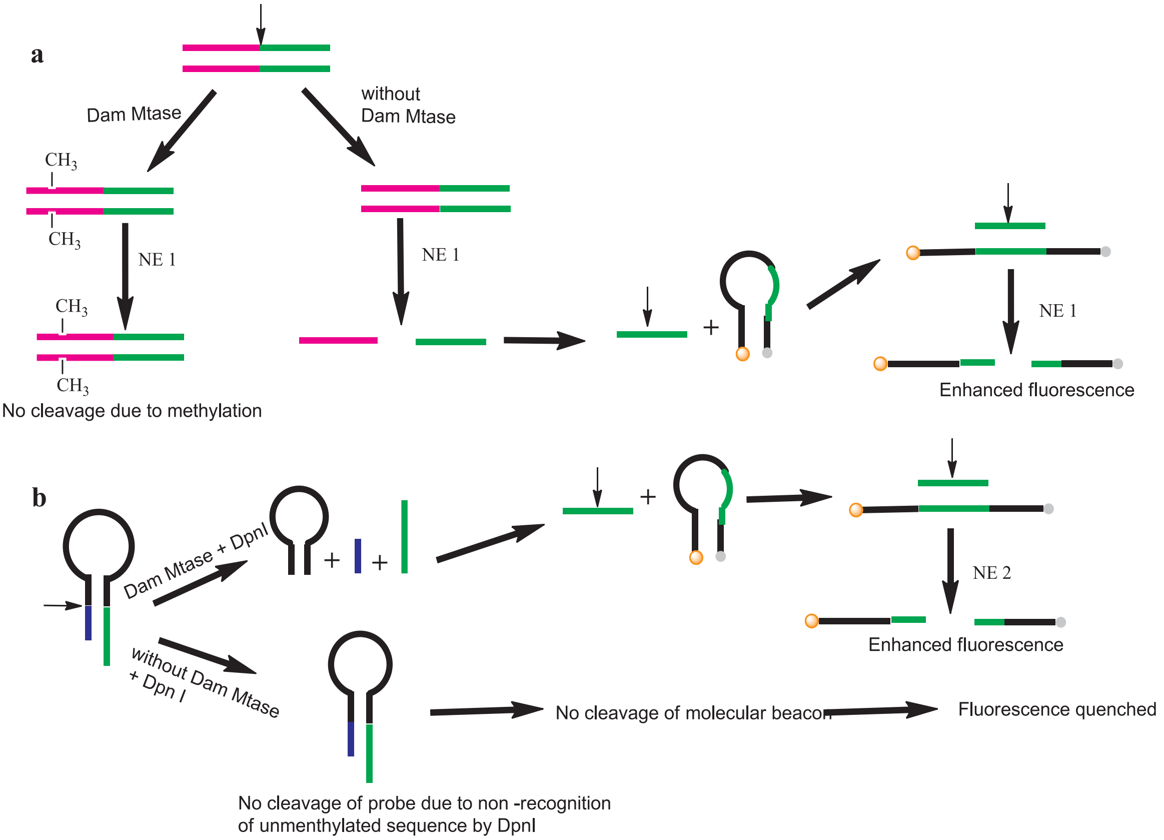


Fig. 5. a): Dam MTase assay depends on the NEases activity (NE1 that cleaves unmethylated sequences) that results in cleavage of probe and MB and hence enhanced fluorescence in the absence of Dam MTase, but quenches fluorescence in its presence. **b)** Dam MTase assay dependent of an endonuclease DpnI (which cleaves methylated sequences) and NEase NE 2 activity that results in enhanced fluorescence in the presence of Dam MTase and quenched fluorescence in its absence. Arrows around sequences points the recognition sequences for NEases (NE1&NE2) and endonuclease (DpnI).

including cancer biomarkers. Bioassays for the detection of miRNA, methyl transferase, ATP, thrombin, p53 gene etc. have been tremendously reported in the last few years. NESA-based strategies have superior advantages due to their specificity, simple instrumentation and capability for further improvement compared to other analytical methods. NEases have improved the detection limits of such assays to many folds, as reported by numerous studies (Cheng et al., 2016; Cui et al., 2016; Miao et al., 2015b; Zhao et al., 2014). This section is dedicated to discuss few important NEase assisted bioassays designed for clinical diagnosis of cancer biomarkers- Dam MTase and miRNAs.

4.1. DNA adenine methylation methyl transferase (Dam MTase)

Dam MTase is a key enzyme involved in the transfer of methyl group from S- adenosine methionine to the adenine or cytosine nucleotide of the recognition sequence. Methylation play vital role in cellular functions such as chromosome stability, genomic imprinting, X-chromosome inactivation and regulation of gene expression. Aberrant DNA methylation has been associated with development of various human pathologies including cancer. Hence, analysis of DNA methylation and Dam MTase activities are the essential factors and thus, Dam Mtase has been regarded as a potential biomarker for early diagnosis and prognosis of cancer. Dam MTase has not only earned an explosive attention due to its plausible role as cancer biomarker, but also as potential target for anticancer therapy (Chen and Zhao, 2013; Zhang et al., 2016c; Zhou et al., 2016a). Due to its high importance, it becomes highly essential to detect this enzyme sensitively in highly complex matrices such as serum samples. Their altered amount could directly be linked with diverse cancer types depending upon hypo or hyper-methylation extent of the target genes (Ehrlich, 2002; Lambert et al., 2015). This indicates that, accurate estimation of Dam MTase in real clinical samples is an opportunity to configure disease status. A number of studies have reported diverse assays for detection of Dam MTase which has been thoroughly compiled in a comprehensive review (Poh et al., 2016), however, only few studies on NESA based detection assays have been covered in the same. Therefore, it is an effort to advocate the role and significance of NEases in detection of Dam MTase in this section.

Estimation of Dam MTase is generally done by fluorescence based assays employing NEases as signal amplification tools (Zhang et al., 2015b). The simplest method is the use of a heteroduplex recognition probe equipped with recognition sequences for both, Dam MTase and NEase (Chen and Zhao, 2013). Fig. 5**a** illustrates NEase assisted mechanism proposed for detection of Dam MTase. In the absence of Dam MTase, unmethylated ds DNA probe is cleaved by NEase, which provides opportunity to cleaved probe to hybridize with the MB leading to improved fluorescence. The cleavage of linearized MB by NEase further enhances fluorescence. This same assay may be further modified by using enzymes Dam MTase and DpnI along with a HP probe harbouring recognition sequences for NEases. In this case, DpnI mediated cleavage of methylated HP probe (methylated by Dam MTase) generates ssDNA fragments that may hybridize with MB leading to improved fluorescence which could further be enhanced by NEase mediated cleavage of MB as illustrated in Fig. 5**b** (Zhao et al., 2013b). Similarly a number of investigations have reported employment of Nb.BsmI, Nb.BbvCI or Nt.BstNBI with DpnI to achieve highly sensitive Dam MTase detection through NESA (Xue et al., 2015a, 2015b; Zeng et al., 2013).

Cui et al., introduced a more sensitive fluorescence based method for detection of Dam MTase (Cui et al., 2016). The study used Zn^{2+} sensitive DNAzyme to quantify Dam MTase. A trifunctional recognition probe with a methylation site for MTase and a nicking site for Nb.BbvCI was fabricated. The method employed three enzymes, two probes and a MB. The sensitivity and selectivity achieved in the method is commendable and the method is applicable to complex biological matrices such as human serum samples. The developed assay has also displayed successful application in screening of potential anticancer drugs. Some researchers have also used nanoparticles in NESA based assays to achieve high sensitivity. Li et al. employed Raman probes prepared by gold nanoparticles and magnetic separation of signal producing probes that helped to remove background noise. The assay with three displacement reactions facilitated generation of large number of signal probes that were able to anneal with Raman probes and produce surface enhanced Raman scattering (SERS) signals (Li et al., 2016e).

Instead of one way reaction system, combination of SDA and exponential RCA have also been reported to provide better sensitivity for Dam MTase detection (Cui et al., 2017). In this investigation, nicking activity of Nt.BbvCI on hybrid of long stem loop probe and HP probe was able to generate primers for exponential RCA and produced enhanced fluorescence in the presence of Dam MTase. Recently, another report demonstrated exponential NEase mediated RCA strategy using dumbbell shaped probes (Huang et al., 2017). The C-rich dumbbell probe was designed with recognition sites for Hpa II, Dam MTase and NEase Nb.BbvCI which enabled the destruction of probe by Hpa II in the absence of MTase, however in the presence of MTase, RCA took place and resulted in NEase mediated production of G-quadruplexes. Thioflavin was used as the fluorescent probe in this case to produce enhanced fluorescence on interaction with G-quadruplexes (Huang et al., 2017). This study clearly indicated the advantage of simple operation, enhanced fluorescence and real time monitoring through inclusion of NEase in the assay over the traditional linear RCA (without NEase) performed by Zhao and co-workers (Zhao et al., 2016). In addition to exponential RCA, NEase mediated hybridization chain reaction (HCR) has also been reported to facilitate Dam MTase detection (Jiang et al., 2017). The study employed NEase to step wise generate hemin/G-quadruplex super sandwich structures through HCR that were able to quench electro-chemiluminescence signal of oxygen/persulfate system. However, in the presence of Dam MTase, NEase was unable to digest methylated probe and generate G-quadruplexes which resulted in suppression of quenching effect, subsequently enhanced electro-chemiluminescence was observed. The detection limits of few most important assays for Dam MTase have been tabulated in Table 2.

Another method of Dam MTase estimation is colorimetric assay. One such colorimetric assay has reported disappearance of green colour with increasing amount of Dam MTase (Zhao et al., 2014). The working principle was same as that of Chen and Zhao (2013), in addition, this assay involved re-synthesis of nicked probe by a polymerase in a cyclic process. The cleaved fragments folded into a G-quadruplex which catalysed the oxidation of colourless ABTS^{2-} to green ABTS^{-} by hemin and H_2O_2. In the presence of Dam MTase, the nicking reaction was constraint due to methylation which also restricted the formation of G-quadruplex and hence no colour change was observed. Owing to the naked eye identification, these methods provided rapid, cost effective and efficient point of care (POC) testing in cancer diagnosis.

Table 2
Characteristics of NESA based studies for detection of Dam MTase.

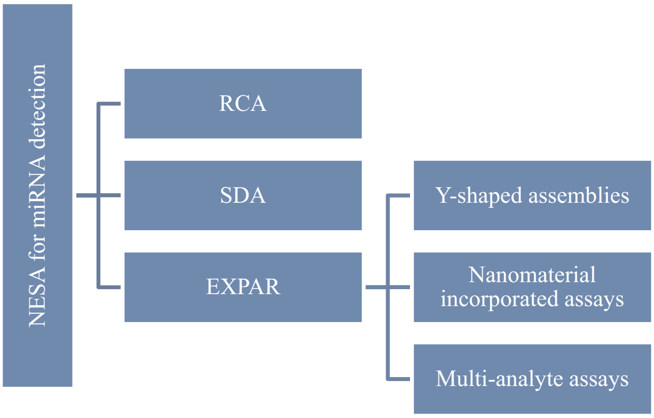
References	Assay time (min)	Detection limit (U ml^{-1})	Endonuclease used
Zheng et al. (2013)	120	1.29×10^{-4}	Nb.BsmI
Chen and Zhao (2013)	65	0.05	Nt.AlwI
Zhao et al. (2014)	77	0.4	Nt.AlwI
Xue et al. (2015a)	210	8.6 × 10–5	Nt.BstNBI
Xue et al. (2015b)	60	1.5×10^{-4}	Nb.BbvCI
Cui et al. (2016)	135	8.2×10^{-3}	Nb.BbvCI
Liu et al. (2016d)	160	2.57×10^{-4}	Nb.BbvCI
Cui et al. (2017)	360	8.1×10^{-15}	Nt.BbvCI
Huang et al. (2017)	150	1.1×10^{-3}	Nb.BbvCI
Jiang et al. (2017)	300	6.4×10^{-3}	Nt.AlwI

4.2. Micro RNAs

Micro RNAs (miRNAs) are the gene expression controlling, evolutionary-conserved, single-stranded non-coding RNAs, whose aberrant expression has been associated with various types of cancer. Hence, they have the potential to be used as promising and ideal biomarkers for classification, diagnosis, prognosis and therapy of various cancers (Kilic et al., 2018; Mittal et al., 2017; Zhang et al., 2016a). However, the specific and sensitive detection of miRNAs using conventional techniques (PCR, microarray and Northern blotting) is quite difficult and challenging because of its intrinsic properties like low plasma expression levels, high sequence homology, precise control of temperature cycling, expensive instrumentation and requirement of large amount of sample. Hence, new improved isothermal amplification strategies complementing currently available techniques for sensitive miRNA quantification are required (Yin et al., 2013). Chen et al. have given an elaborative account of signal amplification techniques for miRNA detection, but the review lacks discussion on the role of NEases (Chen et al., 2018). Therefore, the present review is an endeavour to focus the significance and importance of NEases in bioassays designed for the detection of miRNA.

NEases have been profoundly utilized in cascade bioassays for accurate quantification of miRNAs in diverse types of cancers (Bi et al., 2013; Cheng et al., 2016; Dong et al., 2014; Li et al., 2016a; Miao et al., 2015b; Wang et al., 2013). Generally, the NEase mediated bioassays utilized for miRNA can be divided into three broad categories as summarized in the Scheme 2.

As illustrated in the scheme, RCA, SDA and EXPAR are the main domains under which most of the miRNA detection assays can be categorized. A simple RCA may contribute a large number of DNA “triggers”, short oligonucleotides upon nickase action and may lead to enhanced signal (Chen et al., 2018; Jiang et al., 2016; Wen et al., 2012), but, it could further be amplified with the integration of padlock probe based exponential amplification (He et al., 2017; Liu et al., 2013; Wang et al., 2016d). He et al. has demonstrated combination of SERS technology with padlock probe based exponential RCA process to achieve sensitive and effective detection of miRNA-155 in clinical samples (He et al., 2017). The only limitation of RCA in miRNA detection is the requirement of pre-ligation step involving second enzyme, and the 3′- 5′ exonuclease activity of the involved Phi 29 DNA polymerase which may lead to false negative results (Zhang et al., 2014). Similarly SDA has also been integrated with isothermal exponential amplification to achieve sensitive and specific detection of miRNAs (Bi et al., 2016; Yue et al., 2017; Zhang et al., 2015a). But, SDA also suffers with constraints of relatively complex designs and poor selectivity (Yin et al., 2013;



Scheme 2. Categories of NESA based bioassays for miRNAs detection.

Table 3
The detection limits, and other characteristics of NESA based assays, designed for the detection of miRNAs.

Target miRNA	Method	Endonuclease employed	Readout	Linear range (M)	Limit of Detection (M)	References
Let 7a	EXPAR	Nt.BstNBI	Fluorescence	0.1×10^{-21}–10^{-15}	0.1×10^{-21}	Jia et al. (2010)
Let 7a	QD based two stage EXPAR	Nt.BstNBI	Fluorescence	0.1×10^{-18}–10^{-14}	0.1×10^{-18}	Zhang and Zhang (2011)
miRNA - 141	DNA-Scaffolded Silver-Nanocluster Probe Based on Isothermal Amplification	Nt.BstNBI	Fluorescence	10^{-18}–10^{-9}	2×10^{-18}	Liu et al. (2012)
Let 7d	Cascade RCA- NESA DNAzyme amplification	Nt.BbvCI	Colour change	2×10^{-18}–10×10^{-18}	2×10^{-18}	Wen et al. (2012)
Let 7a	Padlock Probe-Based Exponential Rolling Circle Amplification	Nt.BbvCI	Fluorescence	0.3×10^{-21}–18×10^{-21}	0.24×10^{-21}	Liu et al. (2013)
Let 7b	EXPAR	Nt.BstNBI	Electrochemical signal	10^{-14}–10^{-8}	98.9×10^{-15}	Yan et al. (2013)
miRNA – 141	One step cascade enzymatic amplification	Nt.BsmAI	Fluorescence	10^{-15}–10^{-8}	1×10^{-15}	Yin et al. (2013)
miRNA - 21	Target-triggered isothermal exponential amplification reaction (TIEAR)	Nt.BstNBI	Silver nanoclusters based fluorescence	0.1×10^{-12}–10^{-8}	10×10^{-15}	Zhang et al. (2013)
miRNA - 141	Target-triggered isothermal exponential amplification reaction (TIEAR)	Nt.BstNBI	Silver nanoclusters based fluorescence	0.1×10^{-12}–10^{-8}	10×10^{-15}	Zhang et al. (2013)
Let 7a	Graphene Fluorescence Switch-Based Cooperative Amplification	Nt.BstNBI	Fluorescence	0.06×10^{-12}–12×10^{-12}	10.8×10^{-15}	Liu et al. (2014a)
miRNA - 21	Arched probe based EXPAR	Nb.BbvCI	Electrochemical signal	20×10^{-15}–50×10^{-12}	5.36×10^{-15}	Yu et al. (2014)
Let 7b	Target-Triggered Three-Way Junction - Enzyme Synergetic Isothermal Quadratic DNA Machine	Nb.BbvCI	Fluorescence	$10^{-15} \times 10^{-12}$	10^{-13}	Zhang et al. (2014)
miRNA- 21	SDA	Nt.BsmAI	Electro-chemiluminescence	10^{-17}–10^{-12}	3.3×10^{-18}	Chen et al. (2015a)
miRNA - 21	DNABio-Bar-Code based Enzyme-Assisted Strand Cycle Exponential Signal Amplification	Nb.BbvCI	Fluorescence	3×10^{-18}–3×10^{-13}	52.5×10^{-21}	Dong et al. (2015)
miRNA - 21	Electrochemical biosensor	Nb.BbvCI	Electrochemical signal	10^{-17}–10^{-9}	30×10^{-18}	Miao et al. (2015b)
Let 7a	Toehold-mediated strand displacement (TMSD)	Nb.Mva1269I	Fluorescence	10^{-14} –10^{-9}	58×10^{-15}	Wang et al. (2015)
miRNA- 203	SERS based cyclical nucleic acid strand displacement polymerization reaction (CNDP)	Nt.AlwI	SERS	10^{-17}–10^{-14}	6.3×10^{-15}	Zhang et al. (2015a)
miRNA - 122	Target triggered cascade recycling amplification (CRA)	Nb.BbvCI	Chemiluminescence	10^{-15} – 10^{-10}	0.82×10^{-15}	Bi et al. (2016)
Let 7a	Real time exponential rolling circle amplification (ERCA)	Nb.BbvCI	Fluorescence	10^{-17} – 10^{-9}	4×10^{-18}	Jing et al. (2016)
miRNA - 21	Functional nucleic acid-basedamplification machine	Nb.BbvCI	Colour change	10^{-14} – 10^{-9}	5×10^{-18}	Li et al. (2016a)
miRNA - 221	Isothermal exponential amplification reaction-assisted gold nanoparticle amplification	Nt.BstNBI	Colour change	50×10^{-15} - 10^{-8}	46×10^{-15}	Li et al. (2016b)
miRNA-21	Graphene oxide integrated isothermal exponential amplification	Nt.BtsI	Fluorescence	10^{-14} X 10^{-11}	3×10^{-15}	Li et al. (2016d)
Let 7b	YJS-mediated SDA-RCA process	Nb.BbvCI	Fluorescence	10^{-11} – 10^{-8}	32×10^{-12}	Wang et al. (2016d)
miRNA - 122	3 WJ based T- EXPEA	Nb.BbvCI	Fluorescence	10^{-17} – 10^{-11}	7.8×10^{-18}	Xu et al. (2016)
Let 7a	Catalyst oligomer mediated enzymatic amplification (CMEA)	Nt.CviPII	Fluorescence	2×10^{-12} –10^{-8}	10×10^{-15}	Zhang et al. (2016a)
miRNA – 27a	Triple cascade- isothermal exponential enzymatic amplification (IEEA)	Nt.BstNBI	Fluorescence	2×10^{-18} – 10^{-8}	0.79×10^{-18}	Zhou et al. (2016b)
miRNA - 155	Combination of SERS platform with padlock probe based exponential rolling circle amplification	Nb.BbvCI	SERS	10^{-16} – 10^{-10}	70.2×10^{-18}	He et al. (2017)
miRNA- 21	Hairpin probes-mediated exponential isothermal amplification	Nt.BstNBI	Fluorescence	10^{-14} - 10^{-10}	3×10^{-15}	Liu et al. (2017)
miRNA-141	3D DNA walker machine based on ‘on-off-super on strategy’	Nt.smAI	Electro-chemiluminescence	10^{-14} – 10^{-10}	3×10^{-15}	Xu et al. (2017)
miRNA-21	DNA strand displacement reaction and Mg^{2+}-dependent DNAzyme cleavage	Nt.BstNBI	Fluorescence	10^{-15} – 5×10^{-11}	0.27×10^{-15}	Yin et al. (2017)
miRNA - 155	Toehold-mediated strand displacement (TMSD) reactions	Nb.BbvCI	Chemiluminescence	10^{-14} – 10^{-10}	7.6×10^{-15}	Yue et al. (2017)
miRNA - 141	Toehold-mediated strand displacement (TMSD) reactions	Nb.BbvCI	Chemiluminescence	10^{-14} – 10^{-10}	5.8×10^{-15}	Yue et al. (2017)
Let 7a	Toehold-mediated strand displacement (TMSD) reactions	Nb.BbvCI	Chemiluminescence	10^{-14} – 10^{-10}	8.3×10^{-15}	Yue et al. (2017)
miRNA - 17	Mismatch- protected-long-stem hairpin probe (mLSHP mediated NESA	Nb.BbvCI	Fluorescence	12.5×10^{-15}–2.5×10^{-12}	2.5×10^{-15}	Huang et al. (2018)

Zhang et al., 2014). As a consequence, EXPAR comes out to be the most preferred and sensitive method for NESA based miRNA detection (Deng et al., 2017; Xu et al., 2016; Yan et al., 2013; Yu et al., 2014).

EXPAR is a unique technique employing exponential synthesis of short oligonucleotides by itself, which acts as primer for amplification process. EXPAR utilizes template containing two copies of sequence complementary to the target DNA. These sequences are separated by a recognition sequence for NEase. EXPAR implicates DNA polymerase strand extension with strand displacement activity and NEase mediated single strand nicking at a temperature of 55 °C. Further, this reaction temperature of EXPAR is close to the estimated T_m value for annealing (according to the length of miRNA) and hence employing EXPAR for miRNA analysis represents a well-suited example. The intrinsic merits of isothermal nature, rapid and high amplification kinetics with 10^6-10^9 fold amplification in relatively less time and expense along with minimal interference by inhibitory components of clinical samples are the attributes of EXPAR that confers distinct advantage and thus, makes it a valuable method (Yu et al., 2014). Therefore, this section is dedicated to discuss some common NESA based EXPAR strategies and designs used to detect miRNA in complex biological matrices such as human serum samples and cell lysates. EXPAR could further be categorized into assays with Y-shaped assemblies, nanomaterials incorporated assays and assays designed for multi-miRNA detection.

4.2.1. EXPAR based on Y-shaped assemblies

A major constraint in utilization of NEase-assisted amplification strategy is requirement of specific sequence containing target analyte. To counteract this limitation Y-shaped probe detection system has been developed. The Y-shaped junction structure contains three complementary branches of oligonucleotides. It has been reported to be based on "template enhanced hybridization process", in which Y-shaped junction structure is formed due to hybridization of target DNA, signal probe and assistant probe (Dong et al., 2014; Kong et al., 2010; Song et al., 2016; Wang et al., 2016d). Dong et al. reported employment of mercury intercalated MB probe and silver nanoclusters to realize signal amplification. A Y-shaped assembly was formed between the three i.e. MB probe, target sequence and an assistant probe. NEase mediated cleavage of MB released intercalated mercury that quenched fluorescence of silver nanoclusters. The nicking activity also regenerated assistant probe and target sequence to carry out another cleavage cycle that further quenched fluorescence (Dong et al., 2014). Recent studies have displayed imperative role of NEases for miRNA detection through electrochemical processes. Miao et al. immobilized a methylene blue labelled capture probe on electrode surface which contained a nicking domain for Nb.BbvCI and was partially complementary to an assistant probe and the target miRNA. Association of the three moieties (capture probe, assistant probe and the target miRNA) on the working electrode produced a Y-shaped configuration which induced NEase assisted cleavage. This resulted in release of assistant probe and target miRNA for recycling and loss of methylene blue from the electrode surface led to differential change in electrochemical signal (Miao et al., 2015b). Similar strategy was utilized by Chen et al. to amplify a segment released after the activity of Nt.BsmA1 on an assembly of capture probe and target miRNA. The amplified segment was incorporated in a Y-shaped junction at the electrode to produce electro-chemiluminescence response, which was quenched in the presence of target miRNA (Chen et al., 2015a). With novel probe designing, Y-shaped junction binary probe strategy seems promising towards improvement of sensing system for effective and amplified detection of various target analytes.

4.2.2. Nanomaterial incorporated EXPAR

Among the various novel methods developed for the purpose of miRNA analysis, the application of nanotechnology employing intracellular nanoprobes offers easy, rapid, reliable, highly sensitive, cost-effective and simple operation option and thus, opens new avenues for improvement of biomarker detection (Chen et al., 2018). Silver nanoclusters have been utilized in assays for miRNA detection due to their inherent fluorogenic properties (Dong et al., 2014; Liu et al., 2012; Zhang et al., 2013). Some highly sensitive method for miRNA detection has been reported by utilization of EXPAR system with DNA bio-barcode (Dong et al., 2015; Zhang et al., 2015a). Gold nanoparticles (AuNPs) have been broadly used in such bio-bar-codes for nucleic acid-based detection strategies. Dong et al. reported inclusion of AuNP labelled probes, LNA probes and NEase Nb.BbvCI to produce specific and sensitive sandwich hybridization structures capable of producing fluorescence in response to miRNA-21 (mediated by cleavage of MB). LNA probes are high affinity RNA analogues, with 10 fold higher efficiency than classical DNA probes (Dong et al., 2013) and hence attributed a detection limit of 52.5 zM exclusively for miRNA-21 (Dong et al., 2015). Utilization of AuNPs labelled probes have also been reported for EXPAR based detection of miRNA-221–3p (Li et al., 2016b). The detection limits, and other characteristics of assays designed for detection of miRNAs mediated through NESA have been tabulated in Table 3.

Incorporation of graphene oxide (GO) in EXPAR is another technique for signal amplification. The extra-ordinary quenching ability of GO has been regarded as the reason for sensitive estimation of miRNA with low background fluorescence (Li et al., 2016d; Liu et al., 2014a). Briefly, the target miRNA was non-covalently immobilized on GO, followed by its amplification through duplex formation with a HP probe and polymerase activity. The large numbers of DNA templates (triggers) were determined through SYBR green as the intercalated fluorescent dye or followed by strand displacement and amplification step leading to circular exponential amplification. Such nanomaterial based NESA assays have been reported to be highly specific which could discriminate between pre and mature miRNAs and even one base mismatch. Their application to cell lysates is the most important attribute contributed by the simple, label free and low-cost assay designs.

Another kind of nanomaterial incorporated in NESA assays are the QD (Niu et al., 2010). A two stage EXPAR equipped with a single QD has been reported for miRNA detection (Zhang and Zhang, 2011). The investigation has employed polymerase and NEase for generating large number of target oligonucleotides. In addition, Cy5 labelled reporter probes and biotin labelled capture probes were utilized to form sandwich between them and target oligonucleotides. These numerous sandwich hybrids were assembled at a single streptavidin labelled QD that resulted in fluorescence resonance energy transfer (FRET) based fluorescence enhancement. The assay claimed zero background signals in the absence of target miRNA, in addition to this, the ability to convert different miRNAs into same reporter oligonucleotides provided the practical flexibility to the proposed assay. This delivered the ease to use the same reporter and capture probes for different miRNAs, without the need to resynthesize specific DNA probes (Zhang and Zhang, 2011). Another recent study employed graphene QD as fluorescence quencher of DAP (which was formed by G-quadruplex mediated oxidation of OPD). The study ruled out the background signal through the incorporation of single walled carbon nanotubes (SWCNTs) which have strong affinity for free hemin and thus reduced their contribution in back ground signal (Wang et al., 2016a). An additional strategy reported employment of 3D DNA walking machine comprising of AuNP, CdS:Mn QDs, protector probe, walker probe and supporter probe to construct an 'on-off-super on' type of biosensor for miRNA detection (Xu et al., 2017). The investigation advocated that NEase mediated generation of intermediate DNA enabled achievement of super on condition that offered advantage of reduced background signal and impressive detection sensitivity. These nanostructures enable highly sensitive detection limits, and thus, seem plausible for miRNA analysis.

4.2.3. EXPAR assays for multi- miRNA detection

Recently researchers are focusing on multi-analyte assays in comparison to single-target assays due to the benefits of simultaneous analysis in less time (Yue et al., 2017). In addition to this, such analytical methods provide the estimation of more than one biomarker and hence confirm the disease status. EXPAR has been modified to assess levels of multi-miRNA in serum samples with incorporation of multicoloured fluorescent probes or chemiluminescence imaging array (Yue et al., 2017; Zhang et al., 2013). Zhang et al. employed sequence specific oligonucleotide templates to synthesize fluorescent/muti-colour DNA/silver nanocluster probes with distinct optical properties for simultaneous detection of miRNA-21 and miRNA-141. Whereas, Yue et al. reported a chemiluminescence imaging array for high throughput and simultaneous detection of miR-155, miR-let-7a and miR-141 based on EXPAR and programmable DNA toehold-mediated strand displacement reaction. Such multi-analyte NESA based EXPAR assays for miRNA are rare, but they are the forthcoming cancer biosensors and would provide reliable and highly efficient POC devices in near future.

In spite of so many advantages being offered by EXPAR, they also suffer from some drawbacks such as non-specific background amplification due to interaction between templates and polymerase in the absence of target miRNA. Secondly they show unsatisfactory specificity towards homologous sequences with mismatch bases at 5′ terminus and in the middle (Zhang et al., 2014). In addition to this, all the EXPAR based methods involve cumbersome procedures which are vulnerable to contamination making it a tough job for clinical diagnosis (Yin et al., 2013). Still NESA based EXPAR methods are the most sensitive approaches developed so far for cancer diagnosis, and further improvements in the designs would definitely potentiate their application as POC tools.

5. Conclusions and future prospects

This review focuses on the recent progress in the application of NEases and describes the basic principles, acting roles of NEases, signalling strategies, and bioassays for detection of cancer biomarkers Dam MTase and miRNA. Although, NEase has facilitated bioassay designs for sensitive and specific estimation of these biomarkers, the assays suffer from few limitations and still need further improvement to develop into reliable POC devices. Firstly, all the investigations discussed showcase the need of a very sophisticated instruments and contamination free zones for analysis that limits their applicability as robust analytical method. Secondly, although some signalling strategies including gold and carbon-based nanomaterials have been employed in NESA, the exploration of other nanomaterials is still in the initial stages. Thirdly, the poor stability and reproducibility of the current bioassays are hardly comparable to those of commercial enzyme-linked immunoassays (ELISAs), which greatly limit their application. In particular, the amplification of undesired sequences should be carefully controlled because nonspecific events on the sensing interface are an important cause of low reproducibility and background noise. A great deal of effort should be devoted to overcome this problem. Finally, simultaneous multi-analyte and high throughput bioassays are particularly valuable for diagnostic screening of miRNAs. However, the related scientific reports are rare and advocates for new interventions in this regard.

Fortunately, along with the constant advances in RCA, SDA and EXPAR methods, there are also great opportunities and bright prospects for the development of NESA based reliable diagnostic tools for cancer detection. Dumbbell probe mediated exponential RCA is a promising strategy for Dam MTase detection and further improvement in probe designing could furnish better results. Moreover, by incorporating photo-electrochemical (PEC) sensing system background signals may be lowered and hence higher sensitivity may be achieved. Further, miniaturization and microfluidic approaches may convert the long and complex analysis protocols into real time monitoring portable devices. This may enable high-throughput assay for large quantities of complex samples. NEases have provided the opportunity to design cyclic processes for minute detection of diverse analytes, however the realization of a POC device is still in infancy and need further research to become reality.

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Declaration of interest

The authors would wish to declare that they have no conflict of interest related to any publishing issue.

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