

SUMMARY OF THE INVENTION

[0011] In one aspect, the present disclosure refers to a target reporter construct (TRC) for detecting a target nucleic acid, the TRC being a closed, partially single-stranded nucleic acid comprising: (a) a target sequence complementary to the target nucleic acid; (b) a bridge sequence forming a double-stranded portion of the TRC; and (c) an accessory sequence comprising a multifunctional probe sequence, wherein the bridge sequence comprises a palindromic sequence for the formation of nanospheres during replication, and wherein the target sequence is further defined as a toehold switch, wherein the toehold switch is a sequence capable of uniquely hybridizing to the target nucleic acid. In one example, there are disclosed ~~there are provided~~ target reporter constructs (TRCs) for detecting a target nucleic acid, the TRCs being closed, partially single-stranded nucleic acid molecules comprising: (a) a target sequence complementary to the target nucleic acid, (b) a bridge sequence forming a double-stranded portion of the TRC; and (c) an accessory sequence comprising a multifunctional probe sequence. In some example embodiments, the TRCs comprise DNA, RNA, or a combination thereof. In some example embodiments, the TRCs comprise standard canonical bases, modified bases, non-natural bases, or any combination of the bases thereof.

[0012] In some example embodiments, the target nucleic acid is single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), or ssRNA. In some example embodiments, the ssRNA is microRNA (miRNA) (*e.g.*, cell-free miRNA). In some example embodiments, the ssRNA is a non-coding RNA (ncRNA). In some example embodiments, the ncRNA is small interfering RNA (siRNA), piwi-interacting RNA (piRNA), miRNA, long non-coding RNA (lncRNA), or small nucleolar RNA (snoRNA).

[0013] In some example embodiments, the target sequence is further defined as a toehold switch, wherein the toehold switch is a sequence capable of uniquely hybridizing to the target nucleic acid. In some example embodiments, the toehold switch comprises a double-stranded switch stem and a single-stranded toehold. In some example embodiments, the toehold is between 5 and 10 nucleotides in length. In some example embodiments, the toehold is 7, 8, or 9 nucleotides in length. In some example embodiments, the toehold switch comprises a portion of the bridge sequence. In some example embodiments, the toehold switch is within 5-10 nucleotides of the bridge sequence. In some example embodiments, the toehold switch is within 10-20 nucleotides of the bridge sequence.

In some exampleembodiments, the toehold switch is within 1-5 nucleotides of the bridge sequence. In some exampleembodiments, the toehold switch and bridge sequence overlap. In some exampleembodiments, the target sequence or toehold switch is structurally independent from the bridge sequence.

5 **[0014]** In some exampleembodiments, the TRCs further comprise a second target sequence complementary to a second target nucleic acid.

[0015] In some exampleembodiments, the bridge sequence comprises a palindromic sequence. In some exampleembodiments, the bridge sequence is between the target sequence and accessory

sequence. In some exampleembodiments, the bridge sequence comprises a low complexity, repetitive sequence, such as di-, tri-, and/or tetra-nucleotide repeats.

[0016] In some exampleembodiments, the TRCs comprise multiple, such as two or more, bridge sequences. In some exampleembodiments, the multiple bridge sequences are identical in sequence. In some exampleembodiments, the multiple bridge sequences comprise different sequences.

[0017] In some exampleembodiments, the accessory sequence further comprises at least a second probe sequence. In some exampleembodiments, the second probe sequence is distinct from the first probe sequence. In some exampleembodiments, the first probe sequence and distinct second probe sequence form a unique multiplex probe signature. In some exampleembodiments, the first probe sequence is specific to a first detectable moiety, such as a fluorophore, and the second probe sequence is specific to a second detectable moiety, such as a second fluorophore. In some exampleembodiments, the unique probe signature further comprised a third, fourth, or fifth probe sequence. In some exampleembodiments, the unique multiplex probe signature comprises a plurality of fluorophores, such as 2, 3, 4, 5, or more fluorophores.

[0018] In some exampleembodiments, the TRCs form a loop structure under hybridization conditions that are well known in the art. In some exampleembodiments, the TRCs form a hairpin structure or dumbbell structure. In some exampleembodiments, the TRCs form a three- or four-looped structure. In some exampleembodiments, the TRCs form a multi-loop structure. In some exampleembodiments, the accessory sequence or portion thereof (*e.g.*, probe sequence) forms the loops structure and the bridge sequence and/or the toehold switch stem forms the stem region of a dumbbell structure.

[0019] In some exampleembodiments, the multifunctional probe sequence comprises a binding sequence. In some exampleembodiments, the binding sequence facilitates localization of replicated TRCs to an oligonucleotide array. In some exampleembodiments, the oligonucleotide array is used to identify and quantify the presence of target nucleic acids.

[0020] In some exampleembodiments, the TRCs are between 50 and 100 nucleotides in length. In some exampleembodiments, the TRCs are between 100 and 500 nucleotides in length.

[0021] In another aspect, ~~there are provided~~ the present disclosure refers to a replicated TRC₁ wherein the replicated TRC comprises ~~comprising~~ concatenated monomer repeats of a TRC sequence as disclosed herein. In some exampleembodiments, the replicated

TRC is produced from rolling circle replication and/or rolling circle amplification of a TRC, such as a TRC of the above ~~embodiments~~examples.

[0022] In a further aspect, ~~there is provided~~the present disclosure refers to a replicated nanosphere comprising a replicated TRC as disclosed herein, wherein the replicated TRC ~~which has~~ undergone intramolecular hybridization between the bridge sequences within the replicated TRC.

[0023] In ~~another aspect~~one example, intramolecular hybridization between the bridge sequences within replicated TRCs is minimal and there is no higher-order structure formed upon replication or amplification of the TRC. In some ~~examples~~embodiments, the replicated TRC may comprise a substantially single-stranded product that can be detected (*e.g.*, by probes or localization) or serve as a substrate for RCA reactions.

[0024] ~~A further aspect provides~~In yet another aspect, the present disclosure refers to a method of detecting a target nucleic acid comprising: (a) obtaining target reporter constructs (TRCs) ~~of the embodiments~~as disclosed herein, (b) contacting the TRCs with a population of target nucleic acids, wherein ~~individual the~~ target nucleic acids ~~molecules~~ within the population hybridize to the complementary target sequence of the target-specific TRC, (c) performing rolling circle replication (RCR), thereby obtaining replicated TRCs, (d) introducing at least one detectable moiety, wherein the at least one detectable moiety binds the probe sequence of the replicated TRC; and (e) detecting the at least one detectable moiety, thereby detecting the target nucleic acid.

[0025] ~~A further aspect provides~~In another example, there is described a method of detecting target nucleic acid comprising: (a) obtaining target reporter constructs (TRCs) of the ~~examples~~embodiments, (b) contacting the TRCs with a population of target nucleic acids, wherein individual target nucleic acid molecules within the population hybridize to the complementary target sequence of the target-specific TRC, (c) performing rolling circle replication (RCR), thereby obtaining replicated TRCs, (d) introducing an intercalating dye that binds the replicated TRC, and (e) detecting the detectable signal from such a bound dye, thereby detecting the target nucleic acid.

[0026] ~~A further aspect provides~~In another example, there is described a method of detecting target nucleic acid comprising: (a) obtaining target reporter constructs (TRCs) of the ~~embodiments~~examples, (b) contacting the TRCs with a population of target nucleic acids, wherein individual target nucleic acid molecules within the population hybridize to the complementary target sequence of the target-specific TRC, (c) performing rolling circle replication (RCR) in the presence of

pH sensitive dyes, thereby obtaining replicated TRCs, and (d) detecting the fluorescent or colorimetric signal from such a dye, thereby detecting the target nucleic acid.

[0027] ~~A further aspect provides~~In yet another example, there is disclosed a method of detecting target nucleic acid comprising: (a) obtaining target reporter constructs (TRCs) of the ~~embodiments~~examples, (b) contacting the TRCs with a population of target nucleic acids, wherein individual target nucleic acid molecules within the population hybridize to the complementary target sequence of the target-specific TRC, (c) performing rolling circle replication (RCR) obtaining replicated TRCs; and (d) introducing the replicated TRCs onto an organized array, and (e) detecting the replicated TRCs with intercalating dyes, fluorescent probes, or by the presence of modified nucleotides that have been incorporated into the replicated TRC, thereby detecting the target nucleic acid.

[0028] ~~A further aspect provides~~In one example, there is described a method of detecting target nucleic acid comprising: (a) obtaining target reporter constructs (TRCs) of the ~~embodiments~~examples, (b) contacting the TRCs with a population of target nucleic acids, wherein individual target nucleic acid molecules within the population hybridize to the complementary target sequence of the target-specific TRC, (c) performing rolling circle replication (RCR) obtaining replicated TRCs; and (d) introducing the replicated TRCs onto an array which has location specific binding probes capable of hybridizing with the accessory sequence of a target-specific replicated TRC, and (e) detecting the replicated TRCs with intercalating dyes, fluorescent probes, or by the presence of modified nucleotides that have been incorporated into the replicated TRC, thereby detecting the target nucleic acid.

[0029] ~~A further aspect provides~~In another example, there is disclosed a method of detecting target nucleic acid comprising: (a) obtaining target reporter constructs (TRCs) of the ~~embodiments~~examples (b) contacting the TRCs with a population of target nucleic acids, wherein individual target nucleic acid molecules within the population hybridize to the complementary target sequence of the target-specific TRC, (c) performing rolling circle replication (RCR), (d) performing rolling circle amplification (RCA) of the replicated TRC with bound or free secondary primers that hybridize with the accessory sequence on the replicated TRC, and (d) detecting the replicated TRCs with intercalating dyes, fluorescent probes, or by the presence of modified nucleotides that have been incorporated into the replicated TRC, thereby detecting the target nucleic acid.

[0030] In another example, there is disclosed ~~A further aspect provides~~ a method of detecting target nucleic acid comprising: (a) obtaining target reporter constructs (TRCs) of the embodiments and attaching them to a solid or porous support, such as but not limited to a styrene bead or microscope slide (b) contacting the TRCs with a population of target nucleic acids, wherein individual target nucleic acid molecules within the population hybridize to the complementary target sequence of the target-specific TRC, (c) performing rolling circle replication (RCR), (d) performing rolling circle amplification (RCA) of the replicated TRC with bound or free secondary primers that hybridize with the accessory sequence on the replicated TRC; and (d) detecting the replicated TRCs with intercalating dyes, fluorescent probes, or by the presence of modified nucleotides that have been incorporated into the replicated TRC, thereby detecting the target nucleic acid.

[0031] In some ~~embodiments~~ examples of the above aspects, performing RCR and/or RCA comprises introducing a strand-displacing polymerase, such as a DNA polymerase or RNA polymerase. In some ~~embodiments~~ examples, the DNA polymerase is ϕ 29, *Bst*, *Bsu*, or Klenow exonuclease.

[0032] In some example ~~embodiments~~, performing RCR and/or RCA comprises introducing a polymerase and accessory proteins like topoisomerases, helicases, single- and double-stranded binding proteins to facilitate strand displacement.

[0033] In some example ~~embodiments~~, step (b) and step (c) of the above aspects are performed in a single tube. In some example ~~embodiments~~, RCR is performed for 45 minutes to 90 minutes. In some example ~~embodiments~~, RCR is performed for 90 minutes to 48 hours.

[0034] In some example ~~embodiments~~, the target nucleic acids have a length greater than the length of the TRC, thereby producing a 3' overhang or a 5' overhang. In some example ~~embodiments~~, the method further comprises introducing an exonuclease. In some example ~~embodiments~~, the exonuclease is exonuclease T. In some example ~~embodiments~~, the exonuclease removes a 3' overhang of the target nucleic acid hybridized to the TRC, thereby presenting a 3'-OH group.

[0035] In some example ~~embodiments~~, obtaining replicated TRCs comprises hybridizing the bridge of at least one of the replicated TRCs within the population to the bridge of at least a second replicated TRC within the population. In some example ~~embodiments~~, the replicated TRCs comprise at least two replicated TRCs interconnected at the bridge. In some example ~~embodiments~~, a

replicated TRCs comprises at least 100, 1000, 5000, or 50000 replicated TRCs interconnected at the bridge.

[0036] In some exampleembodiments, the population of target nucleic acids comprises pathogenic nucleic acids, such as viral pathogenic nucleic acids. In some exampleembodiments, the pathogenic nucleic acids are human immunodeficiency virus (HIV), herpes simplex virus (HSV-1), human papillomavirus (HPV), and/or Epstein-Barr virus (EBV) pathogenic nucleic acids. In some exampleembodiments, the target sequence for the pathogenic nucleic acids comprises a length of 50-100 nucleotides.

[0037] In some exampleembodiments, the population of target nucleic acids comprise one or more miRNAs. In some exampleembodiments, the one or more miRNAs are tumor-associated miRNAs. In some exampleembodiments, the tumor-associated miRNAs are selected from the group consisting of miR-let-7, miR-10b, miR-21, miR-25, miR-106b, miR-11, miR-196a, miR-210, miR-212, and miR-221.

[0038] In some exampleembodiments, the population of target nucleic acids comprise diabetes-associated miRNAs. In some exampleembodiments, the diabetes-associated miRNAs are selected from the group consisting of miR-146a, miR-195, miR-320, miR-29, miR-192, miR-377, miR-126, miR-203, and miR-503.

[0039] In some exampleembodiments, the population of target nucleic acids comprise psychiatric disorders-associated miRNAs. In some exampleembodiments, the psychiatric disorders-associated miRNAs can be selected from the group consisting of miR-128, miR-134, miR-182, miR-652, miR-132, miR-15b, miR-let-7b, miR-let-7c, miR-1202, miR-135, miR-124, miR-let-7d, miR-181a, miR-212, miR-207, miR-298, miR-26b, miR-30b, miR-29b, miR-195, miR-92, miR-30a-5p, miR-30d, miR-20b, miR-29c, miR-29a, miR-106b, miR-7, miR-24, miR-30c, miR-9-3p, let-7g, miR-181b, miR-185, miR-674, miR-532, miR-673, miR-224, miR-491, miR-93, miR-383, miR-422b, miR-708, miR-540, miR-106b, miR-140, miR-194, miR-325, miR-494, miR-362, miR-409, miR-323, miR-669a, miR-151, miR-18, miR-219, miR-596, miR-597, miR-124-1, miR-598, miR-320, miR-486, miR-219, miR-346, miR-219, miR-1202, miR-135, and miR-16.

[0040] In some exampleembodiments, the population of target nucleic acids comprise gynecological-associated miRNAs. In some exampleembodiments, the gynecological-associated miRNAs can be selected from the group consisting of miR-451a, miR-20a, miR-29c, miR-

145, miR-200a, miR-191, miR-543, miR-141, miR-let-7b, miR-126, miR-17, miR-210, miR-202, miR-122, miR-183, miR-196a, miR-2, miR-22, miR-let-7d, miR-143, miR-195, miR-200b, miR-21, miR-940, miR-4634, miR-100, miR-10b, miR-128, miR-1, miR-215, miR-23a, miR-143, miR-195, miR-200b, miR-21, miR-940, miR-4634, miR-100, miR-10b, miR-1215, miR-23a, miR-23b, miR-26a-1, miR-135b, miR-196b, miR-503, miR-504, miR-629, miR-10a, miR-100, miR-184, miR-193a, , miR-297, miR-625, miR-let-7a/f/d/g, miR-146b, miR-5p, miR-155, miR-193a, miR-297, miR-602, miR-888, miR-212, miR-662, miR-299, miR-339, miR-5p, miR-486, miR-5p, miR-768, miR-5p, miR-376a, miR-15a, miR-29a, miR-30d, miR-93*, miR-125miR-a, miR-320a, miR-7*, miR-425, miR-744, miR-146, miR-7d, miR-202, miR-7e, miR-233b, miR-523amiR-3p, miR-188, miR-10a, miR-105, miR-182, miR-372, miR-27b, miR-339, miR-3p, miR-345, miR-25, miR-302c, miR-196a2, miR-181a, miR-372, miR-645, miR-9, miR-18b, miR-19b, miR-27b, miR-30c, miR-93, miR-103, miR-132, miR-135a, miR-146a, miR-155, miR-222, miR-224, miR-320, and miR-383.

[0041] In some exampleembodiments, the population of target nucleic acids comprise neurodegenerative disease-associated miRNAs. In some exampleembodiments, the neurodegenerative disease-associated miRNAs can be selected from the group consisting of miR-195, miR-30d, miR-451, miR-328, miR-92a, miR-486, miR-505, miR-362, miR-151, miR-20a, miR-let-7, miR-106a*, miR-133b, miR-153, miR-184*, miR-205, miR-21*, miR-224, miR-26b, miR-301b, miR-34b/c, miR-373, miR-433, miR-64, miR-65, miR-7, miR-9, miR-106a, miR-106b, miR-107 miR-124a, miR-132, miR-146a, miR-153, miR-181c, miR-29a, miR-29b, miR-29c, miR-34a, miR-429, miR-106b, miR-124a, miR-125b, miR-146a, miR-150, miR-200a, miR-200c, miR-34b, miR-9, miR-9*, miR-206, miR-21, miR-31, miR-30c-1, miR-340, miR-208b, miR-499a, miR-4538, miR-4539, miR-208a, miR-95, miR-486, miR-1, miR-539, miR-606, miR-454, miR-124a, miR-146a*, miR-206, miR-338, miR-3p, miR-9, miR-146a, miR-106b, miR-134, miR-155, miR-935, miR-149, miR-196amiR-2, miR-203a, miR-219a, miR-1, miR-301a, miR-30a, miR-34a, miR-499a, miR-876, miR-1295a, miR-10b, miR-15a, miR-193a, miR-204, miR-21, miR-27a, miR-378a, and miR-487a.

[0042] In some exampleembodiments, the population of target nucleic acids comprise autoimmune disease-associated miRNAs. In some exampleembodiments, the autoimmune disease-associated miRNAs can be selected from the group consisting of miR-146a, miR-155, miR-16, miR-101, miR-21, miR-130b, miR-146a, miR-26-1, miR-125a, miR-150, miR-638, miR-198, miR-29c, miR-30b, miR-155, miR-371a, miR-422a, miR-423, miR-410, miR-663a,

miR-127, miR-221, miR-222, miR-380, miR-132, miR-141, miR-17, miR-200a, miR-30a, miR-572, miR-181c, miR-196a-2, miR-22, miR-27a, miR-499a, miR-1915, miR-106a, miR-126, miR-214, miR-320a, miR-328, miR-381, miR-422a, miR-633, miR-922, miR-let-7e, miR-let-7g, miR-106b, miR-197, miR-199a-2, miR-19a, miR-19b-1, miR-210, miR-215, 5 miR-219a-1, miR-23b, miR-29-1, miR-9-2, miR-93, miR-338, miR-372, miR-375, miR-491, miR-146b, miR-614, miR-645, miR-648, miR-99a, miR-375, miR-424, miR-20b, miR-411, miR-629, miR-146a, miR-31, miR-29b-1, miR-122, miR-155, miR-19b-1, miR-106b, miR-203a, miR-223, miR-1246, miR-106a, miR-14, miR-200b, miR-21, miR-215, miR-29a, miR-320a, miR-595, miR-1286, miR-let-7d, miR-107, miR-124-1, miR-125a, miR-125b-1, miR-10 126, miR-130a, miR-148a, miR-17, miR-18a, miR-196a-2, miR-19a, miR-20a, miR-200c, miR-206, miR-23a, miR-26b, miR-30c-1, miR-9-3, miR-98, miR-155, miR-586, miR-146a, miR-214, miR-29a, miR-326, miR-199a-2, miR-26b-9, miR-3, miR-374a, miR-377, miR-423, miR-100, miR-200b, miR-30a, miR-489, miR-146b, and miR-411.

[0043] In some example embodiments, the population of target nucleic acids 15 comprise cardiovascular disease-associated miRNAs. In some example embodiments, the cardiovascular disease-associated miRNAs can be selected from the group consisting of miR-126, miR-210, miR-21, miR-214, miR-30d, miR-150, miR-221, miR-208a, miR-423, miR-499a, miR-208b, miR-145, miR-155, miR-22, miR-25, miR-29a, miR-29b-1, miR-340, miR-378a, miR-146a, miR-181c, miR-19b-1, miR-30a, miR-320a, miR-34a, miR-650, miR-665, 20 miR-134, miR-137, miR-182, miR-192, miR-195, miR-199a-2, miR-199b, miR-19a, miR-223, miR-328, miR-377, miR-92b, miR-744, miR-940, miR-1292, miR-1296, miR-1825, miR-1228, miR-1293, miR-663b, miR-3148, miR-3155a, miR-3175, miR-3713, miR-4491, miR-100, miR-107, miR-10b, miR-130b, miR-142, miR-185, miR-206, miR-216a, miR-23a, miR-27b, miR-30b, miR-34b, miR-34c, miR-302c, miR-425, miR-451a, miR-146b, miR-25 494, miR-518e, miR-568, miR-583, miR-595 and miR-652.

[0044] In some example embodiments, a single nucleic acid is the target of a TRC in a single reaction. In some example embodiments, multiple nucleic acids are targeted in a single reaction with multiple, unique, target-specific TRCs in which each target nucleic acid is the target of a target-specific TRC. In some example embodiments, the TRCs target 30 between 1 and 2500 target nucleic acids. In some example embodiments, the TRCs target between 1 and 100 target nucleic acids. In some example embodiments, the TRCs target between 10 and 50 target nucleic acids.

[0045] In some example embodiments, the TRCs are at concentrations ranging from 1 zeptomolar to 1 molar in step (b). In some example embodiments, the TRCs are at individual concentrations that are dependent on the target nucleic acid.

[0046] In some example embodiments, the TRCs comprise TRCs with identical target sequences. In some example embodiments, the TRCs comprise subsets of TRCs with different target sequences. In some example embodiments, the TRCs comprise TRCs with multiple target sequences for a viral pathogen. In some example embodiments, the TRCs comprise TRCs with target sequences for more than one viral pathogen. In some example embodiments, the TRCs comprise TRCs with target sequences for HIV, HSV-1, and/or EBV. In some example embodiments, the TRCs comprise TRCs with target sequence for more than one miRNA (*e.g.*, multiple miRNAs that form a miRNA signature).

[0047] In some example embodiments, the method further comprises applying the replicated TRCs of step (c) to an array prior to step (d). In some example embodiments, the array is a grid-patterned array. In some example embodiments, the grid-patterned array is functionalized with chemical or biological moieties to direct replicated TRC positioning. In some example embodiments, the chemical moieties rely on hydrophobic, hydrophilic, and/or electrostatic forces to position replicated TRCs on the grid patterned array. In some example embodiments, the grid-patterned array is silanized by an aminosilane. In some example embodiments, the grid patterned array is functionalized with epoxy silane. In some example embodiments, the grid patterned array is functionalized with isothiocyanate. In some example embodiments, the grid patterned array is functionalized with aminopropyl-derivatized or aminophenyl. In some example embodiments, the grid patterned array is functionalized with mercaptosilane. In some example embodiments, the grid patterned array is functionalized with aldehyde or epoxide.

[0048] In some example embodiments, the detectable moiety is a fluorophore, chromophore, or radioisotope. In some example embodiments, the detectable moiety is soluble or insoluble. In some example embodiments, the detectable moiety is a fluorophore. In some example embodiments, detecting the at least one detectable moiety comprises normalizing to a control replicated TRC. In some example embodiments, step (d) comprises introducing 2, 3, or 4 distinct detectable moieties. In some example embodiments, step (e) comprises detecting the 2, 3, or 4 distinct detectable moieties. In some example embodiments, detecting the 2, 3, or 4 distinct detectable moieties is further defined as detecting the unique multiplex probe signature of a replicated TRC. In some example embodiments, each unique multiplex probe signature comprises a plurality of fluorophores (*e.g.*, 2, 3, 4, 5,

or more). In some example embodiments, detecting a multiplex probe signature comprises hybridizing a first detectable moiety, detecting the first detectable moiety, and washing of the array followed by hybridizing a second detectable moiety, and detecting the second detectable moiety, such that the combination of the first and second detectable moieties identifies a unique multiplex probe signature. In some example embodiments, detecting is performed using an epifluorescence microscope.

[0049] In some example embodiments, detecting is performed using fluorometric or colorimetric analysis of the RCR or RCA reaction, such as but not limited to pH sensitive dyes.

[0050] In some example embodiments, detecting is performed by detecting the presence of modified nucleotides which have been incorporated into the replicated TRC. In some example embodiments, these modified nucleotides include fluorescently labeled nucleotides, such as but not limited to Cy3, Cy5, fluorescein, or other fluorescent dyes described herein.

[0051] In some example embodiments, replicated TRCs are applied to arrays and detected by modified nucleotides or by dye staining (*e.g.*, Sybr).

[0052] In some example embodiments, replicated TRCs are applied to arrays and bind to specific locations based on the accessory sequence which can then be used to detect and quantify the target nucleic acid(s).

[0053] In some example embodiments, replicated TRCs are detected using the methods described herein using a plate reader, RT-PCR instrument, or flow cytometer.

[0054] In some example embodiments, TRCs are bound to solid supports like slides or beads, and RCR and/or RCA produces replicated TRCs that can be detected using the methods described herein.

[0055] Other objects, features and advantages of the present disclosure will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

WHAT IS CLAIMED IS:

1. A target reporter construct (TRC) for detecting a target nucleic acid, the TRC being a closed, partially single-stranded nucleic acid comprising:

(a) a target sequence complementary to the target nucleic acid;

5 (b) a bridge sequence forming a double-stranded portion of the TRC; and

(c) an accessory sequence comprising a multifunctional probe sequence,

wherein the bridge sequence comprises a palindromic sequence for the formation of nanospheres during replication, and

10 wherein the target sequence is further defined as a toehold switch, wherein the toehold switch is a sequence capable of uniquely hybridizing to the target nucleic acid.

2. The TRC of claim 1, wherein: (a) the TRC comprises DNA, RNA, or a combination thereof; (b) the TRC is a TRC according to part (a) of this claim which comprises standard

canonical bases, modified bases, non-natural bases, or any combination of the bases thereof;

15 (c) the TRC is a TRC according to part (b) of this claim, wherein the target nucleic acid is single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), or single-stranded RNA

(ssRNA); (d) the TRC is a TRC according to part (c) of this claim, wherein the ssRNA is

microRNA (miRNA); (e) the TRC is a TRC according to part (c) of this claim, wherein the

ssRNA is a non-coding RNA (ncRNA); or (f) the TRC is a TRC according to part (e) of this

20 claim, wherein the ncRNA is small interfering RNA (siRNA), piwi-interacting RNA (piRNA), miRNA, long non-coding RNA (lncRNA), or small nucleolar RNA (snoRNA).

~~3. The TRC of claim 2, wherein the TRC comprises standard canonical bases, modified bases, non-natural bases, or any combination of the bases thereof.~~

~~4. The TRC of claim 3, wherein the target nucleic acid is single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), or single-stranded RNA (ssRNA).~~

25 ~~5. The TRC of claim 4, wherein the ssRNA is microRNA (miRNA).~~

6. — The TRC of claim 4, wherein the ssRNA is a non-coding RNA (ncRNA).

7. — The TRC of claim 6, wherein the ncRNA is small interfering RNA (siRNA), piwi-interacting RNA (piRNA), miRNA, long non-coding RNA (lncRNA), or small nucleolar RNA (snoRNA).

8. — The TRC of claim 1, wherein the target sequence is further defined as a toehold switch, wherein the toehold switch is a sequence capable of uniquely hybridizing to the target nucleic acid.

9.3. — The TRC of claim 18, wherein: (a) the toehold switch comprises a double-stranded switch stem and a single-stranded toehold; (b) the TRC is a TRC according to part (a) of this claim, wherein the toehold is between 5 and 10 nucleotides in length, or is 7, 8, or 9 nucleotides in length; or (c) the toehold switch and the bridge sequence overlap.

10. — The TRC of claim 9, wherein the toehold is between 5 and 10 nucleotides in length.

11. — The TRC of claim 10, wherein the toehold is 7, 8, or 9 nucleotides in length.

12. — The TRC of claim 8, wherein the toehold switch and the bridge sequence overlap.

13. — The TRC of claim 8, wherein the target sequence or toehold switch is structurally independent from the bridge sequence.

14. — The TRC of claim 13, wherein the toehold switch is within 5–10 nucleotides of the bridge sequence.

15.4. — The TRC of claim 1; (a) further comprising a second target sequence complementary to a second target nucleic acid; (b) wherein the bridge sequence is between the target sequence and accessory sequence; (c) wherein the bridge sequence comprises a low complexity, repetitive sequence; (d) wherein the TRC comprises two or more bridge sequences; (e) wherein the TRC comprises two or more bridge sequence that are identical in sequence; or (f) wherein the TRC comprises two or more bridge sequences that comprise different sequences.

16. — ~~The TRC of claim 1, wherein the bridge sequence comprises a palindromic sequence.~~

17. — ~~The TRC of claim 1, wherein the bridge sequence is between the target sequence and accessory sequence.~~

18. — ~~The TRC of claim 1, wherein the bridge sequence comprises a low complexity, repetitive sequence.~~

19. — ~~The TRC of claim 1, wherein the TRC comprises two or more bridge sequences.~~

20. — ~~The TRC of claim 19, wherein the two or more bridge sequence are identical in sequence.~~

21. — ~~The TRC of claim 19, wherein the two or more bridge sequences comprise different sequences.~~

22. 5. The TRC of claim 1; (a) wherein the accessory sequence further comprises at least a second probe sequence; (b) wherein the accessory sequence further comprises at least a second probe sequence, and the second probe sequence is distinct from the first probe sequence; (c) wherein the TRC is a TRC according to part (b) of this claim, and wherein the first probe sequence and distinct second probe sequence form a unique multiplex probe signature; (d) wherein the TRC is a TRC according to part (c) of this claim, and wherein the unique multiplex probe signature further comprises a third, fourth, or fifth probe sequence; (e) wherein the TRC forms a loop structure under hybridization conditions; (f) wherein the TRC forms a loop structure under hybridization conditions and the loop structure is a hairpin structure, a dumbbell structure, or a multi-loop structure; (g) wherein the multifunctional probe sequence comprises a binding sequence; (h) wherein the TRC is a TRC according to part (g) of this claim, and wherein the binding sequence facilitates localization to an oligonucleotide array; or (i) wherein the TRC is between 50 and 100 nucleotides in length, or between 100 and 500 nucleotides in length.

23.——The TRC of claim 22, wherein the second probe sequence is distinct from the first probe sequence.

24.——The TRC of claim 23, wherein the first probe sequence and distinct second probe sequence form a unique multiplex probe signature.

5 25.——The TRC of claim 24, wherein the unique multiplex probe signature further comprises a third, fourth, or fifth probe sequence.

26.——The TRC of claim 1, wherein the TRC forms a loop structure under hybridization conditions.

10 27.——The TRC of claim 26, wherein the loop structure is a hairpin structure, a dumbbell structure, or a multi-loop structure.

28.——The TRC of claim 1, wherein the multifunctional probe sequence comprises a binding sequence.

29.——The TRC of claim 28, wherein the binding sequence facilitates localization to an oligonucleotide array.

15 30.——The TRC of claim 1, wherein the TRC is between 50 and 100 nucleotides in length.

31.——The TRC of claim 1, wherein the TRC is between 100 and 500 nucleotides in length.

32.6. A replicated TRC, wherein the replicated TRC comprises comprising concatenated monomer repeats of a TRC according to any one of claims 1-305.

20 33.——The replicated TRC of claim 32, wherein the replicated TRC is produced from rolling circle replication (RCR) and/or rolling circle amplification (RCA).

34.7. A replicated nanosphere comprising a replicated TRC of claim 326, wherein the replicated TRC has undergone intramolecular hybridization between the bridge sequences within the replicated TRC.

35.8. A method of detecting a target nucleic acid comprising:

25 (a) obtaining target reporter constructs (TRCs) according to any one of claims 1-305;

(b) contacting the TRCs with a population of target nucleic acids, wherein the target nucleic acids within the population hybridize to the complementary target sequence of a target-specific TRC;

(c) performing rolling circle replication (RCR), thereby obtaining replicated TRCs;

5 (d) introducing at least one detectable moiety, wherein the at least one detectable moiety binds the probe sequence of the replicated TRC; and

(e) detecting the at least one detectable moiety, thereby detecting the target nucleic acid.

36-9. The method of claim 358, wherein: (a) performing RCR comprises introducing a
10 strand-displacing polymerase; (b) the method is a method according to part (a) of this claim, and wherein the strand-displacing polymerase is a DNA polymerase or RNA polymerase; (c) the method is a method according to part (b) of this claim, wherein the DNA polymerase is ϕ 29, *Bst*, or *Bsu*; (d) step (b) and step (c) are performed in a single tube; (e) RCR is
15 performed for 45 minutes to 90 minutes; (f) the target nucleic acids have a length greater than the length of the TRC, thereby producing a 3' overhang or a 5' overhang; (g) the method is a method according to part (f) of this claim, and the method further comprises introducing an
20 exonuclease; (h) the method is a method according to part (g) of this claim, and wherein the exonuclease is exonuclease T; (i) the method is a method according to part (g) of this claim, and wherein the exonuclease removes a 3' overhang of the target nucleic acid hybridized to
the TRC, thereby presenting a 3'-OH group; (j) obtaining replicated TRCs comprises
hybridizing the bridge of at least one of the replicated TRCs within the population to the
bridge of at least a second replicated TRC within the population; (k) the replicated TRCs
comprise at least two replicated TRCs interconnected at the bridge; or (l) the replicated TRCs
comprises at least 100, 1,000, 5,000, or 50,000 replicated TRCs interconnected at the bridge.

37. — ~~The method of claim 36, wherein the strand-displacing polymerase is a DNA polymerase or RNA polymerase~~

38. — ~~The method of claim 36, wherein the DNA polymerase is ϕ 29, *Bst*, or *Bsu*.~~

39. — ~~The method of claim 35, wherein step (b) and step (c) are performed in a single tube.~~

5 40. — ~~The method of claim 35, wherein RCR is performed for 45 minutes to 90 minutes.~~

41. — ~~The method of claim 35, wherein the target nucleic acids have a length greater than the length of the TRC, thereby producing a 3' overhang or a 5' overhang.~~

42. — ~~The method of claim 41, further comprising introducing an exonuclease.~~

43. — ~~The method of claim 42, wherein the exonuclease is exonuclease T.~~

10 44. — ~~The method of claim 42, wherein the exonuclease removes a 3' overhang of the target nucleic acid hybridized to the TRC, thereby presenting a 3' OH group.~~

45. — ~~The method of claim 35, wherein obtaining replicated TRCs comprises hybridizing the bridge of at least one of the replicated TRCs within the population to the bridge of at least a second replicated TRC within the population.~~

15 46. — ~~The method of claim 35, wherein the replicated TRCs comprise at least two replicated TRCs interconnected at the bridge.~~

47. — ~~The method of claim 35, wherein the replicated TRCs comprises at least 100, 1,000, 5,000, or 50,000 replicated TRCs interconnected at the bridge.~~

48. 10. The method of claim ~~35~~8, wherein (a) the population of target nucleic acids comprises
20 pathogenic nucleic acids; (b) the method is a method according to part (a) of this claim, and
wherein the pathogenic nucleic acids are human immunodeficiency virus (HIV), herpes
simplex virus (HSV-1), human papillomavirus (HPV), and/or Epstein-Barr virus (EBV)
pathogenic nucleic acids; or (c) the method is a method according to part (a) of this claim,
and wherein the target sequence for the pathogenic nucleic acids comprises a length of 50-
25 100 nucleotides.

49. — ~~The method of claim 48, wherein the pathogenic nucleic acids are human immunodeficiency virus (HIV), herpes simplex virus (HSV-1), human papillomavirus (HPV), and/or Epstein Barr virus (EBV) pathogenic nucleic acids.~~

50. — ~~The method of claim 48, wherein the target sequence for the pathogenic nucleic acids comprises a length of 50-100 nucleotides.~~

51.11. ~~The method of claim 358, wherein: (a) the population of target nucleic acids comprise one or more miRNAs; (b) the method is a method according to part (a) of this, and wherein the one or more miRNAs are tumor-associated miRNAs; or (c) the method is a method according to part (b) of this claim, and wherein the tumor-associated miRNAs are selected from the group consisting of miR-let7, miR-10b, miR-21, miR-25, miR-106b, miR-11, miR-196a, miR-210, miR-212, and miR-221.~~

52. — ~~The method of claim 51, wherein the one or more miRNAs are tumor-associated miRNAs.~~

53. — ~~The method of claim 52, wherein the tumor-associated miRNAs are selected from the group consisting of miR-let7, miR-10b, miR-21, miR-25, miR-106b, miR-11, miR-196a, miR-210, miR-212, and miR-221.~~

54.12. ~~The method of claim 358, wherein: (a) the population of target nucleic acids comprise diabetes-associated miRNAs; (b) the method is a method according to part (a) of this claim, and wherein the diabetes-associated miRNAs are selected from the group consisting of miR146a, miR-195, miR-320, miR29, miR192, miR-377, miR-126, miR-203, and miR-503.~~

55. — ~~The method of claim 54, wherein the diabetes-associated miRNAs are selected from the group consisting of miR146a, miR-195, miR-320, miR29, miR192, miR-377, miR-126, miR-203, and miR-503.~~

56.13. ~~The method of claim 358, wherein: (a) TRCs comprise TRCs with identical target sequences; (b) TRCs comprise subsets of TRCs with different target sequences; (c) TRCs~~

comprise TRCs with multiple target sequences for a viral pathogen; (d) TRCs comprise TRCs with target sequences for more than one viral pathogen; (e) the population of TRCs comprises TRCs with target sequences for HIV, HSV-1, and/or EBV; (f) the method further comprises applying the replicated TRCs of step (c) of claim 8 to an array prior to step (d) of claim 8; (g) the method is a method according to part (f) of this claim, wherein the array is a grid-patterned array; (h) the method is a method according to part (g) of this claim, wherein the grid-patterned array is functionalized with chemical or biological moieties to direct replicated TRC positioning; (i) the method is a method according to part (g) of this claim, wherein the grid-patterned array is silanized by an aminosilane, epoxy silane, isothiocyanate, aminophenyl, mercaptosilane, aldehyde or epoxy; or (j) the method is a method according to part (g) of this claim, wherein the grid-patterned array is silanized by an aminosilane.

57. — ~~The method of claim 35, wherein TRCs comprise subsets of TRCs with different target sequences.~~

58. — ~~The method of claim 35, wherein TRCs comprise TRCs with multiple target sequences for a viral pathogen.~~

59. — ~~The method of claim 35, wherein TRCs comprise TRCs with target sequences for more than one viral pathogen.~~

60. — ~~The method of claim 35, wherein the population of TRCs comprises TRCs with target sequences for HIV, HSV-1, and/or EBV.~~

61. — ~~The method of claim 35, further comprising applying the replicated TRCs of step (c) to an array prior to step (d).~~

62. — ~~The method of claim 61, wherein the array is a grid-patterned array.~~

63. — ~~The method of claim 62, wherein the grid-patterned array is functionalized with chemical or biological moieties to direct replicated TRC positioning.~~

64. — ~~The method of claim 62, wherein the grid-patterned array is silanized by an aminosilane, epoxy silane, isothiocyanate, aminophenyl, mercaptosilane, aldehyde or epoxy.~~

65. — ~~The method of claim 62, wherein the grid-patterned array is silanized by an aminosilane.~~

5 ~~66.14.~~ The method of claim ~~35~~8, wherein: (a) the detectable moiety is a fluorophore, chromophore, or radioisotope; (b) the detectable moiety is a fluorophore; (c) the detectable moiety is soluble or insoluble; (d) the detectable moiety is an intercalating dye or pH sensitive dye; (e) the detectable moiety comprises modified nucleotides; (f) the detectable moiety comprises modified nucleotides and the modified nucleotides are fluorescently
10 labeled nucleotides; or (g) the detectable moiety comprises modified nucleotides and the detecting comprises detecting the presence of modified nucleotides that have been incorporated into the replicated TRC.

67. — ~~The method of claim 35, wherein the detectable moiety is a fluorophore.~~

68. — ~~The method of claim 35, wherein the detectable moiety is soluble or insoluble.~~

15 ~~69. — The method of claim 35, wherein the detectable moiety is an intercalating dye or pH sensitive dye.~~

70. — ~~The method of claim 35, wherein the detectable moiety comprises modified nucleotides.~~

20 ~~71. — The method of claim 70, wherein the modified nucleotides are fluorescently labeled nucleotides.~~

72. — ~~The method of claim 70, wherein detecting comprises detecting the presence of modified nucleotides that have been incorporated into the replicated TRC.~~

25 ~~73.15.~~ The method of claim ~~35~~8, wherein: (a) detecting the at least one detectable moiety comprises normalizing to a control replicated TRC; (b) step (d) of Claim 8 comprises introducing 2, 3, or 4 distinct detectable moieties; (c) step (e) of Claim 8 comprises detecting

the 2, 3, or 4 distinct detectable moieties; (d) the method is a method according to part (c) of this claim, wherein detecting the 2, 3, or 4 distinct detectable moieties is further defined as detecting the unique multiplex probe signature of a replicated TRC; (e) the method is a method according to part (c) of this claim, wherein detecting is performed using an epifluorescence microscope; or (f) the method is a method according to part (c) of this claim, wherein detecting is performed using colorimetric analysis.

74. — ~~The method of claim 35, wherein step (d) comprises introducing 2, 3, or 4 distinct detectable moieties.~~

75. — ~~The method of claim 74, wherein step (e) comprises detecting the 2, 3, or 4 distinct detectable moieties.~~

76. — ~~The method of claim 75, wherein detecting the 2, 3, or 4 distinct detectable moieties is further defined as detecting the unique multiplex probe signature of a replicated TRC.~~

77. — ~~The method of claim 75, wherein detecting is performed using an epifluorescence microscope.~~

78. — ~~The method of claim 75, wherein detecting is performed using colorimetric analysis.~~