# Supplementary material Figure and Table legends

## Sup. Table 1

Time to positive (TTP) signal in the detection of low copy number of ZIKV synthetic RNA template 5 (from $5x10^{1}$ to $5x10^{-1}$ copies per reaction) and compared to the non-template control (NTC) using the ZIKV RT-RPA assay. For comparison, the time necessary to detect the target using the published Zika RT-PCR is also shown.

## Sup. Table 2

Confirmation of the specificity of the ZIKV RT-RPA assay using a selection Dengue-positive and 4 Chikungunya-positive clinical samples. The Dengue-positive and Chikungunya-positive status of the samples was confirmed by Dengue-specific and Chikungunya-specific RT-PCR, respectively, with outcomes and Ct values indicated.

## Sup. Fig. 1

Sequences of the synthetic fragments used in the ZIKV RPA assay development and ZIKV RPA amplified region within utilised extracted ZIKV genomes.

a. Synthetic fragments 1 (Template 1 – EU545988), 2 (Template 2 – KJ776791), 3 (Template 3 – KU321639), 4 (Template 4 – KU365778) and 5 (Template 5 – KU365780) sequences shown 5'→3' direction. Underlined within each sequence are the T7 promoter at the 5'-end and the SP6 promoter at the 3'-end, used to amplify and synthesize the synthetic RNA fragments.

b. Sequences of the ZIKV RPA region of the African ZIKV (Strain MP1751, KY288905, from Uganda, 1962) and the South American ZIKV (Strain PRVABC59, KU501215, from Puerto Rico, 2015)

**Sup. Fig. 2**

Agarose gel analysis of the sizes of fragments generated in the ZIKV RT-RPA assay using synthetic fragments (Templates 1-5) and extracted ZIKV nucleic acid (African and South American Zika virus strains). Nuclease-free water instead of nucleic acid was used as a non-template control (NTC). Relative fragment sizes were confirmed using the E-GelÆ Low Range Quantitative DNA Ladder and marker sizes are indicated on the gel image.

**Sup. Fig. 3**

ZIKV RT-RPA assay sensitivity and performance using low copy number of template in comparison to ZIKV RT-PCR. Fluorescent signal generated from the amplification of 50, 5 and 0.5 copies per reaction of Zika synthetic RNA template 5 (KU365780) and compared to the non-template control (NTC) using the ZIKV RT-RPA assay. Amplification curves show the average total fluorescence values from five independent ZIKV RT-RPA assays; standard deviations are represented as error bars.

**Sup. Fig. 4**

Detection of cultured extracted Spondweni virus nucleic acid using the ZIKV RT-RPA assay. Signal from dilutions of $1x10^{-2}$ and $1x10^{-4}$ of the neat extracted Spondweni viral RNA are compared to signal generated using $1x10^{-4}$ dilution of neat extracted ZIKV RNA. Nuclease-

45 free water used in place of viral nucleic acid represents the non-template control (NTC).
46 Amplification curves show the average total fluorescence values from three independent
47 ZIKV RT-RPA assays, standard deviations are represented as error bars.

48

# Sup. Fig. 1

## Template 1 (EU545988)

**GCGC**<u>**TAATACGACTCACTATAGGG**</u>TGCTATGAGGCATCAATATCGGACATGGCTTCGGACAGCCGCTGCCCAACACAAGGTGAAGCCTACCTTGACAAGCAGTCAGA
CACTCAATATGTCTGCAAAAGAACGTTAGTGGACAGAGGCTGGGGAAATGGATGTGGACTTTTTGGCAAAGGGAGCCTGGTGACATGCGCTAAGTTTGCATGCTCCAA
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CCGGAACTCCACATTGGAACAACAAAGAAGCATTGGTAGAGTTCAAGGACGCACATGCCAAAAGGCAAACTGTCGTGGTTCTAGGGAGTCAAGAAGGAGCAGTTCACA
CGGCCCTTGCTGGAGCTCTGGAGGCTGAGATGGATGGTGCAAAGGGAAGGCTGTCCTCTGGCCACTTGAAATGTCGCCTGAAAATGGATAAACTTAGATTGAAGGGC
GTGTCATACTCCTTGTGTACCGCAGCGTTCACATTCACCAAGATCCCGGCTGAAACACTGCACGGGACAGTCACAGTGGAGGTACAGTACGCAGGGACAGATGGACC
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TGGGTTCGGGGTATTTCA<u>**TTCTATAGTGTCACCTAAAT**</u>**GCGC**

## Template 2 (KJ776791)

**GCGC**<u>**TAATACGACTCACTATAGGG**</u>TGCTATGAGGCATCAATATCGGACATGGCTTCGGACAGCCGCTGCCCAACACAAGGTGAAGCCTACCTTGACAAGCAATCAGA
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CATGGGTTCGGGGTATTTCA<u>**TTCTATAGTGTCACCTAAAT**</u>**GCGC**

## Template 3 (KU321639)

**GCGC**<u>**TAATACGACTCACTATAGGG**</u>TGCTATGAGGCATCAATATCAGACATGGCTTCGGACAGCCGCTGCCCAACACAAGGTGAAGCCTACCTTGACAAGCAATCAGA
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GCACTTCGTCAGAGCAGCAAAGACAAATAACAGCTTTGTCGTGGATGGTGACACACTGAAGGAATGCCCACTCAAACATAGAGCATGGAACAGCTTTCTTGTGGAGGA
TCATGGGTTCGGGGTATTTCA**TTCTATAGTGTCACCTAAAT**<u>**GCGC**</u>

## Template 4 (KU365778)

**GCGC**<u>**TAATACGACTCACTATAGGG**</u>TGCTATGAGGCATCAATATCAGACATGGCTTCGGACAGCCGCTGCCCAACACAAGGTGAAGCCTACCTTGACAAGCAATCAGA
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TCATGGGTTCGGGGTATTTCA**TTCTATAGTGTCACCTAAAT**<u>**GCGC**</u>

## Template 5 (KU365780)

**GCGC**<u>**TAATACGACTCACTATAGGG**</u>TGCTATGAGGCATCAATATCAGACATGGCTTCGGACAGCCGCTGCCCAACACAAGGTGAAGCCTACCTTGACAAGCAATCAGA
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TCATGGGTTCGGGGTATTTCA<u>**TTCTATAGTGTCACCTAAAT**</u>**GCGC**

## African Zika virus (Strain MP1751, KY288905, from Uganda, 1962) RPA target

CCAACACAAGGTGAAGCCTACCTTGACAAGCAATCAGACACTCAATATGTTTGCAAAAGAACATTGGTGGACAGAGGTTGGGGAAATGGGTGTGGACTCTTTGGCAAA
GGGAGTTTGGTGACATGTGCTAAGTTCACGTGCTCCAAGAAGATGACTGGGAAGAGCATTCAGCCGGAGAA

## South American Zika virus (Strain PRVABC59, KU501215, from Puerto Rico, 2015 ) RPA target

CCAACACAAGGTGAAGCCTACCTTGACAAGCAATCAGACACTCAATATGTCTGCAAAAGAACGTTAGTGGACAGAGGCTGGGGAAATGGATGTGGACTTTTTGGCAAA
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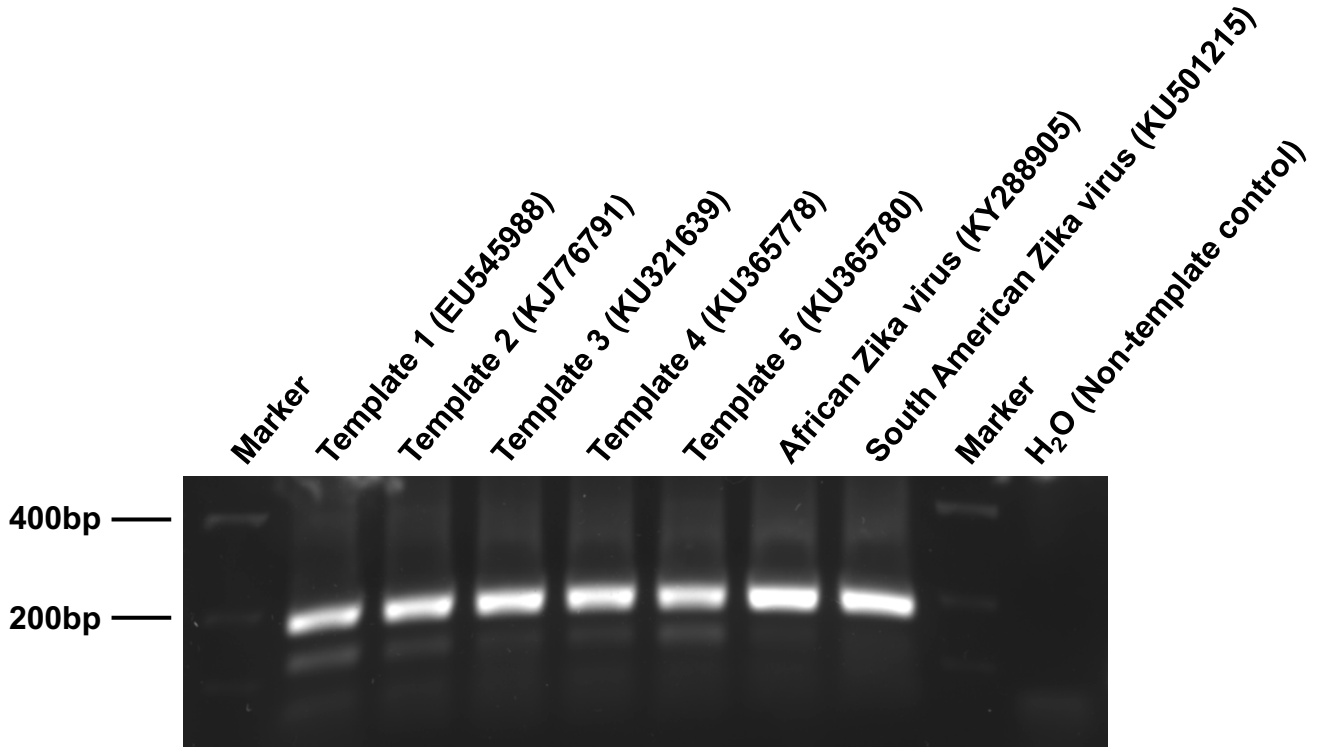
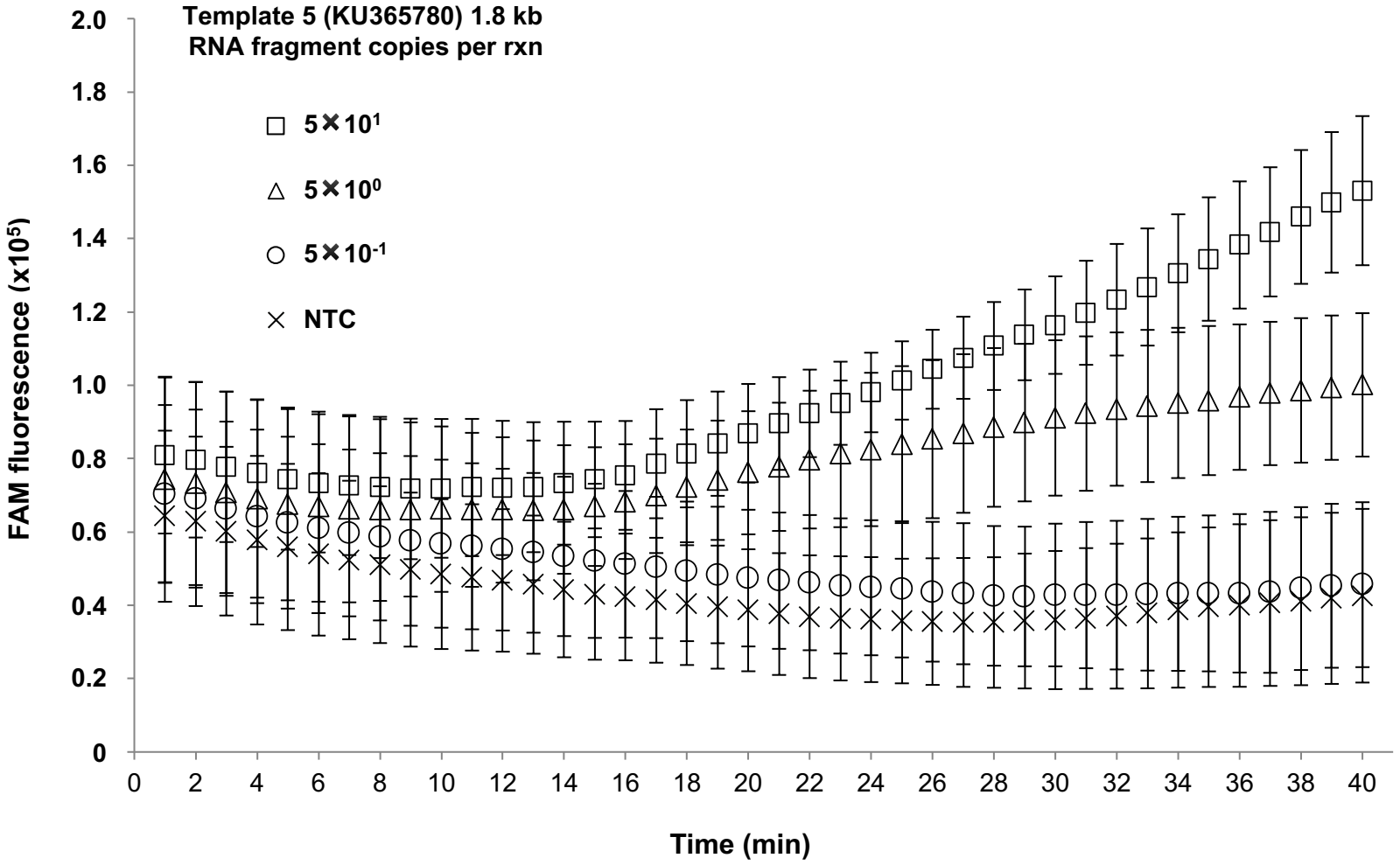
Sup. Fig. 2


Marker
Template 1 (EU545988)
Template 2 (KJ776791)
Template 3 (KU321639)
Template 4 (KU365778)
Template 5 (KU365780)
African Zika virus (KY288905)
South American Zika virus (KU501215)
Marker
H2O (Non-template control)
400bp
200bp

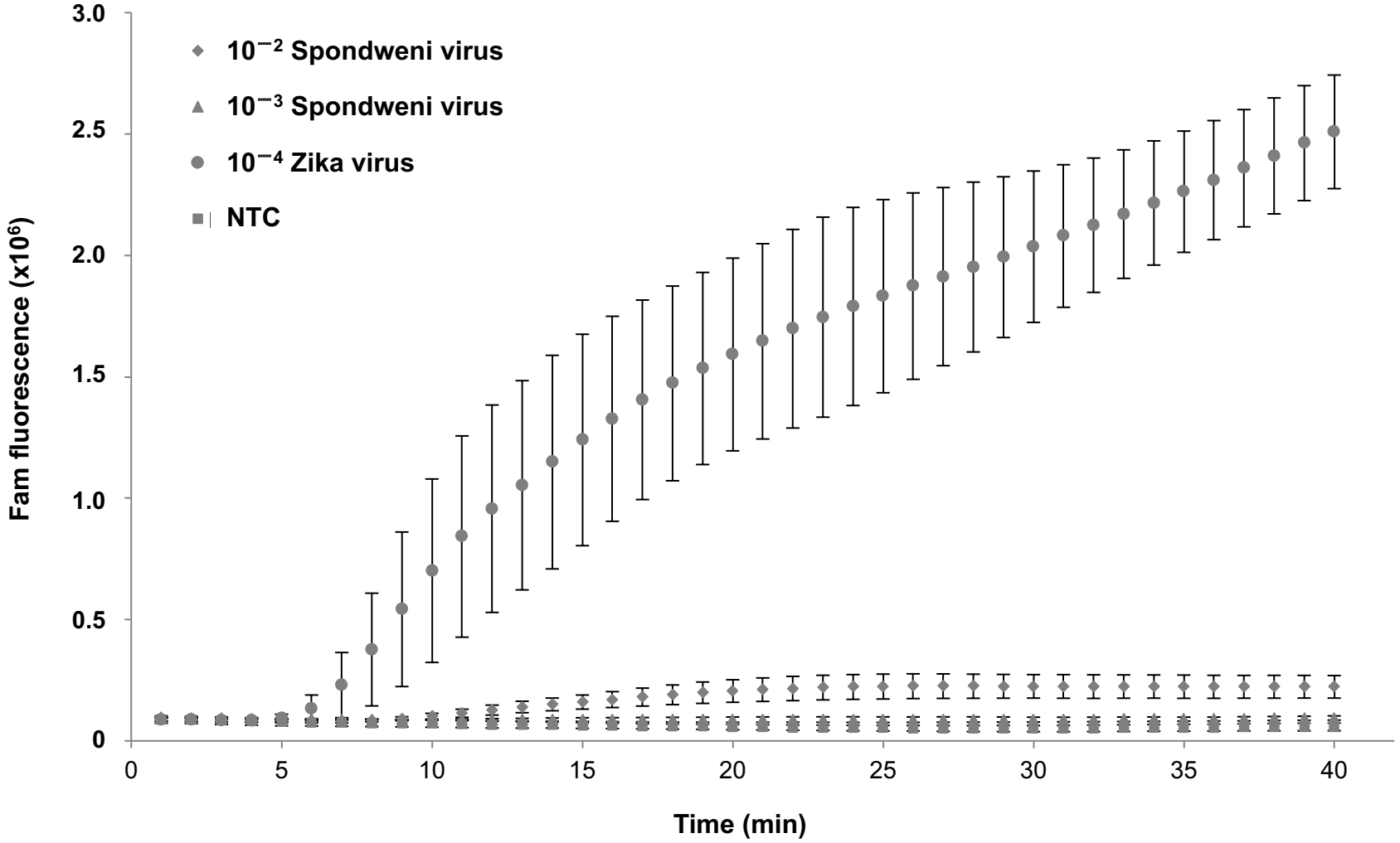
Sup. Fig. 3


Template 5 (KU365780) 1.8 kb
RNA fragment copies per rxn
5×10¹
5×10⁰
5×10⁻¹
NTC
FAM fluorescence (x10⁵)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Time (min)

# Sup. Table 1

| Template 5 (KU365780) copies/rxn | RT-RPA TTP (mins) | RT-RPA result | RT-PCR TTP (mins) | RT-PCR result |
|---|---|---|---|---|
| $5x10^{1}$ | 21.73 | + (3/5) | 60.33 | + (3/3) |
| $5x10^{0}$ | 22.20 | + (3/5) | Not detected | - (3/3) |
| $5x10^{-1}$ | Not detected | - (5/5) | Not detected | - (3/3) |
| NTC | Not detected | - (5/5) | Not detected | - (3/3) |

Sup. Fig. 4


3.0
2.5
2.0
1.5
1.0
0.5
0
Fam fluorescence (x10⁶)
10⁻² Spondweni virus
10⁻³ Spondweni virus
10⁻⁴ Zika virus
NTC
0
5
10
15
20
25
30
35
40
Time (min)

# Sup. Table 2

| Sample No | Sample description | RT-RPA result | RT-PCR Ct | RT-PCR result |
|---|---|---|---|---|
| 1 | Dengue clinical | - | 27.069 | + |
| 2 | Dengue clinical | - | 32.610 | + |
| 3 | Dengue clinical | - | 35.749 | + |
| 4 | Dengue clinical | - | 24.517 | + |
| 5 | Dengue clinical | - | 36.176 | + |
| 6 | Dengue clinical | - | 24.403 | + |
| 7 | Dengue clinical | - | 22.330 | + |
| 8 | Dengue clinical | - | 23.800 | + |
| 9 | Dengue clinical | - | 26.660 | + |
| 10 | Dengue clinical | - | 33.898 | + |
| 11 | Dengue clinical | - | 34.870 | + |
| 12 | Chikungunya clinical | - | 30.412 | + |
| 13 | Chikungunya clinical | - | 30.264 | + |
| 14 | Chikungunya clinical | - | 39.618 | + |
| 15 | Chikungunya clinical | - | 37.512 | + |