# A Novel Conversion Strategy using an Expanded Genetic Alphabet to Assay Nucleic Acids

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## SUPPLEMENTARY DATA

**Table S1, S2, S3, S4, and S5**

**Enzymatic incorporation of dZTP opposite template dG in the absence of dCTP.**

**Figure S1**. Polymerase screening for incorporation of dZTP opposite template dG in the absence of dCTP.

**Microsphere Bead Coupling according to Luminex's carbodiimide coupling procedure.**

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**Figure S2.** Detection of biotinylated analyte using Luminex® microspheres.

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**Figure S3.** Biotinylated primer extension architectures without (top) and with conversion (bottom).

**Figure S4.** Agarose gel (3%) resolving monoplex PCR (lane 1-5) and five-fold multiplex PCR (lane 6) amplification of five target genes from human genomic DNA (Promega, Male).

**Figure S5.** The dose-response behavior of Luminex® xTAG assays.

**Figure S6**. Non-linear dose response for a PCR amplicon obtained by reverse transcriptase-PCR of West Nile RNA using the standard amplicon capture architecture (direct hybridization, Figure 2A).

**Table S1. Oligonucleotides used in hybridization experiments**

| Name of Capture Probes | Amino-modified probes (5` to 3`) (attached to Luminex beads) | Biotinylated Analytes (3` to 5`) (complementary to capture probe) | Name of Analytes |
|---|---|---|---|
| Probe1_Std (Bead21) | $NH_2$-$C_{12}$-GATTGTAAGATTTGATAAAGTGTA-3` | 3`-CTAACATTCTAAACTATTTCACAT-Biotin | Analyte1_Std24 |
| Probe1_5P (Bead21) | $NH_2$-$C_{12}$-GATTPTAAPATTTPATAAAPTPTA-3` | 3`-CTAAZATTZTAAAZTATTTZAZAT-Biotin | Analyte1_5Z |
| Probe2_Std (Bead25) | $NH_2$-$C_{12}$-CTAGGACGACGGACTGC-3` | 3`-GATCCTGCTGCCTGACG-Biotin | Analyte2_Std17 |
| Probe2_4P (Bead25) | $NH_2$-$C_{12}$-CTAPGACPACGPACTPC-3` | 3`-GATZCTGZTGCZTGAZG-Biotin | Analyte2_4Z |

**Table S2. Oligonucleotide sequences used as target-specific primers in PCR**

| Name of targeted gene | | Primer sequences (5′ to 3′) |
|---|---|---|
| GAPDH | Forward | CCTGACCTGCCGTCTAGAAAA |
| | Reverse | CTCCGACGCCTGCTTCAC |
| FLT3 | Forward | CGGGAAAGTGGTGAAGATATGTGAC |
| | Reverse | CCCTGACAACATAGTTGGAATCACT |
| HBEGF | Forward | CGGACATACTCTGTTTGGCACTT |
| | Reverse | CCCCAGTTGCCGTCTAGGA |
| KIT | Forward | CTCCTTACTCATGGTCGGATCACAA |
| | Reverse | TGTCAAGCAGAGAATGGGTACTCAC |
| TSHR | Forward | CCGCAGTACAACCCAGGGGACAAAG |
| | Reverse | ATGAGAGGCTTGTTCAGAATTGCTG |

**Table S3. 5`-biotinylated primer and anti-TAG sequences in the ZiP-TAG architecture with conversion (extension with dA,T,G,Z/TPs)**

| Name of biotin-primer [1] | Biotinylated target-specific primers sequence followed by extension sequence (5`-3`) [2] | Target specific anti-TAG Sequence (3`-5`) [3] | Name of Anti-TAG |
|---|---|---|---|
| Biotin-FLT3-prim-18 | 5`-Biotin-*GACTTTGGATTGGCTCGA*-(GATATZATGAGTGATTZZAAZTATGTTGTZAGGG)-3` | 3`-CTAAPPTTPATACAACAPTCCC-$C_{12}$-$NH_2$ | FLT-22-4P-$NH_2$ |
| Biotin-GAPDH-prim-21 | 5`-Biotin-*CTGCTTCACCACCTTCTTGAT*-(GTZATZATATTTGGZAGGTTTTTZTAG)-3` | 3`-CAPTAPTATAAACCPTCCAAAAAPATC-$C_{12}$-$NH_2$ | GAP-27-4P-$NH_2$ |
| Biotin-HBEGF-prim-21 | 5`-Biotin-*ATTGGGCCTCCCATAATTGCT*-(TTGZZAAAATAZZAGAGZZTTZAA)-3` | 3`-AACPPTTTTATPPTCTCPPAAPTT-$C_{12}$-$NH_2$ | HBE-24-7P-$NH_2$ |
| Biotin-KIT-prim-25 | 5`-Biotin-*ACCACATAATTAGAATCATTCTTGA*-(TGTZTZTGGZTAGAZZAAAAT)-3` | 3`-ACAPAPACCPATCTPPTTTTA-$C_{12}$-$NH_2$ | KIT-21-5P-$NH_2$ |

| Biotin-TSHR-prim-19 | 5`-Biotin-*CATGGCCCCAATCTCATTC*-(TATGZTZTGTZAGZAATTZTGAA)-3` | 3`-ATACPAPACAPTCPTTAAPACTT-$C_{12}$-$NH_2$ | TSHR-23-5P-$NH_2$ |
|---|---|---|---|

1. FLT3, GAPDH, HBEGF, KIT, and TSHR are the names of the targeted genes.

2. The biotinylated target-specific primer (indicated in *Italics*) is extended in primer extension assay with Z replacing C, giving the 3`extension sequence (the sequence in parentheses containing Zs), which hybridizes with microsphere-bound target specific P-containing anti-TAG sequence.

3. Microsphere-bound target specific anti-TAG sequences are designed based on the down-stream sequence of biotinylated primer binding site with P replacing G.

## Table S4. 5`-biotinylated primer and anti-TAG sequences in the "new" architecture without conversion (extension with four standard dNTPs)

| Name of biotin-primer [1] | Biotinylated target-specific primers sequence followed by extension sequence (5`-3`) [2] | Target specific anti-TAG Sequence (3`-5`) [3] | Name of Anti-TAG |
|---|---|---|---|
| Biotin-FLT3-prim-18 | 5`-Biotin-*GACTTTGGATTGGCTCGA*-(GATATCATGAGTGATTCCAACTATGTTGTCAGGG)-3` | 3`-CTAAGGTTGATACAACAGTCCC-$C_{12}$-$NH_2$ | FLT-22-4G-$NH_2$ |
| Biotin-GAPDH-prim-21 | 5`-Biotin-*CTGCTTCACCACCTTCTTGAT*-(GTCATCATATTTGGCAGGTTTTTCTAG)-3` | 3`-CAGTAGTATAAACCGTCCAAAAAGATC-$C_{12}$-$NH_2$ | GAP-27-4G-$NH_2$ |
| Biotin-HBEGF-prim-21 | 5`-Biotin-*ATTGGGCCTCCCATAATTGCT*-(TTGCCAAAATACCAGAGCCTTCAA)-3` | 3`-AACGGTTTTATGGTCTCGGAAGTT-$C_{12}$-$NH_2$ | HBE-24-7G-$NH_2$ |
| Biotin-KIT-prim-25 | 5`-Biotin-*ACCACATAATTAGAATCATTCTTGA*-(TGTCTCTGGCTAGACCAAAAT)-3` | 3`-ACAGAGACCGATCTGGTTTTA-$C_{12}$-$NH_2$ | KIT-21-5G-$NH_2$ |
| Biotin-TSHR-prim-19 | 5`-Biotin-*CATGGCCCCAATCTCATTC*-(TATGCTCTGTCAGCAATTCTGAA)-3` | 3`-ATACGAGACAGTCGTTAAGACTT-$C_{12}$-$NH_2$ | TSHR-23-5G-$NH_2$ |

1. FLT3, GAPDH, HBEGF, KIT, and TSHR are the names of the targeted genes.

2. The biotinylated target-specific primer (indicated in *Italics*) is extended in primer extension assay to give the 3`extension sequence (the sequence in parentheses), which hybridizes with microsphere-bound target specific anti-TAG sequence.

3. Microsphere-bound target specific anti-TAG sequences are designed based on the down-stream sequence of biotinylated primer binding site.

## Table S5. TAG-primer and anti-TAG sequences in target-specific primer extension (TSPE) assays (extension with Biotin-14-dCTP)

| Name of TAG-primer [1] | TAG Sequence-Target Specific Sequence (5`-3`) [2] | Universal anti-TAG Sequence (3`-5`) [3] | Name of Anti-TAG |
|---|---|---|---|
| LUA1-FLT3-prim-18 | 5`-CTTTAATCTCAATCAATACAAATC-*GACTTTGGATTGGCTCGA*-3` | 3`-GAAATTAGAGTTAGTTATGTTTAG-$C_{12}$-$NH_2$ | LUA1-FLT3 |
| LUA3-GAPDH-prim-21 | 5`-TACACTTTATCAAATCTTACAATC-*CTGCTTCACCACCTTCTTGAT*-3` | 3`-ATGTGAAATAGTTTAGAATGTTAG-$C_{12}$-$NH_2$ | LUA3-GAPDH |

| | | | |
|---|---|---|---|
| LUA9-HBEGF-prim-21 | 5`-TAATCTTCTATATCAACATCTTAC-*ATTGGGCCTCCCATAATTGCT*-3` | 3`-ATTAGAAGATATAGTTGTAGAATG-$C_{12}$-$NH_2$ | LUA9-HBEGF |
| LUA11-KIT-prim-25 | 5`-TACAAATCATCAATCACTTTAATC-*ACCACATAATTAGAATCATTCTTGA*-3` | 3`-ATGTTTAGTAGTTAGTGAAATTAG-$C_{12}$-$NH_2$ | LUA11-KIT |
| LUA19-TSHR-prim-19 | 5`-TCAATCAATTACTTACTCAAATAC-*CATGGCCCCAATCTCATTC*-3` | 3`-AGTTAGTTAATGAATGAGTTTATG-$C_{12}$-$NH_2$ | LUA19-TSHR |

1. The number followed by LUA refers to the number of the Luminex microsphere. FLT3, GAPDH, HBEGF, KIT, and TSHR are the names of the targeted genes.
2. The 5`-end of the target specific sequence indicated in *Italics* is appended to an universal TAG sequence (TAG, 24mer oligo in blue color) obtained from Luminex FlexMAP sequence.
3. Microsphere-bound universal anti-TAG sequences (24mer oligo in blue color) are obtained from Luminex FlexMAP sequences and complementary to the corresponding TAG sequence.

**Enzyme screening for incorporation of dZTP opposite template dG in the absence of dCTP:**

1. Assay without dZTP (-): dATP, dTTP, and dGTP (0.1 mM of each), and no dCTP.

**F-17-S:** 5`-($^{32}$P)-ACCGCGGTCTCCCATGG-3`

**F-34-Std:** 3`-TGGCGCCAGAGGGTACCCGTCAGGCAGCAGGATC-5`

2. Assay with dZTP (+): dATP, dTTP, dGTP, and dZTP (0.1 mM of each), and no dCTP.

**F-17-S:** 5`-($^{32}$P)-ACCGCGGTCTCCCATGG-3`

**F-34-Std:** 3`-TGGCGCCAGAGGGTACCCGTCAGGCAGCAGGATC-5`

5`-$^{32}$P-labeled primer, F-17-S, (0.2 pmole) and non-labeled primer (2 pmole, final concentration 220 nM) was annealed to a template sequence, F-34-Std, (3 pmole, final assay concentration 300 nM) in 1X ThermoPol Reaction Buffer (pH 8.8, room temperature) by heating at 95 °C for 5 min and slowly cooling to room temperature over about 30 min. Nucleotide triphosphate mixture (dATP, dTTP, and dGTP, final concentration 0.1 mM of each) with (+) dZTP (final 0.1 mM) or without (-) were added to the above mixture at room temperature. The reaction mixture was pre-incubated at 72 °C for 30 s (for *Bst*, pre-incubated at 65 °C for 30 s), followed by the addition of *Bst (4 units)*, *Taq (2.5 units), Vent (exo-), Deep Vent (exo-), 9 Degree North (modified), Therminator, and Therminator II* DNA polymerase (1 unit for each enzyme) to give a final volume of 20 µL. Aliquots (6 µL) were taken from each reaction after 2 min and 10 min incubation, quenched by PAGE loading/quench buffer (8 µL, 10 mM EDTA in formamide). Samples were resolved by electrophoresis using a 20% PAGE (7 M urea). The gel was analyzed using MolecularImager software.

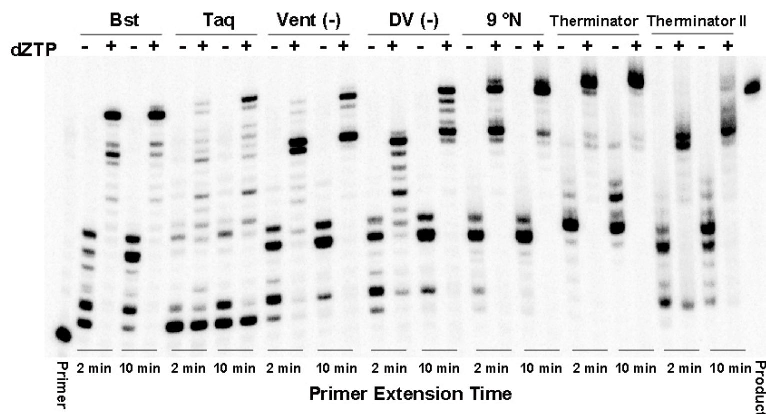



**Figure S1**. Polymerase screening for incorporation of dZTP opposite template dG in the absence of dCTP. In lanes marked "-" (without either dZTP or dCTP): No full-length products were produced by any polymerases tested. Paused bands were caused by misincorporating dT, dA, or, dG opposite template dG in the absence of dCTP and dZTP;
In lanes marked "+" (with dZTP to replace the missing dCTP): Full-length products were produced by most of polymerases tested;
A successful polymerase suppresses the paused bands in "+" assays by incorporating dZ opposite template dG in the absence of dCTP; Therminator DNA Polymerase is the enzyme of choice for incorporating dZTP opposite template dG.

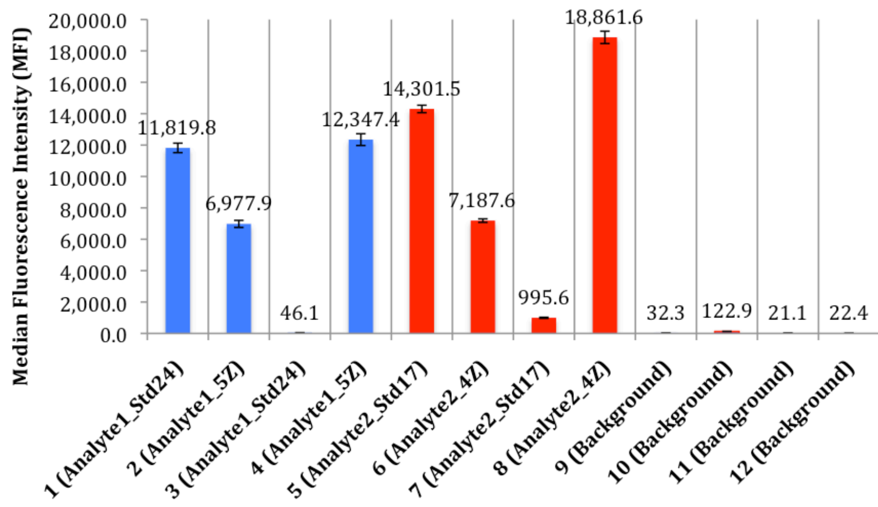
**Microsphere Bead Coupling according to Luminex's carbodiimide coupling procedure**

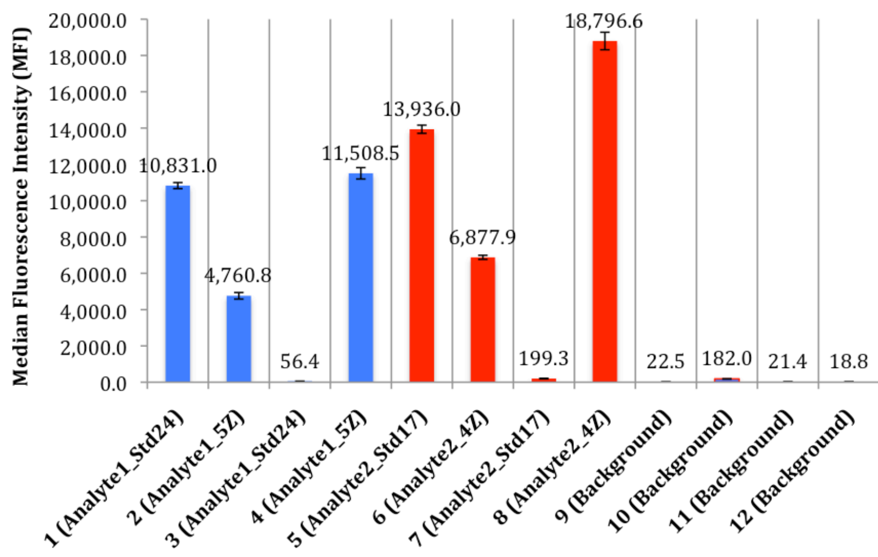
EDC powder (Pierce, dessicated, 2.3 grams) was brought to room temperature. A batch of microspheres, stored at 4 °C, was brought to room temperature. Prior to dispensing, the microspheres were rotated at approximately 20 rpm for 1 minute or mix by gentle inversion for 1 minute. 200 µL (equals $2.5 \times 10^6$ bead populations) of the stock microspheres (original concentration $1.25 \times 10^7$ /mL, also as $1.25 \times 10^4$ /µL) were transferred to a USA Scientific microfuge tube. The microspheres were pelleted by microcentrifugation at 10000 x g for 1 minute. The supernatant in each tube was removed and the pelleted microspheres were re-suspend in 50 µL of 0.1 M MES (pH 4.5) followed by vortexing and sonication for approximately 20 seconds. A 0.1 mM solution of capture probe (5`-amine modified standard oligonucleotide or dP-containing oligonucleotide) in $dH_2O$ was prepared. 4 µL of the capture probe (0.1 mM in $dH_2O$) was added to the resuspended microspheres and mixed by vortex. A fresh solution of 10 mg/mL EDC in $dH_2O$ was prepared (Note: Return the EDC powder to desiccant to re-use for the second EDC addition). Aliquots (5 µL) of fresh

10 mg/mL EDC (10 μg/μL) were added to the microspheres to a final concentration of 1 mg/mL for each coupling reaction and mixed by vortex. Each reaction was incubated for 30 minutes at room temperature in the dark. A second fresh solution of 10 mg/mL EDC in $dH_2O$ was prepared. Aliquots (5 μL) of fresh 10 mg/mL EDC (10 μg/μL) were added to the microspheres to give a final concentration of 1 mg/mL for each coupling reaction and mixed by vortex. The reactions were incubated for 30 minutes at room temperature in the dark. Tween-20 (0.5 mL, 0.02%) was added to the coupled microspheres. The coupled microspheres were pelleted by microcentrifugation at 10000 x g for 1 minute. The supernatant was removed and the pelleted coupled microspheres were re-suspend in 0.5 mL of 0.1% SDS followed by vortex. The coupled microspheres were pelleted by microcentrifugation at 10000 x g for 1 minute. After the supernatant was removed, the pelleted micropsheres were re-suspended in 100 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and enumerated the coupled microspheres by hemacytometer. Each coupled microsphere beads were diluted in TE buffer to a final concentration of 5000 beads / μL and stored at 4°C in the dark.

**Hybridization of GACT duplexes compared with hybridization of GACTZP duplexes**

5`-amino-modified GACT probes and GACTP probes (Supplementary Table S1) were coupled to carboxylated microspheres as described in the “microsphere coupling” section. Each hybridization reaction was carried out with ~5000 microspheres of each probe bearing microsphere populations. The microspheres were combined (mixture of Probe1_Std and Probe2_Std, or mixture of Probe1_5P and Probe2_4P) and pelleted by centrifugation at ≥ 8000 x g for 2 minutes. After removal of the supernatant, the pellets were resuspended to 100 of each microsphere set per μL in 2X Tm Hybridization Buffer (0.4 M NaCl, 0.2 M Tris, 0.16% Triton X-100, pH 8.0), followed by vortex and sonication for approximately 20 seconds. Aliquots (50 μL) of the microsphere mixture were added to each tube. $dH_2O$ was added (50 μL) to each background tube. Each biotinylated analyte (Supplementary Table S1) was added to the sample tubes. The total volume was adjusted to 100 μL by adding the appropriate volume of $dH_2O$ to each sample tube. Each sample was denatured at 95°C for 2 min, then, cooled down to 37°C at a rate of 0.1°C/s, followed by hybridization at 37°C for 10 minutes and then cool to room temperature. 50 μL of 1X $T_m$ Hybridization Buffer containing 6 μg/mL of streptavidin-R-phycoerythrin was added to each tube to give a final volume of 150 μL. Each sample was incubated at 25°C for another 10 minutes, then 50 μL of each sample was analyzed on the Luminex 200$^{TM}$ Systems at three different hybridization temperatures (25 °C, 37 °C, or 50 °C). Results are illustrated in Supplementary Figure S2 and S3.




| Experiment with indicated analyte / Bead | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Probe1_Std (Bead21) | + | + | - | - | - | - | - | - | + | - | - | - |
| Probe1_5P (Bead21) | - | - | + | + | - | - | - | - | - | - | + | - |
| Probe2_Std (Bead25) | - | - | - | - | + | + | - | - | - | + | - | - |

| Probe2_4P (Bead25) | - | - | - | - | - | - | + | + | - | - | - | + |
|---|---|---|---|---|---|---|---|---|---|---|---|---|

Notes: "+" means that the indicated species (bead-bound probe, in left column) is present in the experiment denoted by the left columns. The analyte in each experiment is listed under the vertical bar.

**Figure S2A.** Detection of biotinylated analyte (100 fmoles) using Luminex® beads carrying standard GACT (Lane 1, 2, 5, and 6) and GACTP (Lane 3, 4, 7, and 8) capture probe. See Supplementary Table S1 for sequence information.

Lane 1: The solid phase is Bead21, which carries "Probe1_Std" listed in the left column of the table. The solution contains 5'-biotinylated "Analyte1_Std24" listed under the vertical bar. The strong signal observed is assigned to the hybridization between "Probe1_Std" and its perfectly complementary "Analyte1_Std24".

Lane 2: The solid phase is Bead21, which carries "Probe1_Std". The solution contains 5'-biotinylated "Analyte1_5Z". The weaker signal observed is assigned to a hybridization between "Probe1_Std" and "Analyte1_5Z", which includes five G:Z mismatched pairs, presumably deprotonated. This signal decreases at higher temperature (50 °C).

Lane 3: The solid phase is Bead21, which carries "Probe1_5P". The solution contains 5'-biotinylated "Analyte1_Std24", The absence of signal is interpreted as a failure to form a duplex involving five P:C mismatches.

Lane 4: The solid phase is Bead21, which carries "Probe1_5P". The solution contains 5'-biotinylated "Analyte1_5Z". The strong signal, which is not diminished substantially upon heating, is assigned to the hybridization between "Probe1_5P" and its perfectly complementary "Analyte1_5Z" involving five Z:P pairs.

Lane 5: Bead25-bound "Probe2_Std" hybridized with "Analyte2_Std17" gives strong signal.

Lane 6: Bead25-bound "Probe2_Std" hybridized with "Analyte2_4Z" gives weaker signal.

Lane 7: Bead25-bound "Probe2_4P" hybridized with "Analyte2_Std17" gives negligible signal.

Lane 8: Bead25-bound "Probe2_4P" hybridized with "Analyte2_4Z" gives strongest signal.

Lane 9: Background signal comes from Probe1_Std (Bead 21), hybridized with water (no analyte).

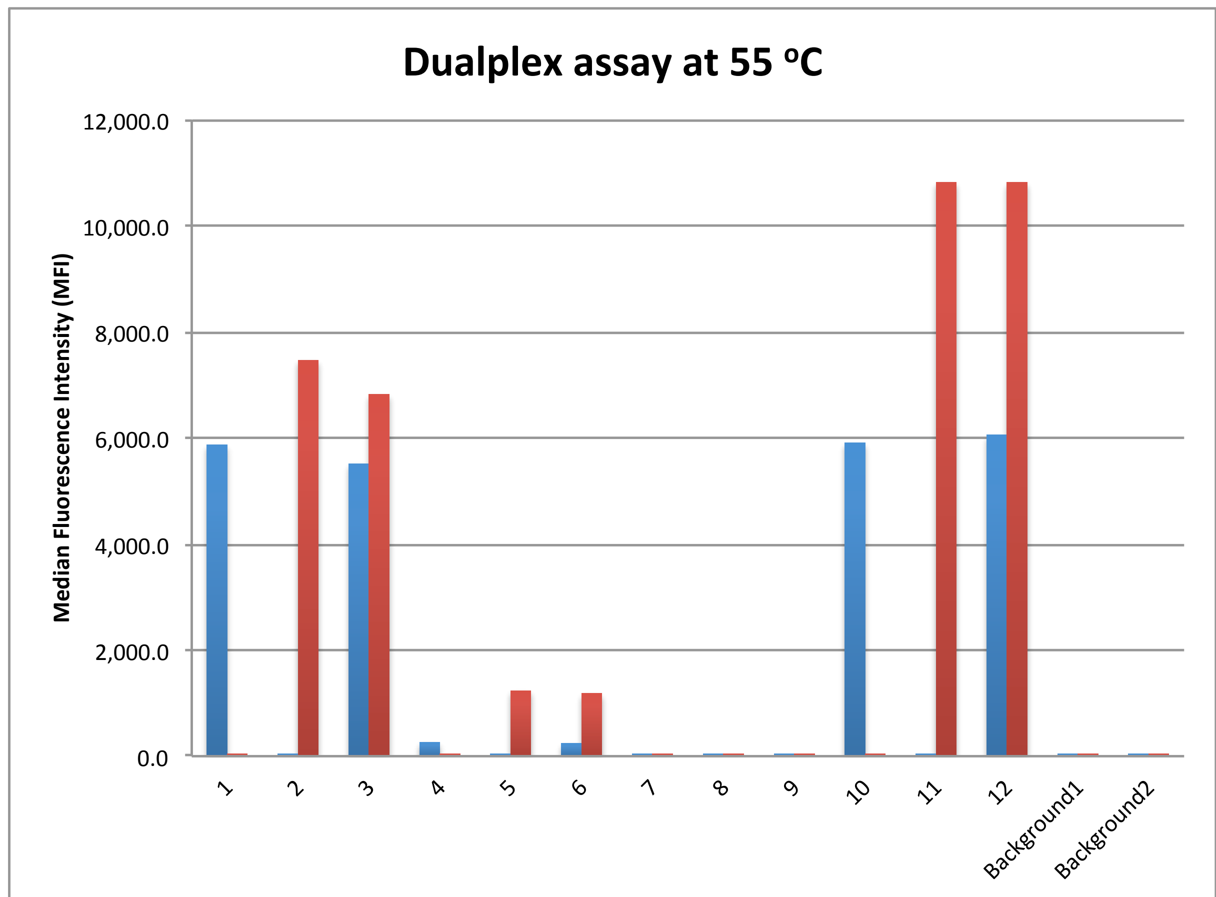
Lane 10: Background signal comes from Probe2_Std (Bead 25), hybridized with water (no analyte).

Lane 11: Background signal comes from Probe1_5P (Bead 21), hybridized with water (no analyte).

Lane 12: Background signal comes from Probe2_4P (Bead 25), hybridized with water (no analyte).

Notice the hybridization of GACTZP oligonucleotides gives higher MFI signal than that of standard GACT oligonucleotides. G:Z mismatch between GACT and GACTZ significantly prevents the hybridization and

gives lower signal; C:P mismatch between GACT and GACTP almost completely prevents the hybridization and gives negligible signal.




| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Bk g1 | Bk g2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Probe1_Std (Bead21) | + | + | + | + | + | + | - | - | - | - | - | - | + | - |
| Probe2_Std (Bead25) | + | + | + | + | + | + | - | - | - | - | - | - | + | - |
| Probe1_5P (Bead21) | - | - | - | - | - | - | + | + | + | + | + | + | - | + |
| Probe2_4P (Bead25) | - | - | - | - | - | - | + | + | + | + | + | + | - | + |
| Analyte1_Std24 | + | - | + | - | - | - | + | - | + | - | - | - | - | - |
| Analyte2_Std17 | - | + | + | - | - | - | - | + | + | - | - | - | - | - |
| Analyte1_5Z | - | - | - | + | | + | - | - | - | + | | + | - | - |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Analyte2_4Z | - | - | - | - | + | + | - | - | - | - | + | + | - | - |
| Water | - | - | - | - | - | - | - | - | - | - | - | - | + | + |

Notes: "+" means that the indicated species (bead-bound probe and analyte, listed in left column) are present in the hybridization experiment. "-" means that the indicated species (bead-bound probe and analyte, listed in left column) are absent in the hybridization experiment.

**Figure S2B.** Detection of biotinylated analytes (10 fmoles per analyte) on Luminex® beads carrying standard GACT (Lane 1- 6) and GACTP (Lane 7-12) capture probes (Supplementary Table S1 lists the analyte and probe sequences).

Experiments 1-6 (lanes 1-6) consist two solid phases (mixture of Beads21 and Bead25) and each bead carrying a standard capture probe, Probe1_Std (Bead21) and Probe2_Std (Bead25).

Lanes 1-3: The solution phase contains standard analyte with GACT sequence: only "Analyte1_Std24" (lane 1, only the blue signal is detected, as expected); only "Analyte2_Std17" (lane 2, only the red signal is seen, as expected); both "Analyte1_Std24" and "Analyte2_Std17" (lane 3, both signals are seen, as expected).

Lanes 4-6: The solution phase contains non-standard analyte with GACTZ sequence: only "Analyte1_5Z" (lane 4, only a weak blue signal is detected); only "Analyte2_4Z" (lane 5, only a weak red signal is seen); both "Analyte1_5Z" and "Analyte2_4Z" (lane 6, both signals are seen).

Experiments 7-12 (lanes 7-12) consist two solid phases (mixture of Beads21 and Bead25) and each bead carrying a non-standard GACTP capture probe, Probe1_5P (Bead21) and Probe2_4P (Bead25).

Lanes 7-9: The solution phase contains analyte with GACT sequence: only "Analyte1_Std24" (lane 7), only "Analyte2_Std17" (lane 8); both "Analyte1_Std24" and "Analyte2_Std17" (lane 9).

Lanes 10-12: The solution phase contains analyte with GACTZ sequence: only "Analyte1_5Z" (lane 10, only the blue signal is detected, as expected); only "Analyte2_4Z" (lane 11, only the red signal is seen, as expected); both "Analyte1_5Z" and "Analyte2_4Z" (lane 12, both signals are seen, as expected).

Background1 (Bkg1) and Background2 (Bkg2) contain water instead of analytes.

Notice the hybridization of GACTZP DNA gives higher MFI signal than that of standard GACT DNA (compare lane 12 to lane 3). G:Z mismatch between GACT and GACTZ significantly prevents the hybridization and gives lower signal (from lane 4 to 6); C:P mismatch between GACT and GACTP almost completely prevents the hybridization and gives negligible signal (from lane 7 to 9).

**Generation of GACTZ oligonucleotides using *Therminator* DNA polymerase and Validation of the ZiP-TAG conversion assay**

**Monitor the primer extension reaction using $^{32}$P-labeled primer:**

5`-$^{32}$P-labeled primer (0.1 pmole) and non-radioactive primer (biotinylated primer, 2 pmole, final assay concentration 210 nM) were annealed to standard DNA template (3 pmole, final assay concentration 300 nM) in 1X ThermoPol Reaction Buffer (pH 8.8, room temperature) by heating at 95 °C for 5 min followed by slow cooling to room temperature over ca. 30 min. Three different nucleotide triphosphate mixtures were added to the resulting reaction mixture, first (negative control, dATP + dTTP + dGTP), second (positive control, dATP + dTTP + dGTP + dCTP), and third (conversion experiment, dATP + dTTP + dGTP + dZTP), respectively. The reaction mixture was pre-incubated at 72 °C for 30 sec, followed by the addition of *Therminator* DNA polymerase (1 unit) to give a final volume of 10 µL. The reaction was quenched by PAGE loading buffer (10 µL, 10 mM EDTA in formamide) after incubated at 72 °C for 1 min. Samples were resolved by electrophoresis using a 16% PAGE (7 M urea). The gel was analyzed using MolecularImager software.

**Extend biotinylated primer under the identical conditions:**

Biotinylated primer (2 pmole, final assay concentration 200 nM) was annealed to standard DNA template (3 pmole, final assay concentration 300 nM) in 1X ThermoPol Reaction Buffer (pH 8.8, room temperature) by heating at 95 °C for 5 min and slowly cool to room temperature in about 30 min. Three different nucleotide triphosphate mixtures were added to the above reaction mixture, first (negative control, dATP + dTTP + dGTP), second (positive control, dATP + dTTP + dGTP + dCTP), and third (conversion experiment, dATP + dTTP + dGTP + dZTP), respectively. The reaction mixture was pre-incubated at 72 °C for 30 sec, followed by the addition of *Therminator* DNA polymerase (1 unit) to give a final volume of 10 µL. The reaction was quenched with 2 µL of 20 mM EDTA, and diluted with 190 µL of $dH_2O$ to give the biotinylated extended standard or dZ-containing oligonucleotide with a final concentration of ca.10 fmoles/µL.

**Hybridization of biotinylated extended-primers (analytes) with standard (type A) or P-containing (type B) anti-TAG probes:**

5`-amino-modified standard anti-TAG probes and 5`-amino-modified P-containing anti-TAG probes were coupled to carboxylated microspheres (Bead10, Bead14, and Bead19). Three types of microspheres carrying standard probes were combined to give Type A bead mixture with ~5000 of each type of microsphere

population per reaction. Type B bead mixture also includes three types of microspheres carrying GACTP probes. The microsphere mixtures were pelleted by centrifugation at ≥ 8000 x g (2 minutes). After removal of the supernatant, the pellets were re-suspended to give a suspension containing 100 of each microsphere set per µL in 2X Tm Hybridization Buffer, followed by vortexing and sonication (~20 seconds). Aliquots (50 µL) of the microsphere mixture were added to each tube. $dH_2O$ was added (50 µL) to the tube, and the background signal was measured. Aliquots (5 µL) of each biotinylated-primer extension reaction mixture (cat. 50 fmoles of biotinylated analyte) were added to the sample tubes. The total volume was adjusted to 100 µL by adding the appropriate volume of $dH_2O$ (45 µL) to each sample tube. Each sample was denatured at 95°C for 2 min, then, cooled to 37°C at a rate of 0.1°C/s, followed by hybridization at 37°C for 10 minutes. The samples were then cooled to room temperature. Aliquots (50 µL) of 1X $T_m$ Hybridization Buffer containing 6 µg/mL of streptavidin-R-phycoerythrin were added to each tube to give a final volume of 150 µL. Each sample was incubated at 25°C for another 10 minutes. Volumes (50 µL) of each sample was analyzed at 25°C on the Luminex 200$^{TM}$ Systems.

**Oligonucleotides used or produced in this study:**

**Type A: amine-modified standard anti-TAG probes were conjugated to carboxylated microshpere beads:**

Bead10-Std: 5`-$NH_2$-$C_{12}$- TGT AGA TTT GTA TGT ATG TAT GAT -3`

Bead14-Std: 5`-$NH_2$-$C_{12}$- AAA GTA TAG TAA GAT GTA TAG TAG -3`

Bead19-Std: 5`-$NH_2$-$C_{12}$- GTA TTT GAG TAA GTA ATT GAT TGA -3`

**Type B: amine-modified dP-containing anti-TAG probes were conjugated to carboxylated microshpere beads:**

Bead10-6P: 5`-$NH_2$-$C_{12}$- TPT APA TTT PTA TPT ATP TAT PAT -3`

Bead14-5P: 5`-$NH_2$-$C_{12}$- AAA PTA TAP TAA PAT PTA TAP TAG -3`

Bead19-6P: 5`-$NH_2$-$C_{12}$- PTA TTT PAP TAA PTA ATT PAT TPA -3`

## 1. Biotinylated TAG10 analytes:

**Negative control (dATP + dTTP + dGTP):**

```
                                   3`-CTGTCGGGGCCTACTCTTG-Biotin-5`
TAG10-Temp-Std:  5`-TGT AGA TTT GTA TGT ATG TAT GAT-GACAGCCCCGGATGAGAAC-3`
```

**Positive control (dATP + dTTP + dGTP + dCTP): produced by polymerase extension without conversion.**

```
TAG10-Std-Biotin: 3`-ACA TCT AAA CAT ACA TAC ATA CTA-CTGTCGGGGCCTACTCTTG-Biotin-5`
TAG10-Temp-Std:   5`-TGT AGA TTT GTA TGT ATG TAT GAT-GACAGCCCCGGATGAGAAC-3`
```

**Experiment (dATP + dTTP + dGTP + dZTP): produced by polymerase extension with conversion.**

```
TAG10-6Z-Biotin:    3`-AZA TZT AAA ZAT AZA TAZ ATA ZTA-CTGTCGGGGCCTACTCTTG-Biotin-5`
TAG10-Temp-Std:     5`-TGT AGA TTT GTA TGT ATG TAT GAT-GACAGCCCCGGATGAGAAC-3`
```

## 2. Biotinylated TAG14 analytes:

**Negative control (dATP + dTTP + dGTP):**

```
                                                        3`-CGGGGTCAACGGCAGATCCT-Biotin-5`
TAG14-Temp-Std:     5`-AAA GTA TAG TAA GAT GTA TAG TAG-GCCCCAGTTGCCGTCTAGGA-3`
```

**Positive control (dATP + dTTP + dGTP + dCTP): produced by polymerase extension without conversion.**

```
TAG14-Std-Biotin:   3`-TTT CAT ATC ATT CTA CAT ATC ATC-CGGGGTCAACGGCAGATCCT-Biotin-5`
TAG14-Temp-Std:     5`-AAA GTA TAG TAA GAT GTA TAG TAG-GCCCCAGTTGCCGTCTAGGA-3`
```

**Experiment (dATP + dTTP + dGTP + dZTP): produced by polymerase extension with conversion.**

```
TAG14-6Z-Biotin:    3`-TTT ZAT ATZ ATT ZTA ZAT ATZ ATZ-CGGGGTCAACGGCAGATCCT-Biotin-5`
TAG14-Temp-Std:     5`-AAA GTA TAG TAA GAT GTA TAG TAG-GCCCCAGTTGCCGTCTAGGA-3`
```

## 3. Biotinylated TAG19 analytes:

**Negative control (dATP + dTTP + dGTP):**

```
                                                        3`-AGGAGGAATACGGAGATAGTA-Biotin-5`
TAG19-Temp-Std:     5`-GTA TTT GAG TAA GTA ATT GAT TGA-TCCTCCTTATGCCTCTATCAT-3`
```

**Positive control (dATP + dTTP + dGTP + dCTP): produced by polymerase extension without conversion.**

```
TAG19-Std-Biotin:   3`-CAT AAA CTC ATT CAT TAA CTA ACT-AGGAGGAATACGGAGATAGTA-Biotin-5`
TAG19-Temp-Std:     5`-GTA TTT GAG TAA GTA ATT GAT TGA-TCCTCCTTATGCCTCTATCAT-3`
```

**Experiment (dATP + dTTP + dGTP + dZTP): produced by polymerase extension with conversion.**

```
TAG19-6Z-Biotin:    3`-ZAT AAA ZTZ ATT ZAT TAA ZTA AZT-AGGAGGAATACGGAGATAGTA-Biotin-5`
TAG19-Temp-Std:     5`-GTA TTT GAG TAA GTA ATT GAT TGA-TCCTCCTTATGCCTCTATCAT-3`
```

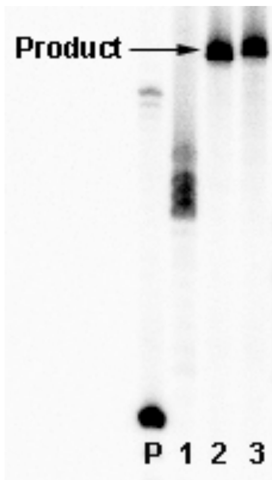


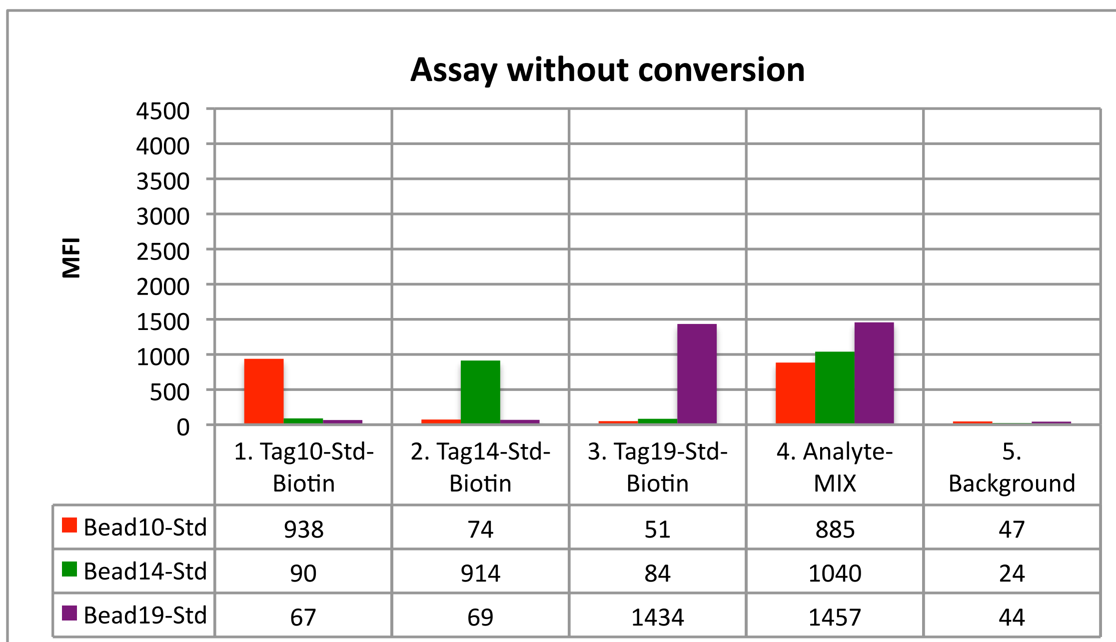

**Figure S3A**. Biotinylated primer extended on standard template using *Therminator* DNA polymerase with various triphosphate mixtures.

Lane 1 (negative control): nucleotide triphosphate mixture contains dATP + dTTP + dGTP;

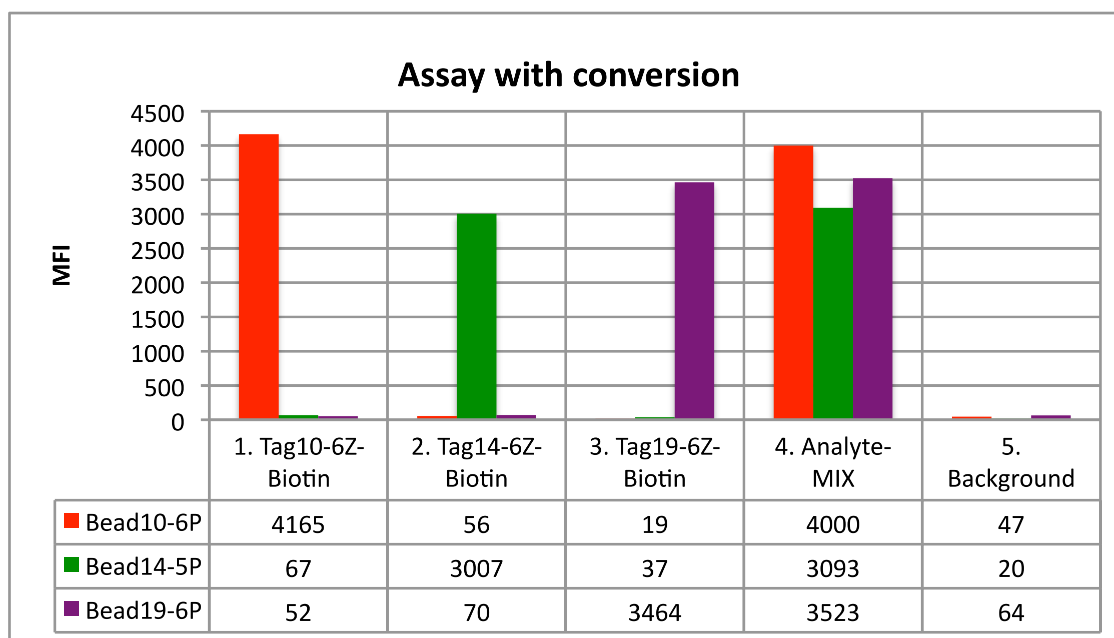
Lane 2 (positive control): nucleotide triphosphate mixture contains dATP + dTTP + dGTP + dCTP;

Lane 3 (conversion experiment): nucleotide triphosphate mixture contains dATP + dTTP + dGTP + dZTP;

P indicates primer; Product indicates extended primer under the described conditions.




| | 1. Tag10-Std-Biotin | 2. Tag14-Std-Biotin | 3. Tag19-Std-Biotin | 4. Analyte-MIX | 5. Background |
|---|---|---|---|---|---|
| Bead10-Std | 938 | 74 | 51 | 885 | 47 |
| Bead14-Std | 90 | 914 | 84 | 1040 | 24 |
| Bead19-Std | 67 | 69 | 1434 | 1457 | 44 |




| | 1. Tag10-6Z-Biotin | 2. Tag14-6Z-Biotin | 3. Tag19-6Z-Biotin | 4. Analyte-MIX | 5. Background |
|---|---|---|---|---|---|
| Bead10-6P | 4165 | 56 | 19 | 4000 | 47 |
| Bead14-5P | 67 | 3007 | 37 | 3093 | 20 |
| Bead19-6P | 52 | 70 | 3464 | 3523 | 64 |

**Figure S3B.** The difference biotinylated primer extension architecture without (top) and with conversion (bottom). All experiments contain all three types of Luminex beads (Bead10, Bead14, and Bead19), each carrying an anti-TAG probe for a different analyte. Experiment 1: only TAG10 analyte present. Experiment 2: only TAG14 analyte present. Experiment 3: only TAG19 analyte present. Experiment 4: all three analytes present. Experiment 5: no analytes present. MFI indicates the median fluorescence intensity. Note the stronger signals obtained with GACTZP compared with GACT, and the proportionality conserved in going from singleplexed to three-fold multiplexed assay. See text for discussion.

**Monoplex and five-fold multiplex PCR amplification of five target genes**

Monoplex and five-fold multiplex PCR were carried out with 200 ng of human genomic DNA (Promega, Male), 200 µM of each deoxyribonucleoside triphosphate, 2.5 Units of JumpStart *Taq* DNA polymerase (2.5 unit/µL, Sigma), and 1x reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, pH 8.3) with a final volume of 50 µL. Each monoplexed reaction consisted of a set of forward and reverse primers (final 0.4 µM for each primer, for primer sequences see Supplementary Table S2), and five-fold multiplexed reaction consisted of five sets of forward and reverse primers (final 0.4 µM for each primer). The following PCR conditions were applied to each reaction on the DNAEngine Peltier Thermal Cycler (Bio-Rad). Initial one cycle at 95 °C for 1 min; followed by 31 cycles of (95 °C for 20 s, 60 °C for 30 s, 72 °C for 30 s); and final 72 °C for 10 min. Reactions were then held at 4 °C until use. Upon the completion of PCR,

samples (8 μL) were taken from each PCR mixture, mixed with 6x agarose loading dye (2 μL, Promega), and analyzed on a 3% agarose gel. See **Figure S4** for gel images.

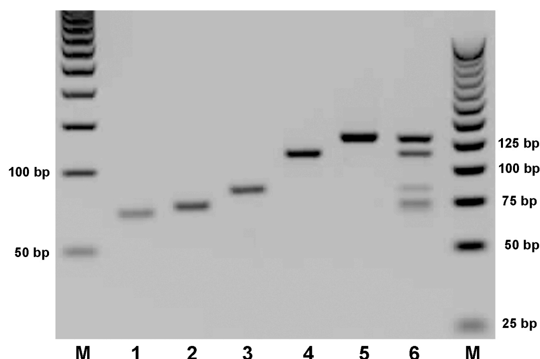
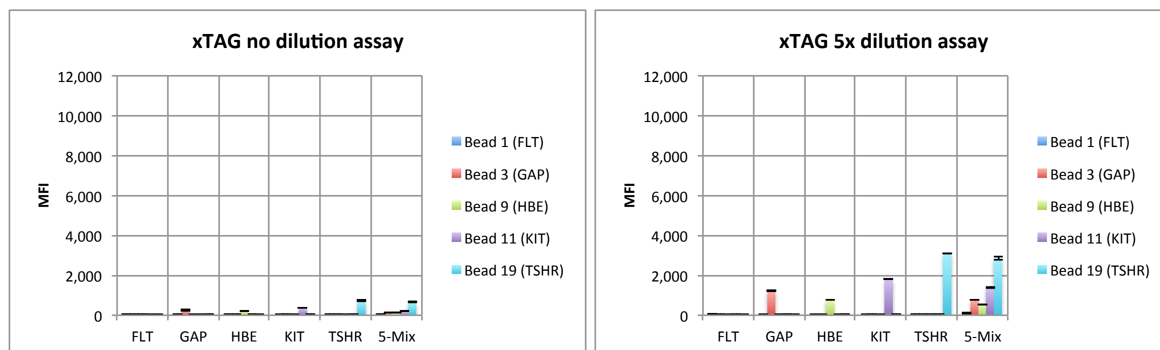



**Figure S4.** Agarose gel (3%) resolving monoplexed PCR (lane 1-5) and five-fold multiplexed PCR (lane 6) amplification of five target genes from human genomic DNA (Promega, Male).
Lane 1: GAPDH; Lane 2: FLT3; Lane 3: HBEGF; Lane 4: KIT; Lane 5: TSHR.
Lane 6: Five-fold multiplexed PCR amplification of the above target genes (note overlap of the fastest two bands). M: DNA Ladders.

**Figure S5A (without filtration)**. The dose-response behavior of Luminex® assays in architectures that extend a sequence-TAGged primer complementary to the amplicon with biotinylated dCTP, without filtration to remove excess biotinylated dCTP. Each assay uses five Luminex® microspheres (indicated by color) each carrying its own analyte-specific probe built from standard nucleotides. Each panel shows median fluorescence intensity (MFI) for the five analytes presented separately. The last panel then shows results from a mixture of analytes (each at the same concentration). Serial dilutions show an apparent increase in signal intensity upon dilution (from 5x to 25x), followed by an apparent decrease in signal intensity upon further dilution (125x).



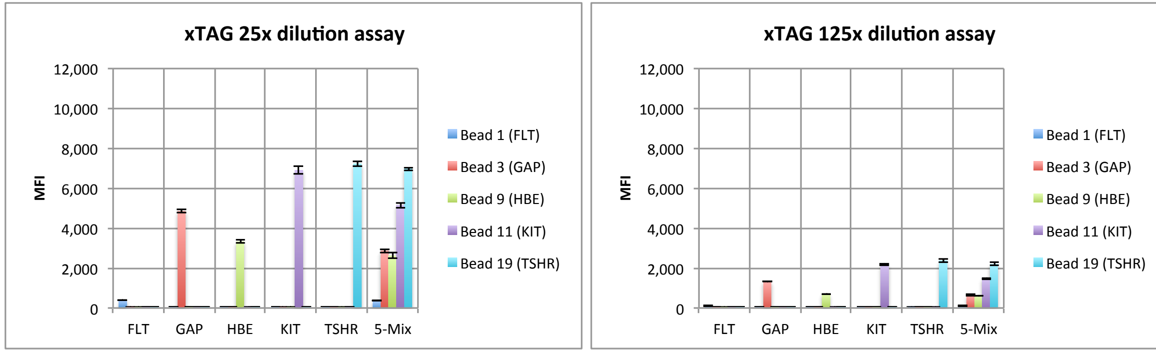



**Figure S5B (with filtration to remove biotin-dCTP)**. The same as above, but with excess biotinylated dCTP removed. The desired dose-response curve was observed.

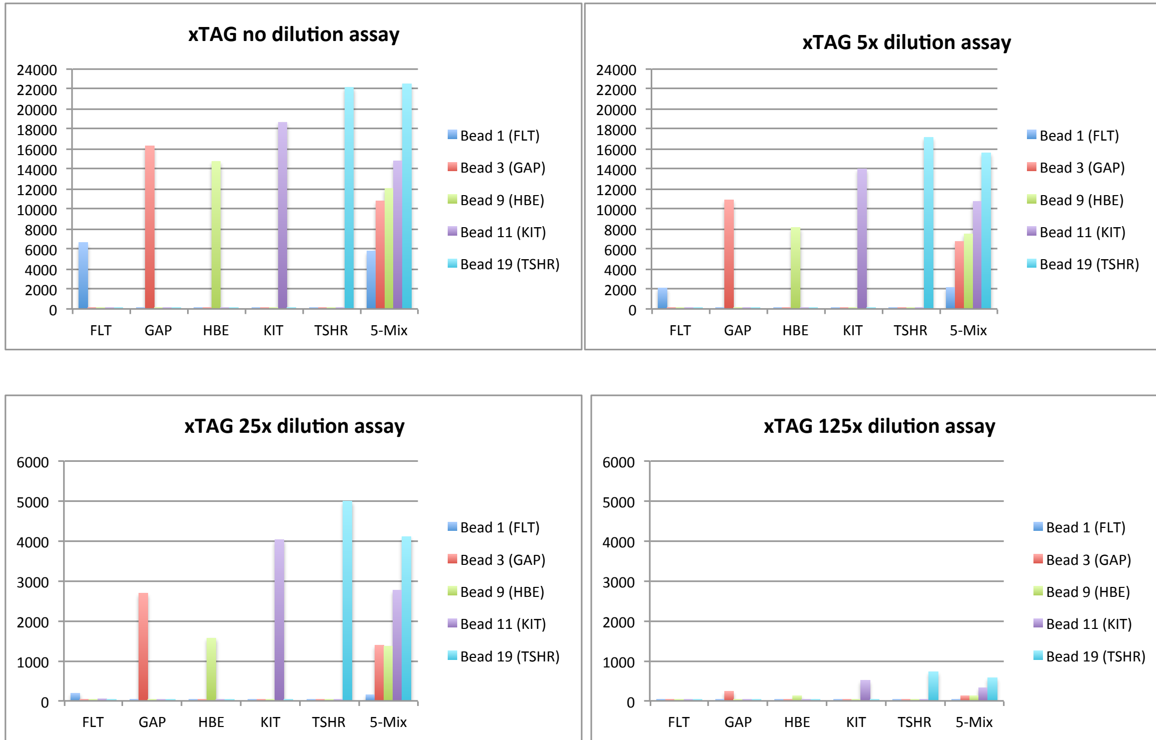
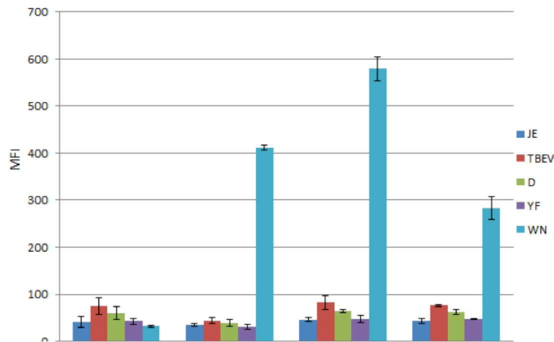


**Figure S6**. Non-linear dose response for a PCR amplicon obtained by reverse transcriptase-PCR of West Nile RNA using the standard amplicon capture architecture (direct hybridization, Figure 3A).




Assay was run with RNA from a single target (West Nile, WN, light blue) in the presence of a mixture of microspheres carrying probes to other targets (JE = Japanese encephalitis; TBEV = tick-borne encephalitis virus; D = dengue; YF = yellow fever). To find the optimal dilution factor that gives the highest MFI signal, PCR products were 10-fold serially diluted and then hybridized with microsphere-carrying capture probes. Cluster 1: Undiluted PCR products; Cluster 2: 1:10 dilution; Cluster 3: 1:100 dilution; Cluster 4: 1:1000 dilution. MFI = median fluorescence intensity.