Electronic Supplementary Material (ESI) for Analyst.

Split G-quadruplex based PfAgo sensing platform for nucleotide mutations discrimination and human genotyping

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Experimental details

1.1 Materials and reagents

All oligonucleotides used in this work were synthesized and modified by JIELI BIOLOGY Co., Ltd (Shanghai, China). PfAgo Endonuclease (microsphere used for RADAR), PfAgo Endonuclease Reaction Buffer, $MnCl_2$ (40mM) were purchased from CHINAPEPTIDES CO., LTD (Wuhan, China). RPA Basic kit (WLB8201KIT) was purchased from Amplification Future (China). Hi-swab DNA kit was obtained from TIANGEN BIOTECH (BEIJING) CO., LTD. Taq PCR Master Mix was bought from BBI CO., LTD. FastPure Gel DNA Extraction Mini Kit was purchased from Vazyme Biotech Co. Ltd (Nanjing, China). ThT was purchased from Aladdin Reagent Corporation (Shanghai, China). Tris-acetic acid-magnesium (1×TAMg) buffer (45 mM Tris-acetic acid and 7.6 mM magnesium acetate, pH 8.0) was used for all nucleic acids self-assembly. All reagents were used as received without further purification.

UV1800PC spectrophotometer (Shanghai, China) was used to quantify all the oligonucleotides. Fluorescence signals were recorded on a RF-5301PC spectrofluorophotometer. All DNA samples were annealed on Applied Biosystems 96-well thermocycler (A37029, Thermo Fisher Scientific, USA). Tanon EPS300 electrophoresis apparatus was used for native polyacrylamide gel electrophoresis (PAGE). Images were obtained with Tanon 4600SF gel imaging system under UV light.

1.2 Native PAGE characterization

8% native PAGE was used in this work. Electrophoresis was performed in 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). A constant voltage of 90 V was used for 80 min of electrophoresis before the gels were stained with 4S Red for imaging.

For the split G4 assembly characterization: equivalent of G4-a, G4-b, and Link (1 μM each) were annealed in 1× TAMg buffer from 95 ℃ to 4 ℃. The resulting samples were then analyzed with 8 % native PAGE.

For the Ago cleavage activity characterization: dsDNA target (0.5 μM), gDNA1 (2 μM), gDNA2 (2 μM), substrate chain (2 μM) and PfAgo endonuclease with $MnCl_2$ were incubated in 10×PfAgo Endonuclease Reaction Buffer at 95℃ for 30 minutes before subjected for native PAGE characterization.

1.4 RPA Reaction

We designed forward primer, reverse primer, and long target sequence to amplify the template with RPA Basic kit. 50 μL RPA reaction system was composed of 4 μL primers (400 nM for each primer), 2 μL synthetic template, 29.4 μL rehydration buffer, 12.1 μL ddH_2O and 2.5 μL MgOAc (280 mM). After incubation at 39°C for 20 min, we added 2 μL amplification products to perform PfAgo endonuclease reaction.

For PAGE characterization, we extracted the products by DNA extraction reagent. Procedures were as follows: the DNA extraction

solution was prepared by combining phenol, chloroform, and isoamyl alcohol in a 25:24:1 ratio. The amplification product was mixed with the solution in 1:1 ratio before being centrifuged at 12,000 rpm for 5 minutes. A total 10 μL of supernatant from the centrifuged product was added to 2 μL DNA up-sampling buffer for native PAGE.

1.5 PfAgo cleavage

For the PfAgo cleavage reaction, the master mix was prepared with the following components: 2.5 μL reaction buffer (10×), 4 μM gDNA1, 4 μM gDNA2, 2 μL amplification target product, 2 μM substrate chain (Link strand), 1.6 mM $MnCl_2$, and one PfAgo endonuclease microsphere were brought up to 25 μL with nuclease-free water. The cleavage reactions were carried out at 95 °C for 30 min. Then 1 μM G4-a, 1 μM G4-b, 100 mM KCl and 20 μM ThT were added to the reaction solution. After incubation at 37℃ for 10 minutes, the fluorescence signals were recorded by RF-5301PC spectrofluorophotometer (λ_{ex}: 405 nm, λ^{em}: 485 nm). Concentrations given above were the final concentrations of each component in the total 25 μL PfAgo cleavage mixture.

1.6 Single nucleotide variation detection

The PfAgo cleavage reaction system was performed as described above, using mutant Link for 0.5 μM WT and Mutant targets detection. After reaction at 95℃ for 30 minutes, 1 μM G4-a, 1 μM G4-b, 100 mM KCl and 20 μM ThT were individually added and incubated at 37℃ for 10 minutes.

The fluorescence signal was recorded with an RF-5301PC spectrofluorophotometer (λ_{ex}: 405 nm, λ_{em}: 485 nm).

1.7 Gene ratio construction

Two types of Links (Link-ACT, Link-ATT) were designed to identify the corresponding polymorphism sites within the rs601338 gene, and the genotype was determined by the fluorescence signal intensity ratio. Using rs601338-G as a target strand of wild-type homozygote and rs601338-A as a target strand of mutant homozygote, mixing wild-type and mutant ssDNA targets (rs601338-G: rs601338-A = 1: 1) to simulate heterozygous gene targets. The PfAgo reaction system was as follows: wild-type homozygous gene target (500 nM), mutant homozygous gene target (500 nM), heterozygous gene target (250 nM each), Link-ACT or Link-ATT (2 μM), 2.5 μL reaction buffer (10×), 4 μM gDNA1, 4 μM gDNA2, 1.6mM $MnCl_2$ and a PfAgo endonuclease microsphere. ddH_2O was supplemented to reach a final volume of 25 μL. The reaction was performed at 95℃ for 30 min, and 1 μM G4-a, 1 μM G4-b, 100 mM KCl and 20 μM ThT were added to the reaction solution. After 10 min at 37℃, the fluorescence signal was recorded with an RF-5301PC fluorescence spectroluminometer (λ_{ex}: 405 nm, λ_{em}: 485 nm).

1.8 Buccal swab sample testing

FUT2 DNA genome extracted from buccal swab samples were provided by the volunteers in the pharmaceutics laboratory of Nantong University.

The FUT2 DNA genomic template was pre-amplified with the RPA kit and the following components were added to the RPA lyophilized powder tube: 29.4 μL complex buffer, 2 μL forward and reverse primers (400 nM each), 5 μL buccal swab sample DNA extraction, 9.1 μL ddH_2O, resulting in a total volume of 47.5 μL. Amplification was initiated by adding 2.5 μL of MgOAc (280 mM). The RPA reactions were performed at 39 ℃ for 20 min. The amplification product was purified with a Fast Pure Gel DNA Extraction Mini Kit (Vazyme Biotech Co. Ltd., Nanjing, China) and 10 μL purified DNA was added into the 25 μL PfAgo reaction mixture containing Link-ACT or Link-ATT (2 μM), 2.5 μL reaction buffer (10×), 4 μM gDNA1, 4 μM gDNA2, 1.6 mM $MnCl_2$ and a PfAgo endonuclease microsphere. The PfAgo cleavage was carried out at 95℃ for 30 min. Then 1 μM G4-a, 1 μM G4-b, 100 mM KCl and 20 μM ThT were added to the reaction solution. After incubation 10 min at 37℃, the fluorescence signal was recorded with an RF-5301PC fluorescence spectroluminometer (λ_{ex}: 405 nm, λ_{em}: 485 nm).

1.9 ASPCR validation

ASPCR was utilized to verify the testing results of buccal swab samples. Genomic DNA was extracted using the efficient buccal swab Genomic DNA kit. Then 2 μL of different reverse primer (PCR-Reverse-Primer-C, PCR-Reverse-Primer-T, 400 nM) were mixed with 2 μL forward primer (PCR-Forward-Primer, 400 nM), 2 μL genomic DNA template, 25 μL Taq

PCR mixture and 19 μL ddH_2O. The solution was divided into two tubes for amplification. In a PCR thermocycler, the DNA underwent pre-duration for 4 min at 94℃, 35 cycles at 94℃ for 30 s, 55℃ annealing for 30s and 72℃ extension for 10s, and 10 min at 72℃. The resulting PCR products were analyzed by 8% native PAGE.

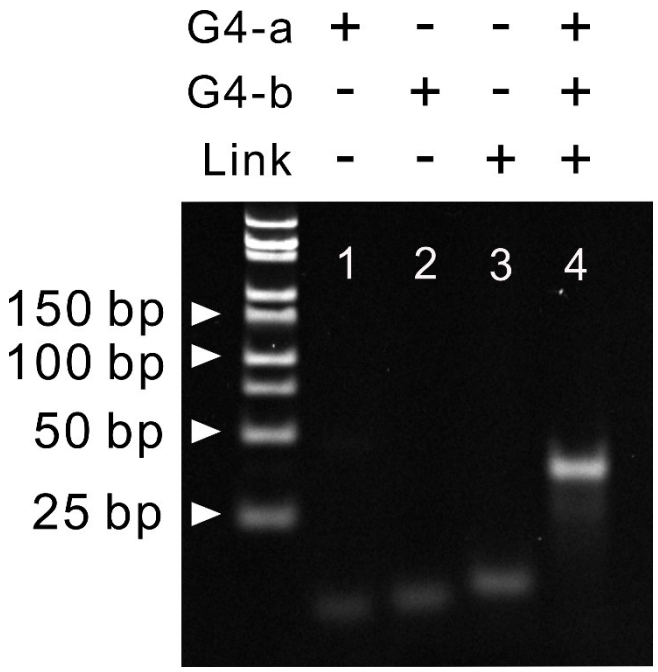

Figure S1 Native PAGE characterization of split G4 formation.

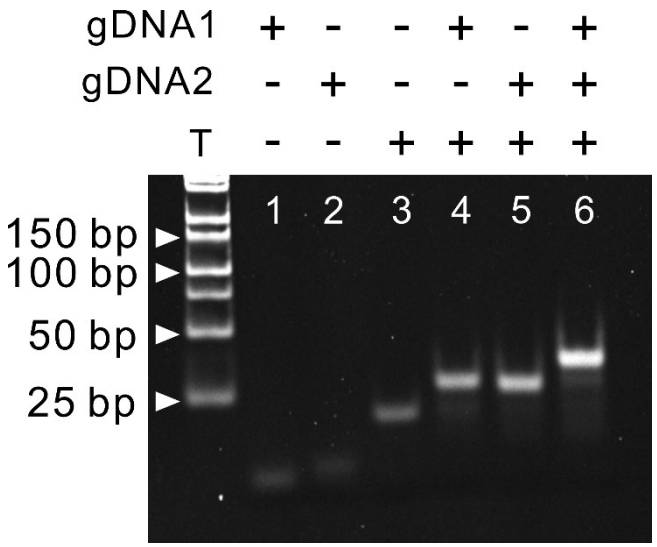

Figure S2 Native PAGE characterization of the hybridization between gDNAs and single stranded target.

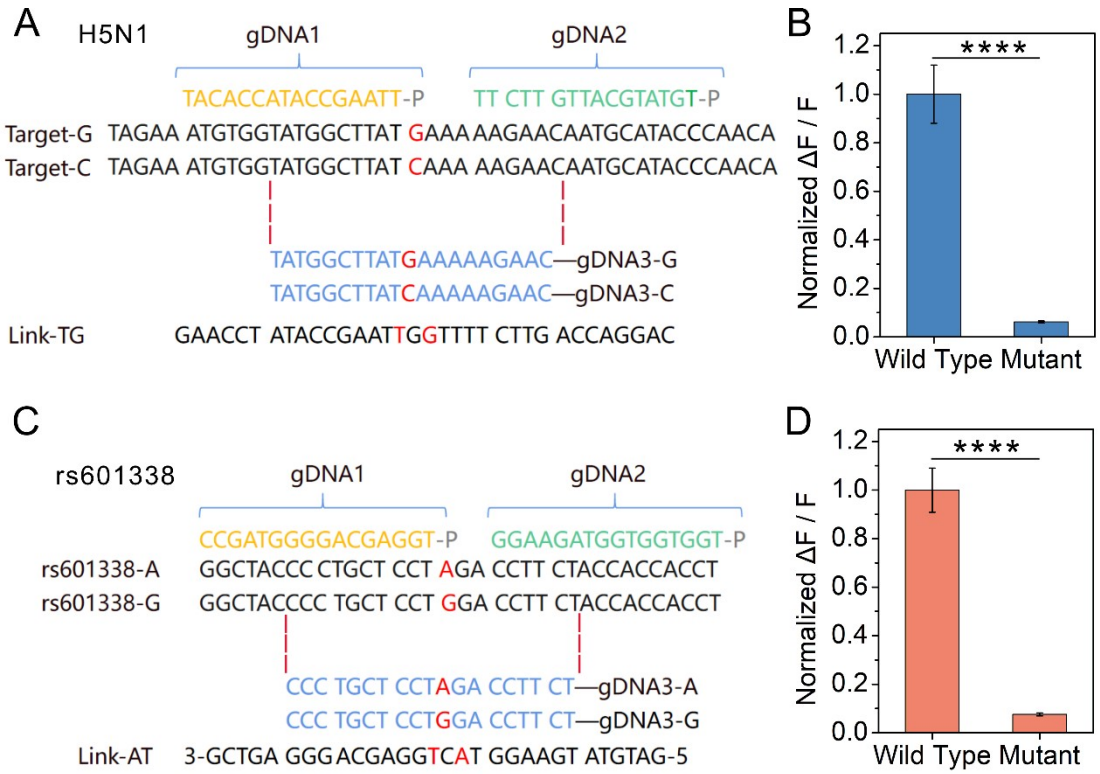

Figure S3 Universality testing of the PfAgo-G4 for SNP discrimination**.** (A) The schematic design of nucleotides for H1N1 mutation discrimination. (B) The discrimination results when utilizing Link-TG with two base mismatches. (C) The schematic design of nucleotides for rs601338 mutation discrimination. (D) The discrimination results when utilizing Link-AT with two base mismatches. n = 3 technical replicates, two-tailed Student's t test; ****P<0.001, bars represent mean ± SD.

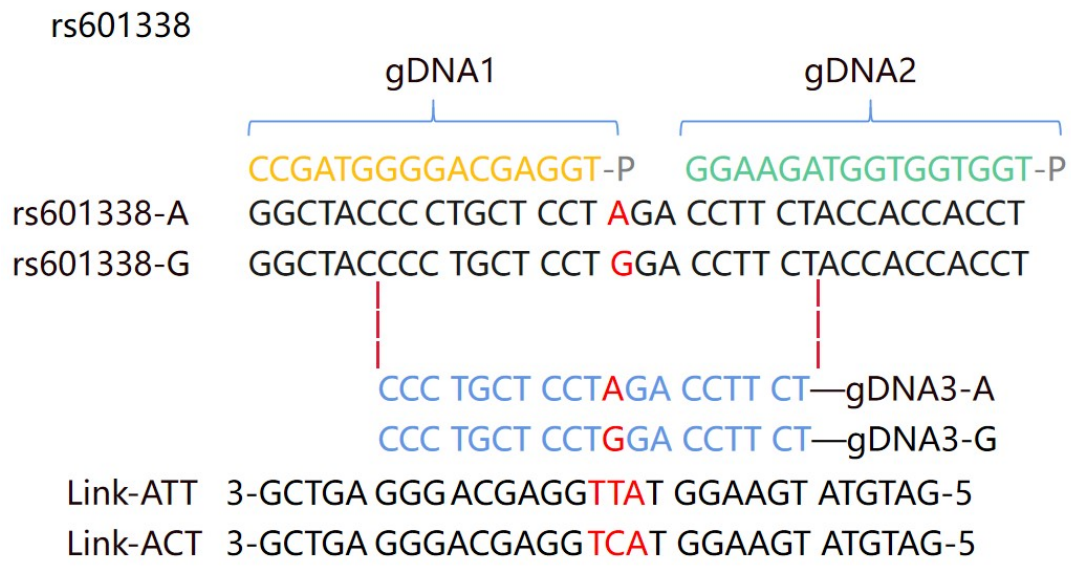

Figure S4 Nucleotides designed for rs601338 SNP discrimination.

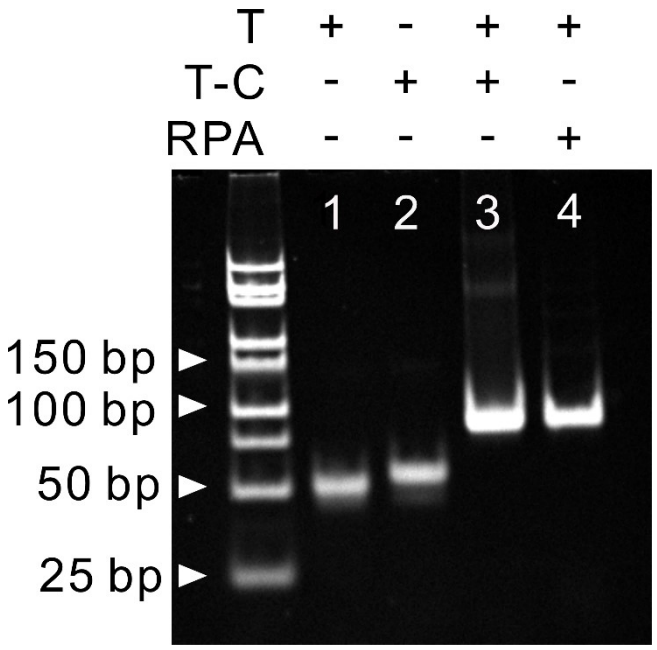

Figure S5 RPA validation with synthetic target standards. T: target sequence; T-C: the sequence complementary to target; RPA: amplification treatment.

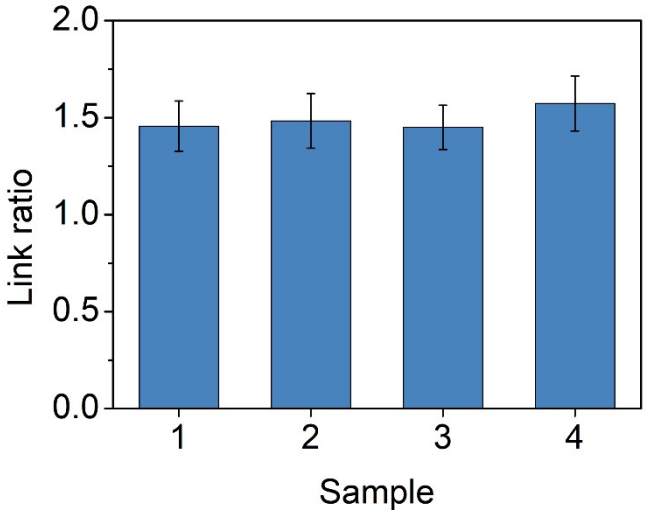

Figure S6 Link ratio results of genome DNA from 4 individuals.

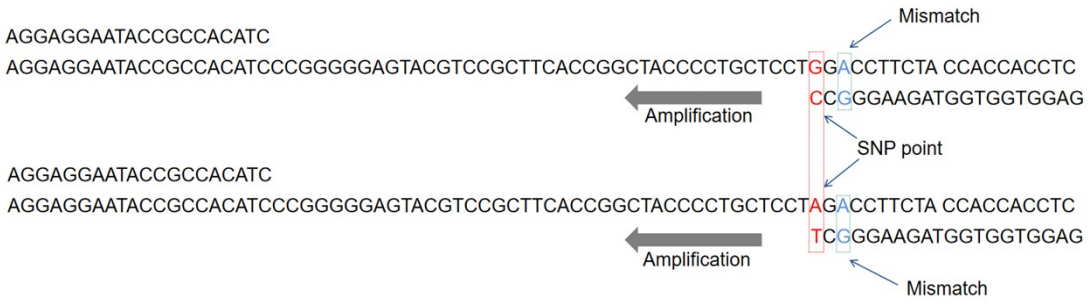

Figure S7 The nucleic acids designed for ASPCR analysis.

Table S1. Nucleic acids used for H1N1 analysis.

Name	Sequences(5'to3')
T-S	GG AAA CTC TTA TCC CAA GCT CAG CAA GTC ATA CA
T-S-C	TGT ATG ACT TGC TGA GCT TGG GAT AAG AGT TTC C
gDNA1	P-TGGGATAAGAGTTTCC
gDNA2	P-TGTATGACTTGCTGAG
gDNA3	TC TTA TCC CAA GCT CAG C
Reporter	FAM-GCATTGACGTGAGCTTGGGATAAGATGCAG-BHQ
A	GGGTGGGT CTC ACG TCA ATGC
B	CTGC ATC TTA TCC TGGGTGGG
Link	GCATTGACGTGAGCTTGGGATAAGATGCAG
Full-T-S	TCG GAA CTT GCT ATG GAT AGT AAA GAA GGG AAA CTC TTA TCC CAA GCT CAG CAA GTC ATA CAC AAA CAA CAA AGG GAA AGA AGT G
Full-T-S-C	CAC TTC TTT CCC TTT GTT GTT TGT GTA TGA CTT GCT GAG CTT GGG ATA AGA GTT TCC CTT CTT TAC TAT CCA TAG CAA GTT CCG A
Forward Primer	TCGGAACTTGCTATGGATAGTAAAGAAGGG

Reverse Primer	CACTTCTTTCCCTTTGTTGTTTGTGTATGA
T-S-16	GG AAA CTC TTA TCC CTA GCT CAG CAA GTC ATA CA
T-S-1	CG AAA CTC TTA TCC CAA GCT CAG CAA GTC ATA CA
T-S-5	GG AAT CTC TTA TCC CAA GCT CAG CAA GTC ATA CA
T-S-9	GG AAA CTC ATA TCC CAA GCT CAG CAA GTC ATA CA
T-S-13	GG AAA CTC TTA TGC CAA GCT CAG CAA GTC ATA CA
T-S-17	GG AAA CTC TTA TCC CAT GCT CAG CAA GTC ATA CA
T-S-21	GG AAA CTC TTA TCC CAA GCT GAG CAA GTC ATA CA
T-S-25	GG AAA CTC TTA TCC CAA GCT CAG CTA GTC ATA CA
T-S-29	GG AAA CTC TTA TCC CAA GCT CAG CAA GTG ATA CA
T-S-33	GG AAA CTC TTA TCC CAA GCT CAG CAA GTC ATA GA

Link-A	GCATTGACGTGAGCATGGGATAAGATGCAG
Link-G	GCATTGACGTGAGCGTGGGATAAGATGCAG
Link-C	GCATTGACGTGAGCCTGGGATAAGATGCAG
Link-GT	GCATTGACGTGAGCGTTGGATAAGATGCAG

Table S2. Nucleic acids used for H5N1 analysis.

Name	Sequences(5'to3')
Full-Target	ACCAG GGAAG GTCCT CCTTT TT TAGAA ATGTG GTATG GCTTA TCA AAAAGAACAA TGCATACCCA ACA ATAAAGA GAAGTTACAA
Full-Target-C	TTGTA ACTTC TCTTT ATTGT TGGGT ATGCA TTGTT CTTTT TGATAAGCCATACCACATTTCTAAAAAAGGAGGA CCTTCCCTGGT
Forward Primer	ACCAG GGAAGGTCCT CCTTTTTTAG AAATG
Reverse Primer	TTG TAA CTT CTC TTT ATT GTT GGG TAT
gDNA1	P-TTAAGCCATACCACAT
gDNA2	P-TGTATGCATTGTTCTT
Reporter	Cy5-CAGGACCAGTTCTTTTTGATAAGCCATATCCAAG-BHQ2
Target-C	TAGAA ATGTGGTATGGCTTAT CAAA AAGAACAATGCATACCCAACA
Target-G	TAGAA ATGTGGTATGGCTTAT GAAA AAGAACAATGCATACCCAACA

A	GGGTGGGTAAAGAACTGGTCCTG
B	CTTGGATATGGCTTATGGGTGGG
Link-GT	CAGGACCAGTTCTTTTGGTTAAGCCATATCCAAG
Link-TA	CAGGACCAGTTCTTTTTGATAAGCCATATCCAAG

Table S3. Nucleic acids used for FUT2 gene analysis.

Name	Sequences(5'to3')
rs601338-G	GGCTACCCCTGCTCCTGGACCTTCTACCACCACCT
rs601338-A	GGCTACCCCTGCTCCTAGACCTTCTACCACCACCT
gDNA1	TGGAGCAGGGGTAGCC
gDNA2	TGGTGGTGGTAGAAGG
A	GGGTGGGAACCTTCATACATC
B	CGACTCCCTGCTCTGGGTGGG
Link	GATGTATGAAGGTCCAGGAGCAGGGAGTCG
Link-T	GATGTATGAAGGTCCTGGAGCAGGGAGTCG
Link-AT	GATGTATGAAGGTACTGGAGCAGGGAGTCG
Link-ATT	GATGTATGAAGGTATTGGAGCAGGGAGTCG
Full-rs601338-G	GGGAGTACGTCCGCTTCACCGGCTACCCCTGCTCCTGGACCTTCTACCACCACCTCCGCCAGGAGATCCTCCAGGAGTTCACCCT
Full-rs601338-A	GGGAGTACGTCCGCTTCACCGGCTACCCCTGCTCCTAGACCTTCTACCACCACCTCCGCCAGGAGATCCTCCAGGAGTTCACCCT
Forward-Primer	GGGAGTACGTCCGCTTCACCGGCTAC

Reverse-Primer	AGGGTGAACTCCTGGAGGATCTCCTG
PCR-Full-rs601338-A	AGGAGGAATACCGCCACATCCCGGGGGAGTACGTCCGCTTCACCGGCTACCCCTGCTCCTAGACCTTCTACCACCACCTC
PCR-Forward-Primer	AGGAGGAATACCGCCACATC
PCR-Reverse-Primer-C	GAGGTGGTGGTAGAAGGGCC
PCR-Reverse-Primer-T	GAGGTGGTGGTAGAAGGGCT