VALIDATION REPORT FOR *MTHFR* USING LIFETECH QUANTSTUDIO

Pharmacogenomics Laboratory

Approvals:

Researcher:_____ Date:_____

Scientific Director: _____ Date:_____

Medical Director: _____ Date:_____

CLINICAL VALIDITY

This laboratory-developed test is intended to identify the c.677C>T and c.1298A>C variants in *MTHFR* from genomic DNA. Information about this variant may be used as an aid to clinicians.

The product monograph for norelgestromin and ethinyl estradiol (EVRA) states that the drug is contraindicated in women with Factor V Leiden mutation, antithrombin-III-deficiency, protein C deficiency, protein S deficiency, hyperhomocysteinemia due to mutations in the *MTHFR* gene and prothrombin mutation G20210A (among other contraindications), due to the risk for arterial or venous thrombosis.

Drugs (refer to https://www.pharmgkb.org/gene/PA245 for most up to date guidelines, accessed 15feb2015).

MTHFR lies at the intersection of the pathways for methylation and DNA synthesis. It catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the substrate for conversion of homeocysteine to methionine. Methionine is then converted to the universal methyl donor, S-adenosylmethionine (AdoMet, SAM) which is used for methylation of DNA and proteins. 5,10-methylenetetrahydrofolate is the substrate for de novo purine synthesis (DNPS).

The *MTHFR* gene is comprised of 11 exons with at least two splice variants, Gaughan et al, 2000; Tran et al, 2002. There are several documented variants in *MTHFR* (data are available on 65 variants at PharmGKB), with the majority of pharmacogenomic studies looking c.677C>T and c.1298A>C. Allele frequencies vary greatly between different racial and ethnic groups and there are over 20 haplotypes that are differentially represented in White (Caucasian), Black or African American (African American), Asian (Han Chinese-American) and Hispanic or Latino (Mexican American) populations, Martin et al, 2006.

Given the role of MTHFR in DNA synthesis, it is part of pathways that are acted on by several chemotherapeutic antineoplastic and antirheumatic drugs, such as methotrexate and 5-fluorouracil, although none act directly on the MTHFR protein, reviewed in Innocenti and Ratain, 2002 and Maring et al, 2005.

MTHFR is also of interest to the nutrigenomics community and there are many studies on the interactions between dietary folate, MTHFR variation and disease development. MTHFR: c.677C>T was the first reported risk factor for Neural Tube Defects, reviewed in van der Linden et al, 2006. There are many studies on cancer incidence and MTHFR, often with conflicting results, reviewed in Schwann and Rozen, 2001. Due to its relationship with homocysteine, there is also relevance for cardiovascular diseases. While severe deficiencies in MTHFR result in hyperhomocysteinurea, and mental retardation (OMIM: 607093), there is still some debate as to whether the common genetic variants are important risk factors for cardiovascular disease, reviewed in Lewis et al, 2005. ACMG guidelines state that there is no clinical utility of *MTHFR* testing in cardiovascular disease (Hickey et al., 2013).

Data regarding the *MTHFR* gene, its variants and its interaction with [folic acid|PA449692] are important from the ethical, legal and social aspects of folate supplementation; the decision in the USA to supplement and in Europe not to supplement. Folic acid is also one of the proposed components of the Polypill, a combination of cardio-beneficial medications, which may prove controversial given the recent conflicts of evidence.

Assay Principle

TaqMan® reagents consist of a pair of unlabeled PCR primers and a TaqMan® probe with a FAM™ or VIC® dye label on the 5' end and minor groove binder (MGB) and nonfluorescent quencher (NFQ) on the 3' end. TaqMan® probes are designed such that they anneal within a DNA region amplified by a specific set of primers. As the *Taq* polymerase extends the primer and synthesizes the nascent strand, the 5' to 3' exonuclease activity of the polymerase degrades the probe that has annealed to the template. Degradation of the probe releases the fluorophore from it and breaks the close proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore. The fluorescence detected in the quantitative PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR. Using TaqMan® probes for both the normal and variant allele enables genotyping (Table 1). Each variant can be tested individually or within a custom array.

Table 1. Variants in *MTHFR* Assay

Allele	variant	dbSNP	Effect on enzyme activity
	c.677C>T	rs1801133	Likely pathogenic
	c.1298A>C	rs1801131	Likely pathogenic

PURPOSE OF VALIDATION STUDY

The purpose of the validation study was to validate a new version of the open array using the LifeTech's QuantStudio 12 K Flex (software v1.2.2) for the performance of the c.677C>T and c.1298A>C variants in the *MTHFR* gene. A custom designed array (on an open array block) was used. Additional instruments and software validated include: Accufill open array (v1.1), Genotyper (v1.3), and Alleletyper (1.0).

METHODOLOGY

The validation experiments were set up both manually and using the Biomek 3000 for the custom array. Various samples types were previously validated for the open array and not included in this study. A total of 18 known DNAs were obtained from Coriell and one blood taken from a donor with known genotype (Table 2).

Table 2. Reference Materials

Reference Materials		
Lab Name	**Coriell DNA #**	**Genotype**
DNA 5	NA16688	C677T het
DNA 6	NA17084	A1298C hom

DNA 8	NA17246	neg
DNA 9	NA12244	A1298C het
DNA 15	NA17285	C677T het
DNA 17	NA09301	C677T/A1298C
DNA 20	NA02016	A1298C het
DNA 21	NA17280	A1298C hom
DNA 22	NA07439	neg
DNA 23	NA17114	A1298C het
DNA 24	NA17300	C677T/A1298C
DNA 27	NA17281	C677T/A1298C
DNA 29	NA10005	C677T het
DNA 30	NA17221	C677T het
DNA 39	NA17201	A1298C het
DNA 40	NA17210	A1298 hom
DNA 41	NA17057	C677T hom
DNA 44	NA17204	C677T/A1298C
XYZ	N/A	C677T/A1298C

RESULTS

Assay Results

The validation data gave consistent and accurate genotype calls.

Validation Samples

The validation samples consisted of 19 known samples: 18 genomic DNAs obtained from Coriell Cell Repository and one DNA extracted from a refrigerated whole blood collected in an EDTA (lavender top) tubes. A total of 260 samples with unknown genotypes were also validated. The unknown samples consisted of 34 genomic DNAs obtained from Coriell Cell repository, 121 genomic DNAs from the CDC GeT-RM project, DNA extracted from 103 refrigerated whole blood collected in EDTA (lavender top) tubes, and DNA extracted from 2 saliva (Oragene collection devices, DNA Genotek Inc, Kanata, Ontario, Canada). All samples were successfully genotyped. Therefore, this assay is sufficiently robust to accommodate a variety of DNA sample types.

Precision

Known and unknown samples were genotyped for inter-run and intra-run validation. A total of 19 samples of known genotypes and 105 samples of unknown genotypes were included in intra-run (within run) validation (See Tables 3 & 4). The 19 known samples and 93 unknowns were included in inter-run (between run) validation (see Tables 5 & 6).

Table 3: Intra (within Run) Variability for Known Samples

Samples	Run Results			Consistent
	Replicate 1	Replicate 2	Replicate 3	
EBM	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
DNA 5	C677T het	C677T het	C677T het	yes
DNA 6	A1298C hom	A1298C hom	A1298C hom	yes
DNA 8	neg	neg	neg	yes
DNA 9	A1298C het	A1298C het	A1298C het	yes
DNA 15	C677T het	C677T het	C677T het	yes
DNA 17	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
DNA 20	A1298C het	A1298C het	A1298C het	yes
DNA 21	A1298C hom	A1298C hom	A1298C hom	yes
DNA 22	neg	neg	neg	yes
DNA 23	A1298C het	A1298C het	A1298C het	yes
DNA 24	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
DNA 27	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
DNA 29	C677T het	C677T het	C677T het	yes
DNA 30	C677T het	C677T het	C677T het	yes
DNA 39	A1298C het	A1298C het	A1298C het	yes
DNA 40	A1298C hom	A1298C hom	A1298C hom	yes
DNA 41	C677T hom	C677T hom	C677T hom	yes
DNA 44	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes

Table 4: Intra (within run) Variability for Unknown Samples				
Samples	Run Results			Consistent
	Replicate 1	Replicate 2	Replicate 3	
DNA 34	A1298C Hom	A1298C Hom		yes
DNA 49	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
T102	A1298C het	A1298C het		yes
T103	C677T/A1298C	C677T/A1298C		yes
T105	neg	neg		yes
721	A1298C het	A1298C het		yes
759	C677T/A1298C	C677T/A1298C		yes
760	C677T/A1298C	C677T/A1298C		yes
761	A1298C het	A1298C het		yes
763	A1298C het	A1298C het	A1298C het	yes
767	A1298C het	A1298C het	A1298C het	yes
768	A1298 hom	A1298 hom	A1298 hom	yes
769	C677T het	C677T het	C677T het	yes
770	neg	neg	neg	yes
771	A1298C het	A1298C het	A1298C het	yes
772	A1298C hom	A1298C hom	A1298C hom	yes

773	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
774	A1298C het	A1298C het	A1298C het	yes
775	C677T het	C677T het	C677T het	yes
776	C677T het	C677T het	C677T het	yes
777	neg	neg	neg	yes
778	C677T het	C677T het	C677T het	yes
779	C677T het	C677T het	C677T het	yes
780	C677T /A1298C	C677T /A1298C	C677T /A1298C	yes
784	C677T het	C677T het	C677T het	yes
785	A1298C het	A1298C het	A1298C het	yes
786	A1298C hom	A1298C hom	A1298C hom	yes
787	A1298C het	A1298C het	A1298C het	yes
788	neg	neg	neg	yes
789	C677T het	C677T het	C677T het	yes
790	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
791	neg	neg	neg	yes
792	neg	neg	neg	yes
793	neg	neg	neg	yes
794	A1298C hom	A1298C hom	A1298C hom	yes
795	A1298C het	A1298C het	A1298C het	yes
796	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
797	neg	neg	neg	yes
798	neg	neg	neg	yes
799	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
800	neg	neg	neg	yes
801	C677T het	C677T het	C677T het	yes
802	C677T het	C677T het	C677T het	yes
803	neg	neg	neg	yes
804	A1298C het	A1298C het	A1298C het	yes
805	C677T hom	C677T hom	C677T hom	yes
806	C677T het	C677T het	C677T het	yes
807	A1298C het	A1298C het	A1298C het	yes
808	C677T het	C677T het	C677T het	yes
809	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
810	C677T het	C677T het	C677T het	yes
811	neg	neg	neg	yes
812	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
813	C677T het	C677T het	C677T het	yes
814	neg	neg	neg	yes
815	neg	neg	neg	yes
816	A1298C hom	A1298C hom	A1298C hom	yes

817	A1298C het	A1298C het	A1298C het	yes
818	C677T het	C677T het	C677T het	yes
819	A1298C het	A1298C het	A1298C het	yes
820	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
821	neg	neg	neg	yes
822	A1298C hom	A1298C hom	A1298C hom	yes
823	A1298C hom	A1298C hom	A1298C hom	yes
824	A1298C het	A1298C het	A1298C het	yes
825	A1298C het	A1298C het	A1298C het	yes
826	C677T het	C677T het	C677T het	yes
828	neg	neg	neg	yes
829	neg	neg	neg	yes
830	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
831	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
832	C677T het	C677T het	C677T het	yes
833	C677T het	C677T het	C677T het	yes
834	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
835	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
836	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
837	neg	neg	neg	yes
838	neg	neg	neg	yes
839	C677T het	C677T het	C677T het	yes
840	neg	neg	neg	yes
841	neg	neg	neg	yes
842	neg	neg	neg	yes
843	neg	neg	neg	yes
844	neg	neg	neg	yes
845	A1298C hom	A1298C hom	A1298C hom	yes
845	C677T het	C677T het	C677T het	yes
847	A1298C hom	A1298C hom	A1298C hom	yes
848	A1298C hom	A1298C hom	A1298C hom	yes
849	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
850	neg	neg	neg	yes
851	neg	neg	neg	yes
852	neg	neg	neg	yes
853	A1298C hom	A1298C hom	A1298C hom	yes
854	neg	neg	neg	yes
855	neg	neg	neg	yes
856	A1298C het	A1298C het	A1298C het	yes
857	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
858	neg	neg	neg	yes

859	A1298C het	A1298C het	A1298C het	yes
860	C677T het	C677T het	C677T het	yes
861	C677T het	C677T het	C677T het	yes
862	A1298C het	A1298C het	A1298C het	yes
863	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
864	A1298C het	A1298C het	A1298C het	yes
865	A1298C hom	A1298C hom	A1298C hom	yes

Table 5: Inter- Assay Verification - Knowns				
Sample	Run 1	Run 2	Run 3	Consistent
DNA 5	C677T het	C677T het	C677T het	yes
DNA 6	A1298C hom	A1298C hom	A1298C hom	yes
DNA 8	neg	neg	neg	yes
DNA 9	A1298C het	A1298C het	A1298C het	yes
DNA 15	C677T het	C677T het	C677T het	yes
DNA 17	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
DNA 20	A1298C het	A1298C het	A1298C het	yes
DNA 21	A1298C hom	A1298C hom	A1298C hom	yes
DNA 22	neg	neg	neg	yes
DNA 23	A1298C het	A1298C het	A1298C het	yes
DNA 24	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
DNA 27	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
DNA 29	C677T het	C677T het	C677T het	yes
DNA 30	C677T het	C677T het	C677T het	yes
DNA 39	A1298C het	A1298C het	A1298C het	yes
DNA 40	A1298C hom	A1298C hom	A1298C hom	yes
DNA 41	C677T hom	C677T hom	C677T hom	yes
DNA 44	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes

Table 6: Inter- Assay Verification - Unknowns				
Sample	Run 1	Run 2	Run 3	Consistent
DNA 1	C677T het	C677T het	C677T het	yes
DNA 2	C677T het	C677T het	C677T het	yes
DNA 3	C677T hom	C677T hom	C677T hom	yes
DNA 4	C677T het	C677T het	C677T het	yes
DNA 7	neg	neg	neg	yes
DNA 10	neg	neg	neg	yes
DNA 11	A1298C het	A1298C het	A1298C het	yes
DNA 12	C677T hom	C677T hom	C677T hom	yes
DNA 13	neg	neg	neg	yes
DNA 14	neg	neg	neg	yes

DNA 16	A1298C het	A1298C het	A1298C het	yes
DNA 18	C677T het	C677T het	C677T het	yes
DNA 19	neg	neg	neg	yes
DNA 25	C677T hom	C677T hom	C677T hom	yes
DNA 26	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
DNA 28	C677T het	C677T het	C677T het	yes
DNA 31	neg	neg	neg	yes
DNA 32	neg	neg	neg	yes
DNA 33	A1298C het	A1298C het	A1298C het	yes
DNA 34	A1298C hom	A1298C hom	A1298C hom	yes
DNA 35	C677T het	C677T het	C677T het	yes
DNA 36	A1298C het	A1298C het	A1298C het	yes
DNA 37	neg	neg	neg	yes
DNA 38	neg	neg	neg	yes
DNA 42	C677T hom	C677T hom	C677T hom	yes
DNA 43	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
DNA 45	C677T hom	C677T hom	C677T hom	yes
DNA 46	neg	neg	neg	yes
DNA 47	A1298C hom	A1298C hom	A1298C hom	yes
DNA 48	neg	neg	neg	yes
DNA 49	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
DNA 50	neg	neg	neg	yes
DNA 51	neg	neg	neg	yes
DNA 52	neg	neg	neg	yes
EBM	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
721	A1298C het	A1298C het	[redacted]	yes
762	C677T hom	C677T hom		yes
767	A1298C het	A1298C het		yes
768	A1298C hom	A1298C hom		yes
769	C677T het	C677T het		yes
789	C677T het	C677T het		yes
826	C677T het	C677T het		yes
831	C677T/A1298C	C677T/A1298C		yes
835	C677T/A1298C	C677T/A1298C		yes
T101	neg	neg	neg	yes
T102	A1298C het	A1298C het	A1298C het	yes
T103	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
T104	A1298C hom	A1298C hom	A1298C hom	yes
T105	neg	neg	neg	yes
T106	C677T hom	C677T hom	C677T hom	yes
T107	C677T hom	C677T hom	C677T hom	yes

T108	C677T het	C677T het	C677T het	yes
T109	neg	neg	neg	yes
T110	C677T hom	C677T hom	C677T hom	yes
T111	C677T het	C677T het	C677T het	yes
T112	C677T hom	C677T hom	C677T hom	yes
T113	A1298C hom	A1298C hom	A1298C hom	yes
T114	neg	neg	neg	yes
T115	C677T het	C677T het	C677T het	yes
T116	A1298C het	A1298C het	A1298C het	yes
T117	neg	neg	neg	yes
T118	A1298C het	A1298C het	A1298C het	yes
T119	neg	neg	neg	yes
T120	neg	neg	neg	yes
T121	neg	neg	neg	yes
T122	C677T het	C677T het	C677T het	yes
T123	C677T het	C677T het	C677T het	yes
T124	neg	neg	neg	yes
T125	neg	neg	neg	yes
T126	C677T het	C677T het	C677T het	yes
T127	C677T hom	C677T hom	C677T hom	yes
T128	C677T het	C677T het	C677T het	yes
T129	neg	neg	neg	yes
T130	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
T131	A1298C het	A1298C het	A1298C het	yes
T132	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
T133	A1298C hom	A1298C hom	A1298C hom	yes
T134	neg	neg	[redacted]	yes
T135	neg	neg	neg	yes
T136	neg	neg	neg	yes
T137	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
T138	neg	neg	neg	yes
T139	neg	neg	neg	yes
T140	neg	neg	neg	yes
T141	C677T het	C677T het	C677T het	yes
T142	C677T het	C677T het	C677T het	yes
T143	C677T/A1298C	C677T/A1298C	C677T/A1298C	yes
T144	A1298C het	A1298C het	A1298C het	yes
T145	neg	neg	neg	yes
T146	C677T/1298C	C677T/1298C	C677T/1298C	yes
T147	neg	neg	neg	yes
T148	C677T hom	C677T hom	C677T hom	yes

T149	A1298C het	A1298C het	A1298C het	yes
T150	A1298C het	A1298C het	A1298C het	yes

Accuracy

A total of 18 blinded DNA samples were obtained from the CDC GeT-RM project. The assay showed complete concordance with reported consensus results for the alleles tested (See Table 7), for 100% accuracy.

Table 7: Blinded Samples

Lab #	Expected	Observed	Consistent
DNA 5	C677T het	C677T het	yes
DNA 6	A1298C hom	A1298C hom	yes
DNA 8	neg	neg	yes
DNA 9	A1298C het	A1298C het	yes
DNA 15	C677T het	C677T het	yes
DNA 17	C677T/A1298C	C677T/A1298C	yes
DNA 20	A1298C het	A1298C het	yes
DNA 21	A1298C hom	A1298C hom	yes
DNA 22	neg	neg	yes
DNA 23	A1298C het	A1298C het	yes
DNA 24	C677T/A1298C	C677T/A1298C	yes
DNA 27	C677T/A1298C	C677T/A1298C	yes
DNA 29	C677T het	C677T het	yes
DNA 30	C677T het	C677T het	yes
DNA 39	A1298C het	A1298C het	yes
DNA 40	A1298 hom	A1298 hom	yes
DNA 41	C677T hom	C677T hom	yes
DNA 44	C677T/A1298C	C677T/A1298C	yes

Stability

Stability was previously validated for the open array and is documented below. For DNA extracted from saliva, product support materials indicate that saliva can be stored at room temperature for at least 5 years, and frozen (<-20°C) indefinitely. DNA was obtained from Coriell Cell Repositories. The oldest extraction date was in 1999. Thus, extracted DNA has a stability of at least 15 years. (Table 8)

Table 8. Stability

Room Temperature: (15º-30ºC)	Whole Blood: 1 week Saliva: 5 years
Refrigerated (2-10°C):	Whole Blood: 2 weeks Extracted DNA: 5 years

Frozen (< -15ºC):	Frozen whole blood: 4 weeks. Extracted DNA: 15 years Saliva: indefinitely

Robustness
Since currently there is only one instrument and one technologist, various runs were performed on different days. Where possible, various disposable supplies were used (eg, pipette tips, 0.2 mL tubes, 96-well plates, plate seals, caps).

Additionally, the limits of input DNA concentration were previously validated. The extracted DNAs ranged from 15.4-50.8ng/µL and were used undiluted. Results show that input DNA from 15.4-50.8ng/µL yield interpretable results.

It is evident that this assay is sufficiently robust to accommodate variations among input DNA and consumables.

Linearity
Not applicable

Reference Interval
Not applicable

Reportable Range
Not applicable

Analytical Sensitivity
The *MTHFR* genotype was known for 18 of the reference materials obtained from Coriell and one sample from an anonymous donor. From the expected genotype, the variant alleles was identified in 38 of 38 alleles, with no false negatives (note while c.677C>T and c.1298A>T can be in *cis*, they were assumed to be in *trans* for the calculations. Phasing is not known for the samples). The analytical sensitivity is 100% (95% CI; 87-100) (Table 9).

Table 9. Analytical Sensitivity and Analytical Specificity

		Reference standard is positive	Reference standard is negative
	Test is positive	26	0
	Test is negative	0	12
Enter the required confidence interval (eg, 95%) here:	95		
RESULT:			
Sensitivity:	1.0000	CI: 0.8713 to 1	
Specificity:	1.0000	CI: 0.7575 to 1	

Analytical Specificity
The *MTHFR* genotype was known for 18 of the reference materials obtained from Coriell and one sample from an anonymous donor. From the expected genotype, the normal/non-variant allele was identified in 12 of 12 alleles, with no false positive results (note while c.677C>T and c.1298A>T can be in *cis*, they were assumed to be in *trans* for the calculations. Phasing is not known for the samples). . The analytical specificity is 100% (95% CI; 76-100), (Table 9).

Interferences
Heparin is a known *Taq* polymerase inhibitor. Specimens collected in heparin (green-top tubes) were previously validated and did not have increased sample failure.

Carryover
Not applicable. Disposable filter tips were used throughout and no tip was used more than one time.

CONCLUSIONS
The validation experiments showed the Taqman® reagents utilizing the QuantStudio fulfill the requirements of a clinical assay from a variety of collected specimens, with ≥99% accuracy and precision. Repeat rate and signal intensity are appropriate for a clinical assay.