M-Test: Concept, Applications, and Frequently Asked Questions

Definition:
M-Test compares DNA methylation on a genome-wide scale to select DNA fragments that have the most consistent difference in methylation in Control and Test groups.

Strategy:
M-Test has the following components: 1. Genome-wide analysis of methylation; 2. Selection of fragments consistently methylated (or unmethylated) in Control group; 3. Selection of fragments consistently unmethylated (or methylated) in Test group; 4. Selection of fragments that are differentially methylated in Control and Test groups; and 5. Combining a desired number of such fragments into a composite biomarker.

Background:
Cell-free DNA (cfDNA) is present in the bloodstream of every mammal, including humans. cfDNA has variable size (~180 bp to >2kb), is present at different concentrations (~5 ng/ml to >1 mg/ml) and in different forms (e.g. complexed with proteins, naked, etc). cfDNA has a half-life of approximately 1-5 days (estimates vary) and is eliminated through kidneys. cfDNA contains methylated cytosines that are stable in the bloodstream.

DNA methylation is associated with cellular processes, including gene transcription and possibly chromatin packaging, replication, and repair. As such, methylation of cfDNA provides an opportunity to assess the status of the organism.

cfDNA can be generated through multiple mechanisms, including cell death. An alternative mechanism, horizontal transfer of genetic information aka genometastasis, is still controversial, albeit supported by indirect evidence (activity of naked DNA vaccines, binding of DNA by a receptor on the cell surface [TOLL9], and the receptor selectivity regarding methylated DNA).

Potential heterogeneity of cfDNA origins is a significant hindrance for its acceptance as a media for biomarker development by the scientific community.

Concept:
A direct approach to biomarker development (find the biomarker in tissue and test it in blood) was only partially successful (SEPT9) due to the incomplete correlation of informative DNA fragments in tissues and in blood (Melnikov et al, J Mol Diagn. 2009;11(1):60-5). A shift in thinking about blood was made: instead of considering blood as a depository of DNA from different organs, we took it as a separate tissue that contained information about the status of different organs in the body. This approach proved fruitful (see PubMed).

Technical overview:
M-Test has two parts: Discovery and Clinical.

M-Test Discovery consists of (a) cfDNA isolation; (b) pre-processing of sample DNA and its separation into TEST and CONTROL parts; (c) digestion of TEST part with a methylation-sensitive restriction enzyme (CONTROL part is incubated without the enzyme); (d) amplification of both parts (separately); (e) detection of methylated fragments using either microarray hybridization or NGS.

Data from each group of samples (disease and control; responders and non-responders; etc) is then processed to identify commonly methylated and unmethylated fragments. DNA fragments that are differentially methylated are selected for the composite biomarker.

M-Test Clinical consists of parts a-c of the Discovery phase, but does not have the amplification; detection is done by qPCR. The latter was selected to facilitate transfer of the assay to a clinical lab.

Proof-of-Principle Studies (summary):
Significant differences were observed in patients with
- breast cancer vs benign disease vs controls
- pancreatic cancer vs chronic pancreatitis vs controls
- ovarian cancer vs benign disease vs controls
- colon cancer vs controls
- lung cancer (ADC) vs lung cancer (SCC) vs controls
- multiple sclerosis (RRMS, remission) vs multiple sclerosis RRMS, exacerbation) vs controls
- ovarian cancer (Pt-Taxane resistant) vs ovarian cancer (Pt-Taxane sensitive)
- breast cancer (pre-treatment) vs breast cancer (post surgery) vs breast cancer (on hormonal therapy)
- RRMS (treatment naive, remission) vs RRMS (Avonex) vs RRMS (Copaxone) vs RRMS (Tysabri) vs controls

Conclusion:
Genome-wide analysis of cfDNA appears to be a valuable tool for selection of easily accessible biomarkers for differential diagnosis; for selection of patients responsive to a specific treatment; for monitoring their response to treatment in anticipation of emerging resistance.

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Frequently asked questions:

What are CpG sites? Where are they located?

CpG sites are dinucleotides CG or cytosine-guanosine designated CpG. In animals these sites can be epigenetically changed by addition of a methyl group to cytosine, which is then called “a methylated cytosine”. CpG sites exist throughout the genome, every 500-700 nucleotides on average, but in some regions the frequency of CpG sites can be very high. These regions are called “CpG islands”. CpG sites – and CpG Islands – are frequent at the beginning of the gene (CpG island of the promoter/first exon), where they participate in gene regulation.

M-Test identifies CpG that are different in disease and health, e.g. methylated in health and UNmethylated in disease. Such disease-specific CpG will serve as biomarkers for the disease.

Can biomarkers be made for any region of DNA?

Biomarkers can be designed for any section of DNA. Currently, we use a methylation-sensitive enzyme (discriminates between methylated and unmethylated cytosines) with GCGC recognition site. Other enzymes, including methylation-dependent ones, can be used as well.

How can you target and analyze specific regions with CpG?

We analyze specific CpG dinucleotides that are parts of the enzyme recognition site GCGC. If a DNA fragment contains UNmethylated site, it will be attacked, and "digested" (cut at the site), so its integrity will be destroyed, and amplification will not generate any product. If the site is methylated (meC), the enzyme will not cut, and amplification will be successful.

Do all DNA fragments contain these GC-rich regions?

Some of them do, others don't. Those that do not have the site for the enzyme will not be informative and will be excluded from analysis.

How do you select GC-rich regions for analysis?

We use genome-wide analysis to analyze every fragment in the genome. Then the most informative sites (20-30) are tested individually by PCR in individual samples and their informative value confirmed.

What arrays can be used?

We use 244k array produced by Agilent Technologies, Inc. For whole genome screening we can use NGS or Affymetrix Human Tiling arrays 2.0, which tests the whole genome at high resolution (35 bp). Importantly, these devices are used ONLY for selection of candidate DNA fragments for the biomarker. The final biomarker is tested in a simple qPCR-based assay.

What are the benefits of this technology for blood-based analysis?

The M-Test technology was developed for comprehensive analysis of DNA methylation in ultra-small samples. This is important because clinical samples contain little DNA and are very precious. For example, healthy people have only 5-6 ng of cfDNA per milliliter of plasma, so comprehensive analysis is difficult without the M-Test. When the biomarker has been developed only a few fragments are included, so a couple drops of blood will provide all DNA required for analysis.

Why can't you amplify DNA first and analyze its methylation after amplification?

Amplification will make more DNA, but this DNA will not be methylated, so all disease-specific methylation marks will be lost. To get information about disease-specific methylation the test has to use the original DNA from the patient and cannot use PCR product.