Optimal Detection of Fetal Chromosomal Abnormalities by Massively Parallel DNA Sequencing of Cell-Free Fetal DNA from Maternal Blood

Bob Barrett: This is the podcast from '*Clinical Chemistry*'. I'm Bob Barrett. The American College of Obstetrics and Gynecology supports the measurement of Nuchal Translucency and Surrogate Biochemical Markers in all pregnant women in the first trimester to assess the risk of Aneuploidy for Down syndrome. However, these tests can provide only an inconclusive risk determination. They have non-optimal detection and high false-positive rates.

Today, only invasive methods like Chorionic Villus Sampling, Amniocentesis or Chordiocentesis provide definite genetic information about the fetus, but these procedures are associated with risks to both mother and fetus. Therefore, a non-invasive means to obtain definite information on fetal chromosomal status is desirable.

Previous studies suggest counting chromosomes by mapping sequence tags as a potential quantification method for detecting Fetal Aneuploidy from Cell-free DNA obtained from maternal blood.

In these studies, massively parallel DNA sequencing of Cell-free DNA obtained from the maternal plasma yielded millions of short-sequenced tags that could be aligned and uniquely mapped to sides from a reference human genome. The depth of sequencing and subsequent counting statistics determine the sensitivity of detection for Fetal Aneuploidy.

In an article published in the July issue of '*Clinical Chemistry'*, Dr. Richard P. Rava, President and Co-Scientific Founder of Verinata Health and his team developed and tested an optimized algorithm for massively parallel sequencing data and demonstrated the potential universality of the Sequence Tag Mapping and Chromosome Quantification Method for the detection of multiple chromosomal abnormalities. Dr. Rava is our guest in this podcast.

Doctor, what's unique in the procedure described in your paper compared to other published work using massively parallel DNA sequencing for detecting Aneuploidy?

Dr. Richard P. Rava: Others have focused on the classification of Trisomy 21 samples and they've removed other Aneuploidies from their analysis. The algorithms in some of the other studies appeared to be unable to effectively detect Aneuploidies beyond Trisomy 21. Of course, in its commercial setting, one would receive samples from a broad spectrum of chromosomal abnormalities, and we were interested in demonstrating the universality of counting of chromosomes by mapping of all of the sequence tags across the genome.

Bob Barrett: What are your processes for sample collection and processing, and can you provide some insight into how it was designed?

Dr. Richard P. Rava: Well, we designed our process to fit easily into the workflow of the doctor. So as we discussed in our publication, we currently collect two tubes of blood in a standard ACD tube, and these tubes are shipped cold overnight to our laboratory in San Carlos. In this particular study, we collected over a thousand samples from 13 different U.S. clinical locations across the country. The whole blood is then processed here to Cell-free plasma, and the Cell-free DNA is extracted from the plasma.

In the publication we utilized the Illumina Genome Analyzer Instrumentation to actually sequence the samples.

Bob Barrett: You mentioned that intra-run and inter-run variability alters the chromosomal distribution of sequence reads for each sample. What are some of the potential causes of such variations among DNA sequencing runs?

Dr. Richard P. Rava: There are lot of factors that could cause small shifts in the chromosome distribution of the sequencing tags. Remember, we're measuring very small changes in these distributions in order to correctly classify samples where the fetal DNA represents an extra copy of a particular chromosome.

Some of the variability can come from the sample handling and/or the DNA extraction procedure itself. The sequence library appears to add additional variability. And finally, the sequencing itself can lead to small shifts in this distribution of tags.

The reproducibility of the massively parallel sequencing methods is really only optimal if these methods utilized can account for any of these random biases that are introduced in all the different steps.

Bob Barrett: Well, how do you go about correcting for the intra-run and inter-run variability sequencing runs?

Dr. Richard P. Rava: Well, we recognized early in our development that the sample itself actually can provide the best internal control to look for variation. So we looked for chromosomes that had similar shifts in the distribution of sequence tags to the chromosome of interest, for example, Chromosome 21 or 18.

Ultimately, after testing many different combinations, we chose a denominator or a normalizing chromosome which had the minimal variation for the different chromosome ratios among all of the unaffected samples in our training set, both within and between sequencing runs. And this paper really demonstrated that our algorithm effectively identified all the Trisomy 21 and Trisomy 18 fetuses in the study doing these corrections.

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Bob Barrett: Well you use a no call zone, how is that determine and what advantage does having a no call zone provide?

Dr. Richard P. Rava: We decided we really wanted to minimize the possibility of false positives or false negatives in our categorization, and we understood that the sensitivity of these methods that is using prenatal detection of Aneuploidy from plasma using sequencing was going to be limited only by counting statistics. So we chose really conservative boundaries in this study that took the counting statistics into account and would help minimize classification.

No call zone is an area where there is a very, very small but finite possibility that a sample could be misclassified.

In our study samples that were positive for Trisomy 21 or Trisomy 18 were substantially above this no call zone, and all of the Trisomy 21 and Trisomy 18 samples were correctly identified. We expect that we will refine our classification scheme based on the results of our ongoing validation study.

Bob Barrett: Well other studies have noted incoming samples, sometimes were not of sufficient quality for examination, did you have any quality control metrics in this study and did you have to reject any samples?

Dr. Richard P. Rava: All the 119 samples that we'd selected for this study we actually determined to have cell-free DNA of adequate quality prior to sequencing them on doing the analysis.

At the moment, we're currently developing and implementing rigorous design controls to ensure high-quality results in our commercial product. Our current process is very robust for sample quality and we observe very, very few sample rejections because of sample quality.

Bob Barrett: The newer generation of sequencing instruments have many more sequence tags available, how do you see utilizing this additional sequencing capacity?

Dr. Richard P. Rava: When we began this study almost two-and-a-half years ago, the sequencers at the time were only capable of about 2-3 million reads per lane. When we completed the work almost a year ago we were getting almost 25 million reads per lane, and today's generation of sequencers can yield almost 200 million reads per lane. All the samples in this paper will run in what's referred to as a Singleplex protocol, only one sample at a time.

But as we discussed earlier the only limitation for sensitivity using massively parallel sequencing is the actual number of unique sequenced tags that we use per sample.

So the additional sequencing capacity will allow us to actually multiplex samples in a lane, that is do more than one sample in a single lane, by using DNA indices to actually label the individual samples.

Multiplexing then reduces the cost per sample using the sequencing methods, and we're currently implementing these multiplexing for our samples in our ongoing study that we're carrying out right now.

Bob Barrett: What do you see is the advantages of your approach over other approaches for measuring non-invasive determination of Fetal Aneuploidy?

Dr. Richard P. Rava: Earlier approach is looking at cell-free DNA, it generally relied on either enriching the fetal DNA itself or specific alleles for measuring these copy number variations. Often these measurements themselves had more variation than the quantity you were trying to measure.

And in addition, whenever you utilize methods that measure specific alleles or and has to be concerned with the genetics of both different populations and/or private mutations that might alter the quantification method.

Finally, although, though the issues might be worked out for an individual aneuploidy, say Trisomy 21, one would need to start that process all over again, if you were going to add Aneuploidy into the test.

The method of using massively parallel sequencing of cell-free DNA to count chromosomes is really only limited by counting statistics and we're always measuring aneuploidies across the entire genome.

In fact, we showed that we observed a rare aneuploidy in Chromosome 9 in one sample and even we're able to detect a small deletion in Chromosome 11 in another sample, because we're always looking at all the chromosomes all the time. We were able to do this again, because we developed

an optimized algorithm that corrected for any of the variation in the sequencing run.

So if non-invasive prenatal diagnostics to become reality it will need to be able to provide information that's similar to a karyotype and really look across all of the chromosomes in the genome.

Bob Barrett: Finally doctor, what are the next steps to make such a test widely available?

Dr. Richard P. Rava: Larger clinical studies need to be carried out to demonstrate the universality of this approach, and we're currently undertaking a blinded clinical validation study to demonstrate the extensibility of the methods in this paper with samples from a broader set of sites, in fact, over 60 clinical sites right now. And so once this study is completed, we would expect that we could make this test available through our clear laboratory.

Bob Barrett: Dr. Richard P. Rava is the President and Co-Scientific Founder of Verinata Health. He has also been our guest in this podcast from '*Clinical Chemistry*'.

I am Bob Barrett. Thanks for listening!

Total Duration: 10 Minutes