

● SMFM Presentation Poster Winter 2007:

www.cffDNA.com

Non-invasive prenatal testing: Technical strategies to achieve testing of cell free fetal DNA (cffDNA) RHD genotype in a clinical lab

Stephen Brown M.D., University of Vermont; Leonard H Kellner M.S., Lenetix MSL Inc.; Marsha Munson B.S., Lenetix MSL, Inc.; Yanfeng Yang Ph.D., Sequenom, Inc.; Brandy Klotzle M.S., Sequenom, Inc.; Allan T Bombard M.D., Sharp Mary Birch Hospital, San Diego CA

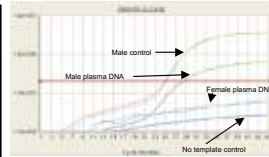
Overview:

Non-invasive determination of fetal Rh genotype based on cffDNA has been available for routine clinical use in Europe for several years; however, this testing has not been available in the US. In order to provide such testing in the US clinical market, it must be extensively validated. We set out to establish and validate non-invasive testing of fetal Rh genotype as a routine clinical test. This has proceeded as follows:

- 1) Establishment of rPCR assays for the detection of SRY and for RHD gene sequences using samples of banked blood.
- 2) Assay validation for use on cffDNA by making use of stored second trimester serum samples from pregnancies where fetal sex was known. These studies showed that correct determination of fetal sex was possible in those samples where sufficient DNA was present.
- 3) Prospective validation of assays on prospectively obtained samples from ongoing pregnancies.
- 4) Securing of New York State approval to offer routine clinical testing fetal Rh genotype. Non-invasive testing of fetal Rh genotype is now commercially available in the US.

Results: Assay Development

rPCR assays for SRY and for exons 4,5 7 and 10 of the RhD gene were based on previously described Taqman® assays. Importantly, the exon 4 and 5 assays were designed to detect the ψ allele that is common in people of African descent. These were optimized by testing a variety of primer and probe concentrations and PCR instruments. Serial dilutions of a standard DNA were used to demonstrate sufficient sensitivity to detect 1 genome equivalent of DNA. Samples of banked blood of known RhD serotype were then used to demonstrate that the correct RhD serotype could be predicted.



An example of rPCR results for the SRY locus. Shown are results from plasma DNA from pregnancies where the fetus was male and others where the fetus was female. In addition, there is a \square no template control \square and a male genomic DNA control.

Results: Stored Serum Samples

Stored second trimester serum samples were provided by Women and Infants' Hospital; Providence, RI. In a series of 78 samples of second trimester stored serum, 32/60 (53%) male samples were correctly identified and, importantly, 0 of 18 females were identified as male.

Following assay optimization, a second series of 50 paired serum and amniotic fluid samples was tested. 28/35 (80%) male samples were correctly identified and no females were identified as male. In those samples where the fetus was male, Rh genotype was consistent with the newborn serotype.

Conclusion: These results proved that when DNA was present, it could be amplified and that sex could be correctly determined.

Results: Prospectively Collected Samples

We obtained IRB approval to collect blood and AF samples from women who had elected to have second trimester amniocentesis. Plasma DNA was prepared within 24 hours of blood collection.

This work is ongoing, but demonstrates that in 39 of 40 samples, the sex was correctly obtained. Moreover, in all of these samples, the maternal Rh genotype was consistent with the serotype and the fetal Rh genotype from cffDNA was consistent with the fetal genotype as determined from the AF sample.

Conclusion: Fetal sex and Rh genotype can be determined from cffDNA.

Conclusions and Future Directions

- 1) Adequate efficiency, sensitivity and specificity for detection of fetal Rh genotype has been achieved.
- 2) NY State approval has been obtained; as a result, non-invasive testing of fetal Rh is now commercially available in the US.
- 3) We are developing methodology to improve detection of cffDNA when the fetus is an Rh negative female.

● Reference Material:

CIRCULATING FETAL NUCLEIC ACID RESEARCH

Y.M., LO. (2000). "Fetal DNA in maternal plasma: biology and diagnostic applications." *Clin Chem* 46:1903-6.

LO, Y.M.D., ZHANG, J., LEUNG, T.N., LAU, T.K., CHANG, A.M., HJELM, N.M. (1999). "Rapid clearance of fetal DNA from maternal plasma." *Am. J Hum Genet* 64:218-24.

LO, Y.M.D., TEIN, M.S., LAU, T.K., HAINES C.J., LEUNG, T.N., POON, P.M. (1998). "Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis." *Am. J Hum Genet* 62:768-75.

LO, Y.M.D., CORBETTA, N., CHAMBERLAIN, P.F., RAI, V., SARGENT, I.L., REDMAN, C.W. (1997). "Presence of fetal DNA in maternal plasma and serum." *Lancet* 350:485-7.

RHD AND GENDER RESEARCH

GEIFMAN-HOLTZMAN, O., GROTEGUT, C.A., GAUGHAN, J.P. (2006). "Diagnostic accuracy of noninvasive fetal Rh genotyping from maternal blood - A meta-analysis." *American Journal of Obstetrics and Gynecology* 195:1163-73

FINNING, K.M., MARTIN, P.G., SCOOHILL, P.W., AVANT, N.D. (2002). "Prediction of Fetal D Status from maternal plasma: introduction of a new noninvasive fetal RHD genotyping service." *Immunohematology* 42:1079-85

ZHONG, X.Y., HOLZGREVE, W., HAHN, S. (2001). "Risk free simultaneous prenatal identification of fetal rhesus D status and sex by multiplex Real-time PCR using cell free DNA in maternal plasma." *Swiss Med Wkly* 131:70-14

LO, Y.M.D., HJELM, N.M., FIDLER, C., SARGENT, I.L., MURPHY, M.F., CHAMBERLAIN, P.F., POON, P.M., REDMAN C.M., WAINSCOAT, J.S. (1998). "Prenatal diagnostics of fetal RhD status by molecular analysis of maternal plasma." *N. Engl J Med* 339:1734-8

TRISOMY RESEARCH

LO, Y.M.D., TSUI, N.B.Y., CHIU, R.W.K., LAU, T.K., LEUNG, T.N., HEUNG, M.S.M., GEROVASSILI, A., JIN, Y., NICOLAIDES, K.H., CANTOR, G. R., DING, C. (2007). "Plasma placental RNA allelic ratio permits noninvasive prenatal chromosomal aneuploidy detection." *Nature Medicine* 13(2):218-23

TONG, Y.K., DING, G., CHIU, R.W.K., GEROVASSILI, A., CHIM, S.S.C., LEUNG, T.Y., LEUNG, T.N., LAU, T.K., NICOLAIDES, K.H., LO, Y.M.D. (2006). "Noninvasive prenatal detection of fetal trisomy 18 by epigenetic allelic ratio analysis in maternal plasma: theoretical and empirical considerations." *Clin Chem* 52:2194-2202

RHD CLINICAL OVERVIEW

P.I. CERRATO (2006). "The battle against Rh disease is about to get a lot easier." *Contemporary OB/GYN*

WAGNER, F.F., AND W.A., FLEGEL (2004). "Review: The molecular basis of the Rh blood group phenotypes." *Immunohematology* 20:23-5

FINNING, K., MARTIN, P., SUMMERS, J., MASSEY, E., POOLE, G., DANIELS, G. (2008). "Effect of high throughput RHD typing of fetal DNA in maternal plasma on use of anti-RhD immunoglobulin in RhD negative pregnant women: prospective feasibility study." *British Medical Journal* -

LENETIX® Medical Screening Laboratory, Inc.

174 Mineola Blvd. • Suite #1 • Mineola, NY 11501

Tel: 516-320-6370 • Fax: 516-248-4436 • Email: testing@lenetix.com • website: www.lenetix.com

The promise of noninvasive RHD genotyping is here **now...**

A Positive Way to Avoid a Negative Outcome[®]

The Problem:

In the United States, there are approximately 400,000 pregnancies in Rh negative women every year and almost all of these could benefit from assessment of the RhD type of the fetus. Until now, this could only be determined by an invasive procedure, such as amniocentesis or CVS, both of which involve risk. Therefore, most Rh negative moms are currently managed without knowing the fetal Rh status, and are routinely treated with RhD immunoglobulin during pregnancy. This is relevant because RhD immunoglobulin is becoming increasingly expensive, and many women today seek to avoid unnecessary injections of human blood products.

The Solution:

LENETIX[®] Medical Screening Laboratory, Inc. is proud to offer an alternative, noninvasive way to assess the fetal RhD status. Using the SEQuReDx[™] technology, we can now determine the fetal RHD genotype from the Mom's blood. LENETIX[®] is approved by the New York Department of Health to perform noninvasive RHD genotyping in pregnancies of 15 weeks' gestational age or greater. Third trimester injections of RhD immunoglobulin are unnecessary in pregnancies in which the fetus is Rh negative. We estimate that approximately 38% of all Rh negative women may avoid third trimester RhD immunoglobulin treatment.

The Clinical Testing Protocol:

Using the SEQuReDx[™] technology, LENETIX[®] has developed a clinical testing protocol that greatly enhances the sensitivity of the assays used in RHD genotyping.

Cell free fetal DNA (cffDNA) is extracted from an aliquot of the patient's blood and evaluated for RHD genotype, as well as for fetal Y chromosome sequences and PSI (Ψ) allele. Each assay is performed in triplicate.

To provide further confidence in the result, cell free fetal DNA (cffDNA) is again extracted from a second aliquot of the patient's blood and the entire procedure is repeated.

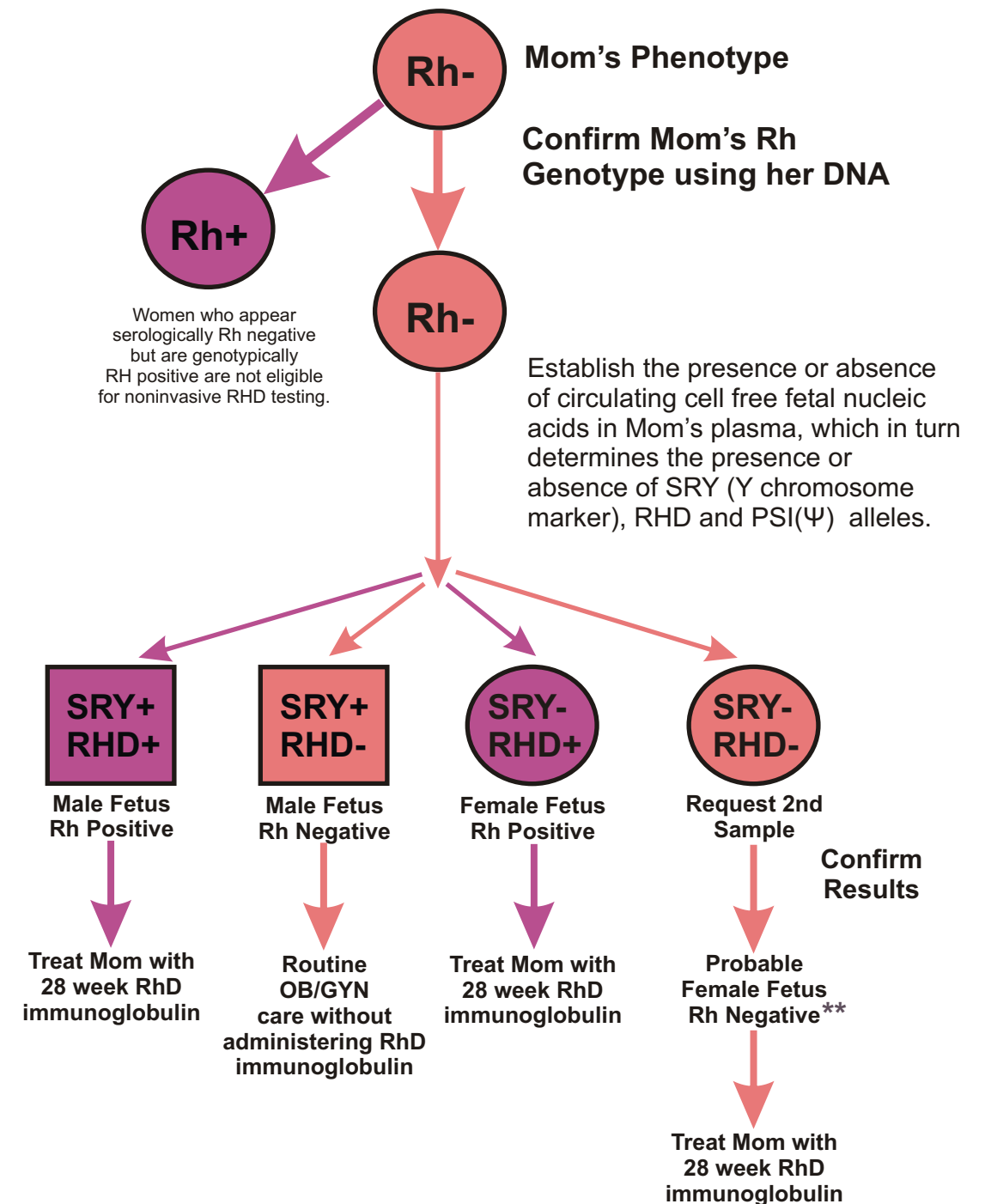
The Sample Submission Requirements:

- To be eligible for testing gestational age must be greater than or equal to 15 weeks
- Completed LENETIX[®] GENETIC Requisition must accompany the sample(s)
- Draw 3 PPT Tubes (White Top Tubes) and spin down, and draw 1 EDTA Tube (Lavender Top Tube)
- Call 516-320-6370 for pick-up and/or ship via FED-EX with LENETIX[®] mailing supplies.
- Store and/or ship at room temperature. **DO NOT ALLOW SPECIMEN TO FREEZE AT ANY TIME.**
- Collections of specimen and shipping **MUST** be done **Monday through Thursday.**

The Report :

The results will be faxed and mailed to your office within 3 to 5 business days of receipt of the sample. FINAL REPORTS ARE GENERATED, except in the case of the female fetus that is Rh negative. In this situation, a second maternal sample is requested (increasing gestational age correlates with increasing circulating cell free fetal nucleic acids), and the entire process is performed again to confirm the probable diagnosis of an Rh negative female fetus.**

USE OF NONINVASIVE RHD GENOTYPING FOR THE Rh NEGATIVE NON-SENSITIZED MOM*



SEQuReDx[™] is a trademark of Sequenom[®], Inc., used under license from Sequenom[®]. Use of the technology, covered by US Patent 6,258,540, is sublicensed to LENETIX[®] by Sequenom[®] Inc., the exclusive licensee. "This service is performed pursuant to an agreement with Roche Molecular Systems, Inc."

* RHD genotyping can also be useful for the Rh sensitized Mom. When the fetus is found to be Rh positive, then careful surveillance for the development of hemolytic disease is warranted, and if the fetus is Rh negative, then it is not in danger of developing hemolytic disease.

**Due to the nature of this testing, these patients will still require a 28 week RhD immunoglobulin shot. Additional markers are currently being evaluated that will allow for a more definitive diagnosis in this group of patients.