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Non-invasive prenatal detection of chromosome aneuploidies using next generation sequencing: First steps towards clinical application

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Prenatal Diagnosis and NGS - Why ?

Dennis Lo

- In 1997 Lo et al. reported that circulating cell-free fetal (ccff) DNA is present in the plasma of pregnant woman.
- ccff DNA can be detected as early as the 32nd day of gestation.
- ccff DNA ranges between 2% and 40% with a mean around 10% across varying gestational ages.

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Massively parallel genomic sequencing

PNAS

Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma

Rossa W. K. Chiu[a,b], K. C. Allen Chan[a,b], Yuan Gao[c,d], Virginia Y. M. Lau[a,b], Wenli Zheng[a,b], Tak Y. Leung[a], Chris H. F. Foo[f], Bin Xie[c], Nancy B. Y. Tsui[a,b], Fiona M. F. Lun[a,b], Benny C. Y. Zee[f], Tze K. Lau[e], Charles R. Cantor[g,1], and Y. M. Dennis Lo[a,b,1]

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Contributed by Charles R. Cantor, October 22, 2008 (sent for review September 29, 2008)

NAS

Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood

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Maternal Plasma DNA Analysis with Massively Parallel Sequencing by Ligation for Noninvasive Prenatal Diagnosis of Trisomy 21

Rossa W.K. Chiu,[1,2] Hao Sun,[1,2] Ranjit Akolekar,[3] Christopher Clouser,[4] Clarence Lee,[4] Kevin McKernan,[4] Daixing Zhou,[4] Kypros H. Nicolaides,[3] and Y.M. Dennis Lo[1,2]*

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From 2008 to 2010 three cohort studies have shown that MPGS of ccff-DNA can identify plasma samples from woman carrying an aneuploid fetus compared to samples from women with euploid fetuses.

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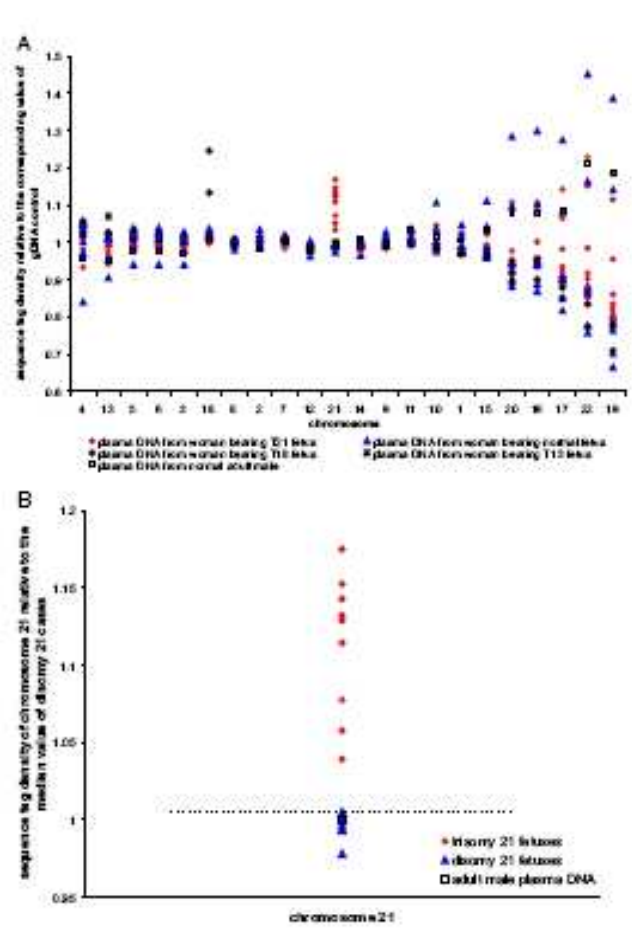

6/6 Euploid ; 9/9 Trisomy 21;

1/1 Trisomy 13; 2/2 Trisomy 18

Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma

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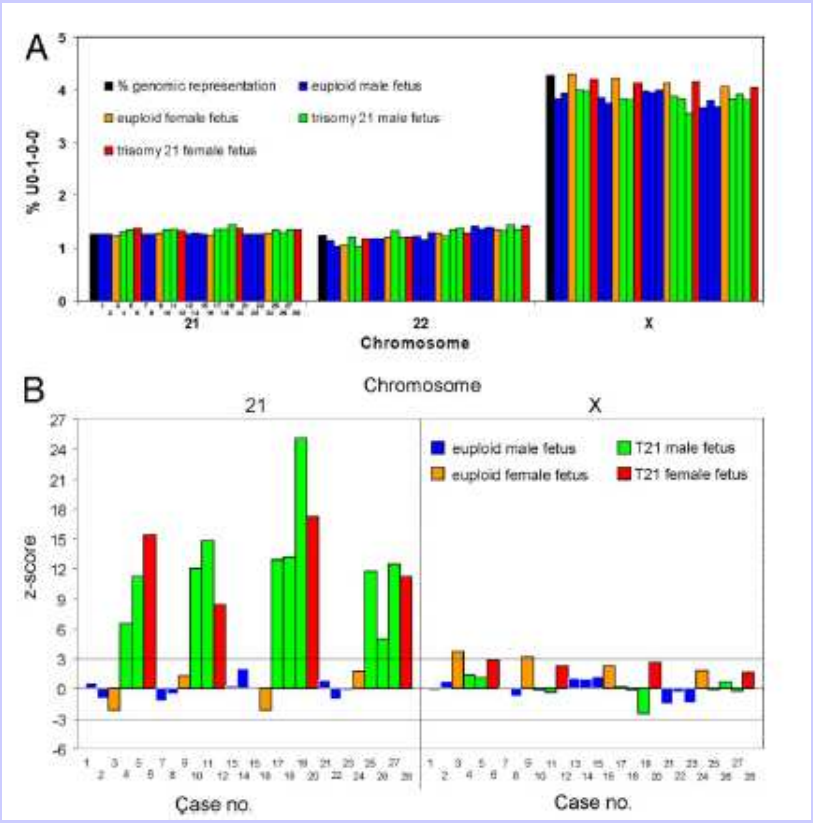

14/14 Euploid

14/14 Trisomy 21

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The Quantification Problem

- Only a small amount of serum DNA is of fetal offspring!
- If a maternal plasma probe contains e.g. 100 copies of chromosome 21 specific sequences, in an euploid pregnancy a maximum of 10 copies are fetal (90 maternal and 10 fetal)
- Accordingly, a trisomy 21 pregnancy contains 105 copies of chromosome 21 specific sequences (90 maternal and 15 fetal)
- In the maternal plasma of a trisomy 21 pregnancy, the amount of chromosome 21 specific DNA is increased only by the factor 1.05
- Can massively parallel genomic sequencing detect that minimal quantitative difference reliably?

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Proof of Principle Study

- Next Generation Sequencing (NGS) in Non Invasive Prenatal Diagnosis (NIPD)
- The diagnostic performance of NGS was compared to full conventional karyotyping
- Positive Vote from the Ethical Committee of the Ärztekammer Berlin in October 2009

- Cooperation: Zentrum für Pränataldiagnostik und Humangenetik Kudamm 199 in Berlin and LifeCodexx AG in Konstanz

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Study Group

- Patient recruitment:
  Zentrum für Pränataldiagnostik und Humangenetik Kudamm-199 Berlin
- Patients inclusion criteria:
  Singleton pregnancies in first and second trimester with clinical indications for chorionic villi sampling or amniocentesis. Patient informed consent was obtained for peripheral blood sampling and for the inclusion of karyotype results.

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Sample processing

- Peripheral venous blood samples were collected (5-10 ml EDTA blood) and plasma samples were harvested and frozen at -20°C
- Plasma samples were sent to LifeCodexx by overnight courier while kept frozen
- Cell free DNA isolation using QIAmp Circulating Nucleic Acid Kit

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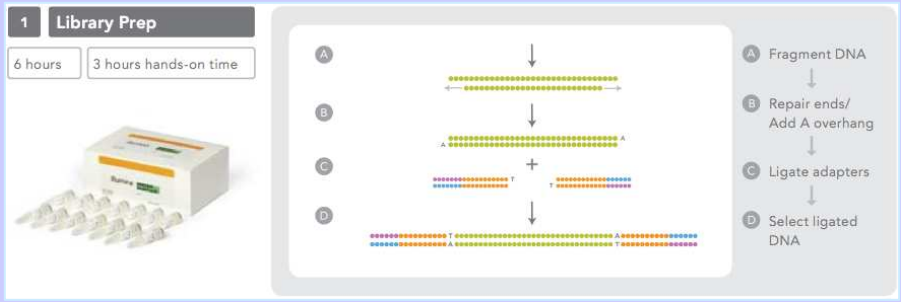
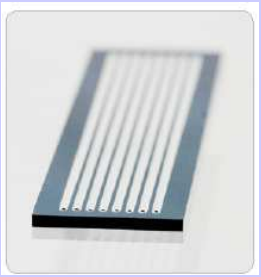
Library Preparation

- Library construction with Ilumina V2-adapters following the protocol for ChIP samples
- Libraries were amplified using a 12 cycle PCR step
- The adapter ligated fragments were size selected in the range of 150-300 bp
- Quality control and quantification using a High Sensitivity DNA Kit on the Agilent 2100 Bioanalyzer
- Molar quantification was done with the Qubit Fluorometer
- Libraries compatible with Paired-End (PE) sequencing were loaded for monoplex analysis on Illumina Paired End Flow Cells





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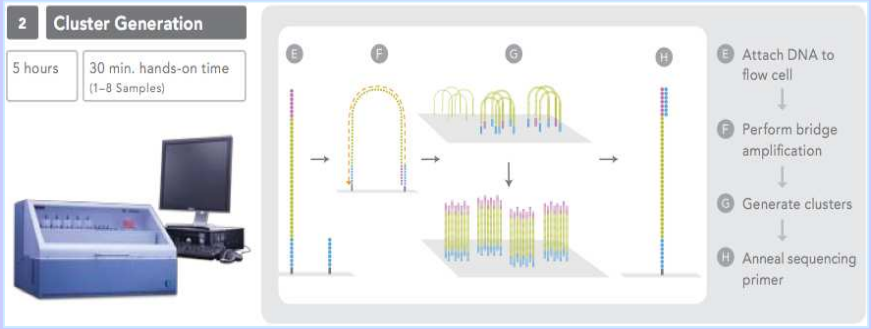
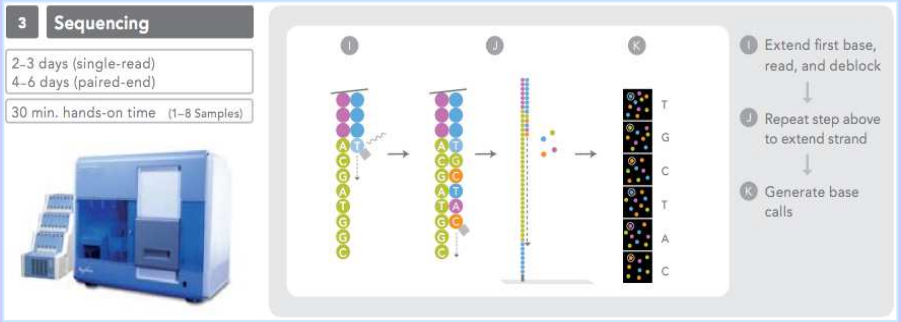
Cell free DNA sequencing

- Cluster Generation with “Bridge Amplification”
- Pyrosequencing on the Illumina Genome Analyzer IIx in single read mode
- Read length 36 bp





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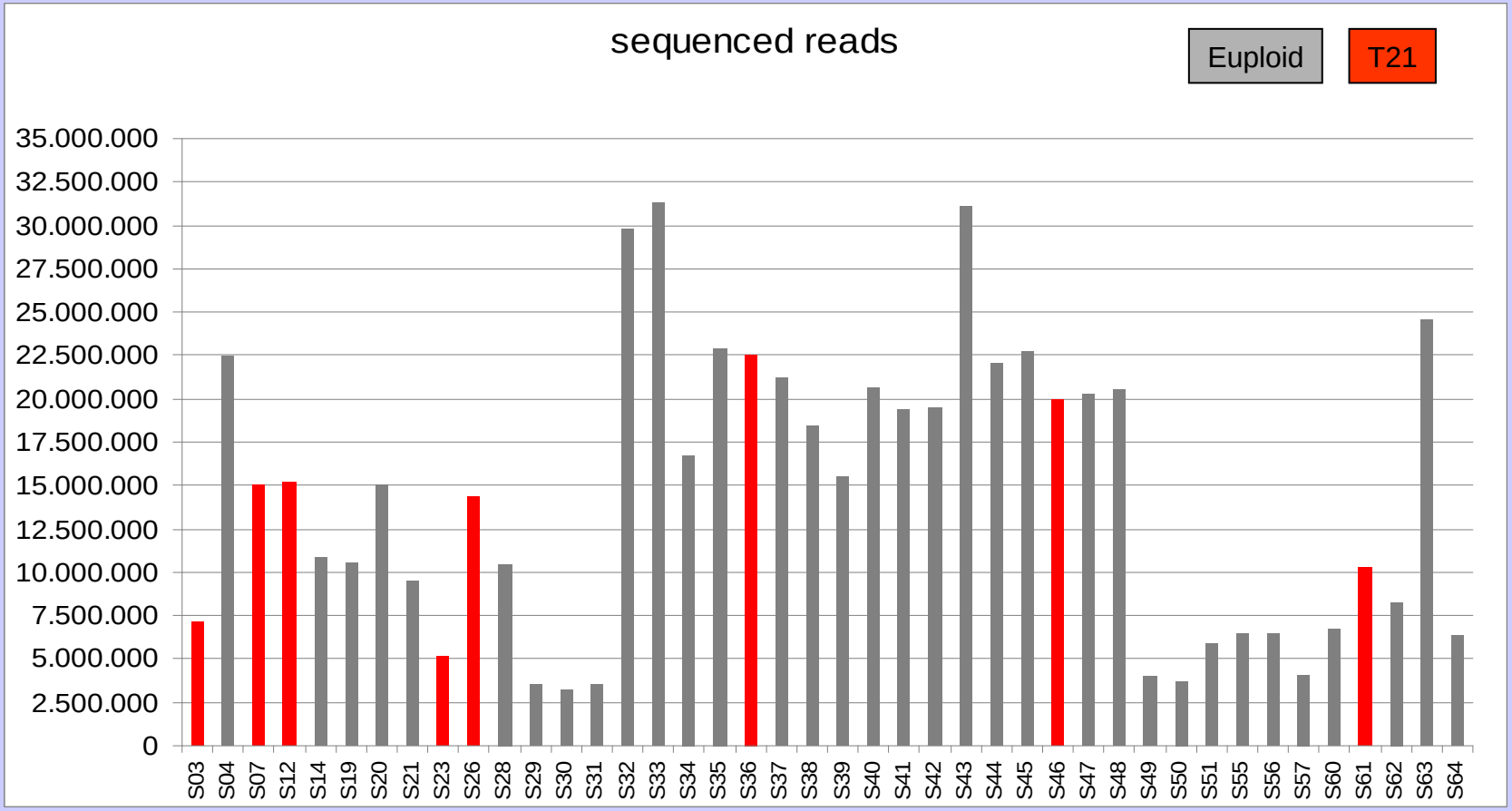
NGS-Results 1

42 samples were sequenced on the Genome Analyzer IIx



- **average of 14.461.145 reads**
- **average genome coverage: ~25%; depth 1**

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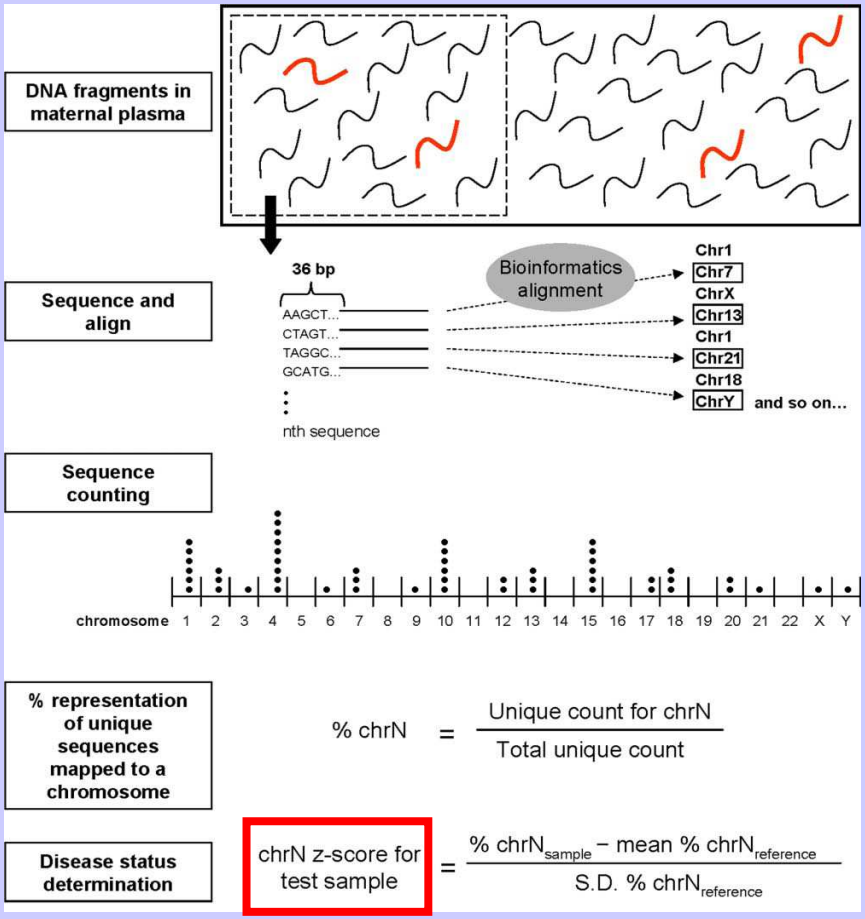
Sequencing data analysis



- Processing of the sequencing data with the use of a bio-informatics algorithm adapted from Chiu et al. (PNAS 2008)
- The first 32 bp of each read were aligned to the repeat-masked human genomic reference sequence NCBI build 36 (hg18)

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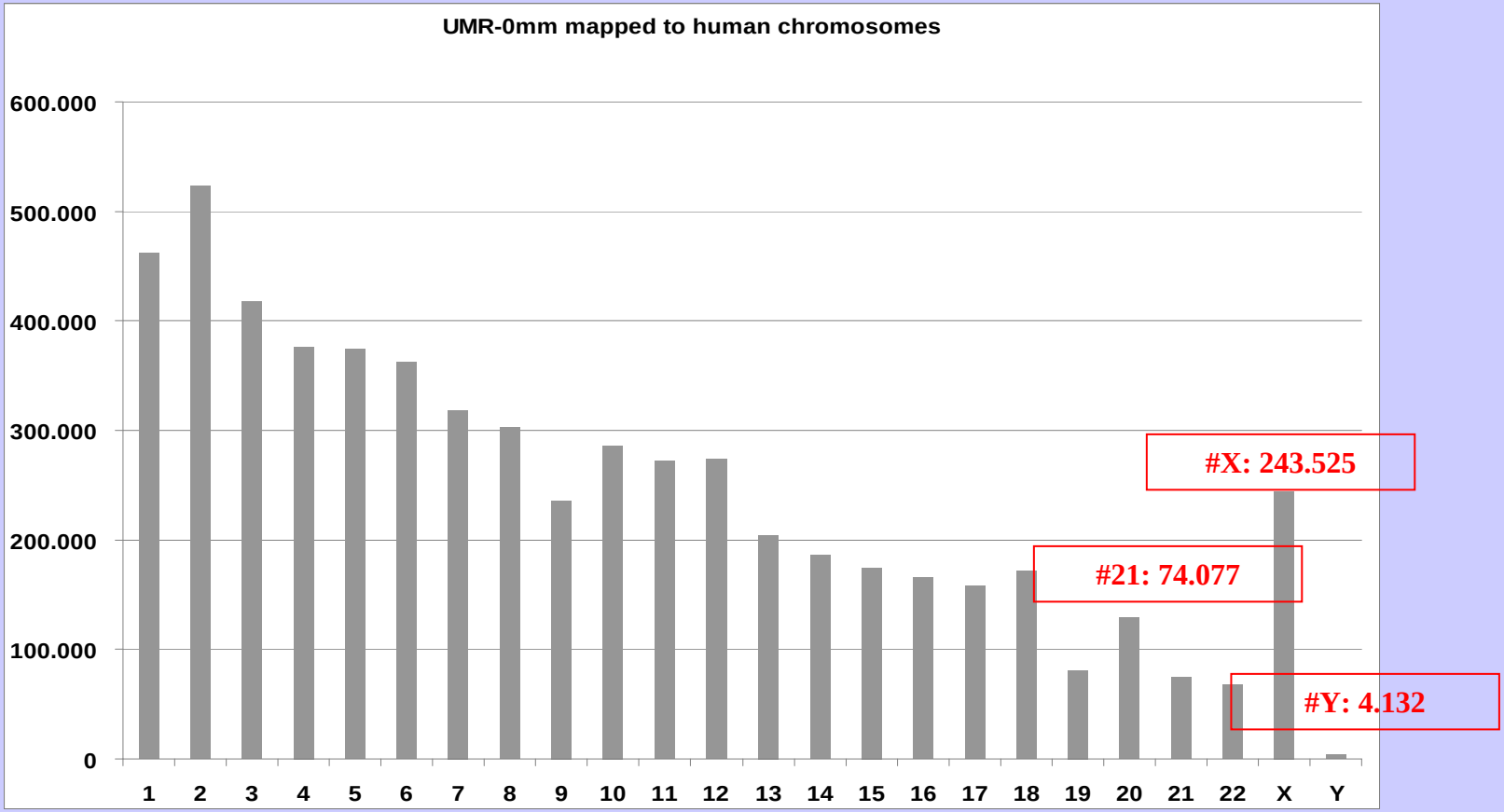
NGS-Results 2

average of 5.857.141 unique mapped reads without any mismatch (UMR-0mm)



Number of counted reads correlates to the size of the chromosomes

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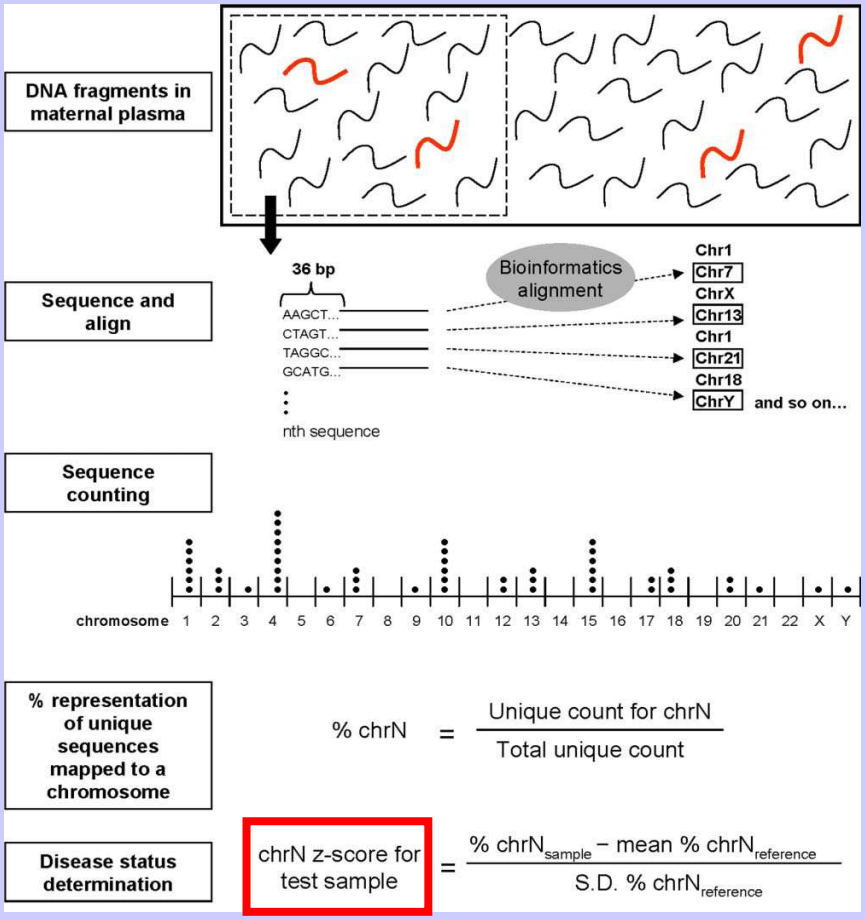
Sequencing data analysis



- Disease status determination by z-score calculation:

  1. the fractional genomic representation of a specific chromosome (% chrN) was determined by dividing the number of unique counts for that specific chromosome by the number of unique counts of all chromosomes

  2. % chrN was standardized by subtracting the mean % chrN of a control group and dividing by the SD of the same control group

- A z-score higher than >3 was used as cut-off value for aneuploid pregnancies

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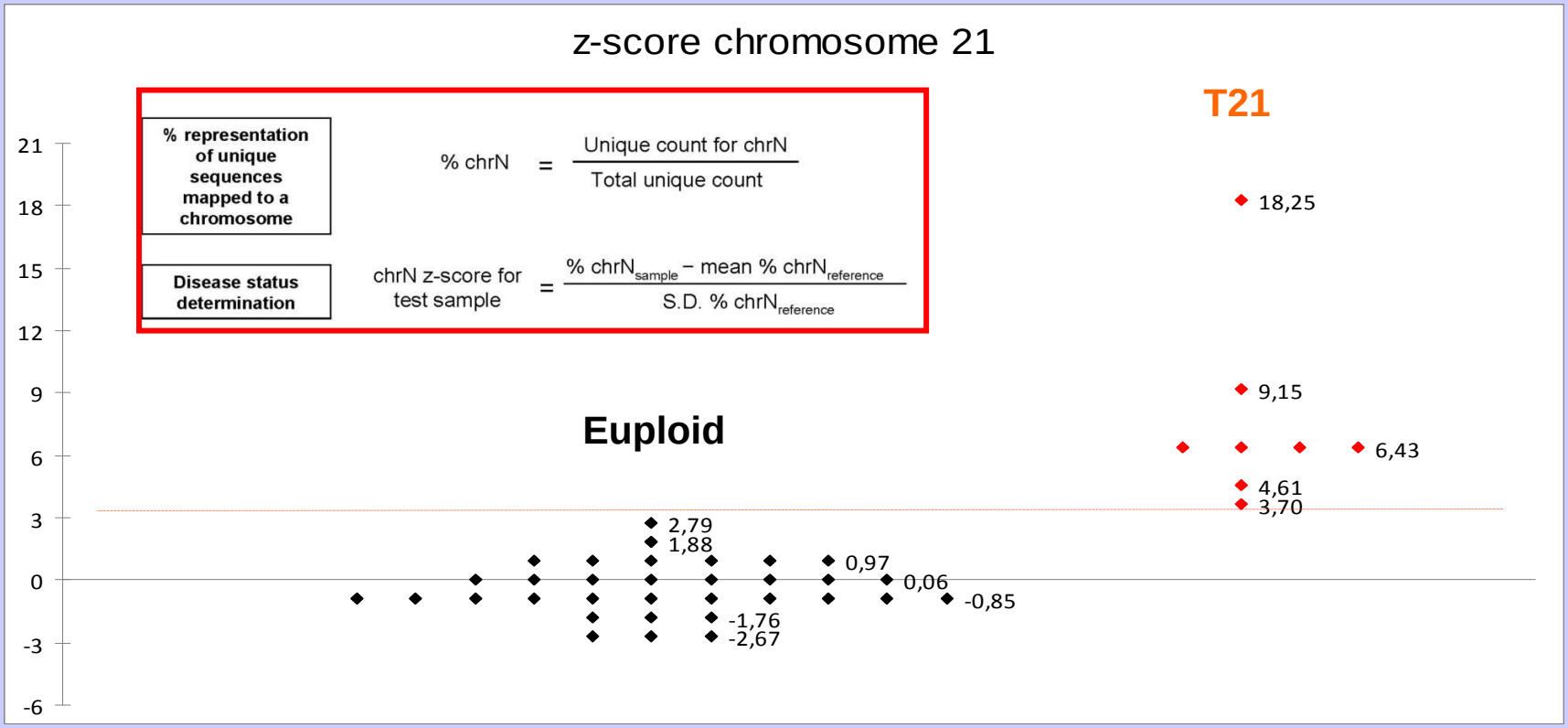
NGS-Results 3



All eight trisomy 21 cases were correctly identified with a z-score >3

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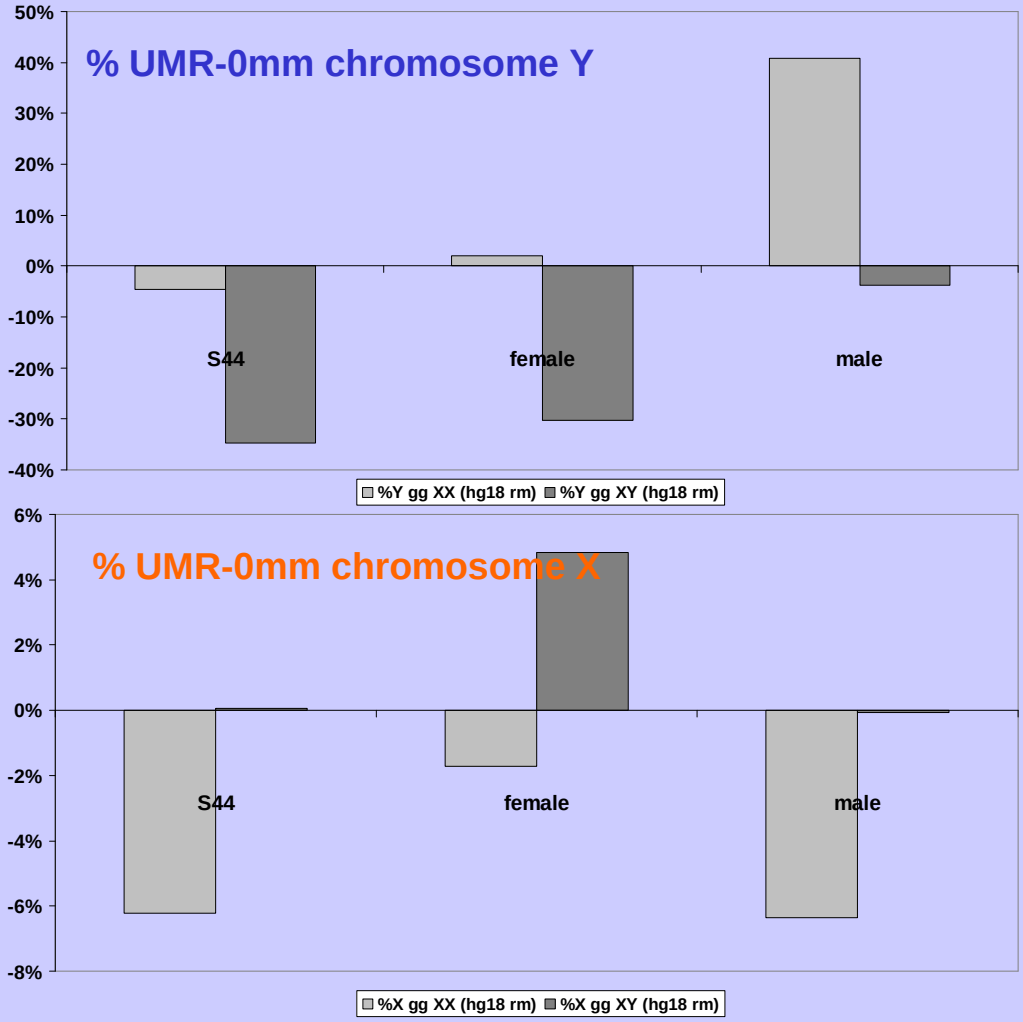
NGS-Results 4



- Comparing the fractional genomic representation of the gonosomes (% chrX and % chrY) allows also the detection of the missing X chromosome in a monosomy X pregnancy.
- % chrY comparable to a female pregnancy combined with a % chrX comparable to a male pregnancy = Monosomy X

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Summary

- 42 cell free serum DNA samples were analysed from singleton pregnancies in first and second trimester with clinical indications for chorionic villi sampling or amniocentesis.

- Eight cases with trisomy 21 cases and one case with monosomy X were detected by next generation sequencing and confirmed by conventional karyotyping.

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Further Improvements

- The detection of other aneuploidies, e.g. trisomy 13 and trisomy 18, is problematical:
  Change to the HiSeq 2000 system and enrichment strategies will increase the number of reads, additional improvements in bioinformatics will further increase the diagnostic sensitivity.
- The determination of the amount of fetal DNA in the maternal plasma sample is necessary for quality control:
  Combined methylation status- and SNP-analyses are in progress
- NGS is currently expensive (~1000€/ sample):
  Probe multiplexing (5x) on the HiSeq 2000 system and further automatic control will reduce the costs.

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Studies	Chiu et al. 2011	Ehrich et al. 2011	LifeCodexx
Patients	232	449	500
Sensitivity	100%	100%	aim: 100%
Specifity	97,9%	99,7%	aim: 99%
Inclusion criteria	increases risk patients phlebotomy prior invasiv procedure full karyotyping	increases risk patients phlebotomy prior invasiv procedure full Karyotyping; FISH, QF-PCR	increased risk patients phlebotomy prior invasive procedure full karyotyping
Sample collection	5-10 ml EDTA-blood 2-4 ml plasma	10 ml EDTA-blood >3,5 ml plasma	10 ml EDTA-blood >3,5 ml plasma
DNA extraction	Qiamp DSP DNA blood mini Kit	QIAmp circulating nucleic acid Kit	QIAmp circulating nucleic acid Kit
Sequencing	GAIIX; 2-plexing	GAIIx; 4-plexing	HiSeq 2000, 5-plexing
Bio-IT analysis	z-score: =/>3 - T21 hg18, repeat-masked U-0-1-0-0 with 0MM	z-score: =/>2,5 - T21 hg 19, nonrepeat-masked 1 MM	z-score: =/>3 - T21 hg18, repeat-masked UMR-0mm
References	82 male euploid	24 euploid	15+ euploid
QC	2,3 Mill. Reads DNA: QRT-PCR: ß-globin	3-5 Mill. reads DNA: fetal fractio: 3,9%	10 Mill. reads 90% UMR-0mm

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Multi-center study in Progress

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