Title

Non-invasive prenatal testing by low coverage genomic sequencing: Detection limits of screened chromosomal microdeletions

Authors

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What's already known about this topic?

Microdeletion detection accuracy, similarly to most common trisomies detection, was found to be dependent mostly on technical and biological parameters of the test and tested samples, such as coverage of target region, fetal fraction, size and positions of the deletions.

What does this study add?

Estimation of relevant regions for five chosen microdeletion syndromes. Confirmation and improvement upon previous methods. Systematic evaluation of sensitivity of microdeletion detection with read counts from 10M to 20M.

Abstract

Objective: To study the detection limits of chromosomal microaberrations in non-invasive prenatal testing with aim for five target microdeletion syndromes, including DiGeorge, Prader-Willi/Angelman, 1p36, Cri-Du-Chat, and Wolf-Hirschhorn syndromes.

Method: We used known cases of pathogenic deletions from ISCA database to specifically define regions critical for the target syndromes. Our approach to detect microdeletions, from whole genome sequencing data, is based on sample normalization and read counting for individual bins. We performed both an *in-silico* study using artificially created data sets and a laboratory test on mixed

DNA samples, with known microdeletions, to assess the sensitivity of prediction for varying fetal fractions, deletion lengths, and sequencing read counts.

Results: The *in-silico* study showed sensitivity of 79.3% for 10% fetal fraction with 20M read count, which further increased to 98.4% if we searched only for deletions longer than 3Mb. The test on laboratory-prepared mixed samples was in agreement with *in-silico* results, while we were able to correctly detect 24 out of 29 control samples.

Conclusion: Our results suggest that it is possible to incorporate microaberration detection into basic NIPT as part of the offered screening/diagnostics procedure, however, accuracy and reliability depends on several specific factors.

Key words: microdeletion syndromes, microdeletion detection, copy number variant detection, accuracy evaluation, low coverage, NIPT enhancement

Introduction

One of the most important milestones in the field of research and clinical use of circulating DNA was the discovery of circulating free fetal DNA (cffDNA) that forms part of the total circulating DNA in the blood of pregnant women. It directly implied the emergence of a new basic and applied research field of non-invasive prenatal testing (NIPT) and has also started high interest in the area of circulating nucleic acids research in general[1]. Recently different commercial companies, e.g. Sequence, BGI, Illumina, Natera, Roche, LifeCodexx, are utilizing results of this research in their services focused on NIPT of T13, T18, T21, and sex chromosome aneuploidies. Furthermore, they are broadening their product portfolio with tests for detection of most common chromosomal microdeletions. Tests of all mentioned companies can be used to identify pregnancies with high risk of DiGeorge syndrome and the first four mentioned all offer also 1p36 syndrome, Cri-Du-Chat syndrome, Prader-Willi/Angelman syndrome, and Wolf-Hirschhorn syndrome. However, validation of methods for detection of

microdeletions is very limited. While sensitivity and specificity of detection of most common trisomies and sex chromosomes aneuploidies were published in large meta-analyses[2-4], these important test parameters are still missing for detection of the above mentioned microdeletions, both in scientific publications and on web-pages of such tests providers. The cause for it is their very low prevalence and, as a consequence, very limited clinical validation data sets available. Therefore, currently available proof of principle studies focused on chromosomal microdeletions detection and corresponding validation studies through analyses of artificial data. These included samples prepared either by *in-silico* massively parallel sequencing data manipulation, or by mixing-up of normal and well defined microdeletion positive DNA samples with subsequent testing on few real clinical samples[5-6]. According to these studies, microdeletion detection sensitivity and specificity, similarly to most common trisomies detection, was found to be dependent at most on technical and biological parameters of the test and tested samples. As most prominent factors, coverage of target region, fetal fraction, size and position of the deletions were identified[6].

In comparison to previously published studies, we tested detection limits for different sizes and positions of known microdeletions causing clinically relevant syndromes. We focused on estimation of the sensitivity of detection in artificially prepared data, mimicking the sizes and positions of pathogenic deletions from the public database of the International Standards for Cytogenomic Arrays (ISCA) Consortium[7], after identification of syndrome specific critical regions (of sizes from 0.9 Mb to 21 Mb) in pooled database data. The experimental part of the study was split into two parts. In the first experiment, analyses were performed on data prepared by artificially “spiking in” reads into sequencing data from physiological pregnancies. In the second experiment, analyses were performed in a blinded manner on artificially prepared sample mix-ups, with defined proportions of normal and well-defined microdeletions containing control DNA, mimicking different fetal fractions from 5% to 20%.

Material and Methods

Retrieval of pathogenic regions

The ISCA database[7] was searched for deletions located in genomic regions associated with five selected microdeletion syndromes (22q11.2 – DiGeorge syndrome, 4p16.3 – Wolf-Hirschhorn syndrome, 15q11 – Angelman/Prader-Willi syndrome, 5p15 – Cri-Du-Chat syndrome, 1p36 – 1p36 deletion syndrome), using the hg19/GRCh37 version of the human reference genome. Coverage was calculated as the number of deletions which included the given nucleotide position, separately for pathogenic (or likely pathogenic) and benign (or likely benign) deletions.

Initially, coverage of 45 pathogenic deletions and higher was used as a cut-off to specify boundaries of the pathogenic regions. Afterwards, the regions were checked for outliers (pathogenic deletions located entirely outside the given region), and the coverage cut-off was gradually decreased until the pathogenic region was defined in such a way, that there were no outliers (in the case of Wolf-Hirschhorn, Angelman / Prader-Willi, and Cri-Du-Chat syndromes), or that the outliers were considered not relevant (in the case of DiGeorge and 1p36 deletion syndromes). The pathogenic region for Wolf-Hirschhorn syndrome defined by coverage cut-off 45 was the only one which did not have any outliers initially (Supplementary Figure 3). The region for DiGeorge syndrome defined by the same cut-off had a considerable amount of outliers located towards the 3'-end, but these corresponded to another well described syndrome, known as 22q11.2 distal deletion syndrome[1]. Therefore, no further adjustment of the region boundaries were needed. In order to address the phenomenon of outliers, the coverage cut-off was reduced to 25 for Angelman/Prader-Willi syndrome, to 28 for Cri-Du-Chat syndrome, and to 3 for 1p36 deletion syndrome. There were still considerable numbers of overhangs from the defined pathogenic regions (pathogenic deletions spanning outside of the regions), but as long as there were no pathogenic deletions located entirely outside of them, it was assumed that the defined regions cover genes responsible for the syndromes. The two outliers found in the 1p36 genomic region (with positions chr1:27,133,503-28,011,702 and chr1:27,927,633-28,215,952 on hg19 human genome assembly) were considered a distinct entity from the deletions responsible for the 1p36 deletion

syndrome and were thus excluded from the analysis.

Three pathogenic deletions in the 22q11.2 genomic region had overlap with DiGeorge syndrome critical region smaller or equal to only 20kb, and in all three cases the overlap was located on the 3'-end of the critical region. These were assumed to be related to the 22q11.2 distal deletion syndrome and were excluded from further analysis.

The final set of the microdeletions are summarized in Table 1. In total, we used 533 microdeletions for evaluation of our methods.

Table 1.

Preparation of artificial NIPT data sets

Sequencing data from healthy NIPT samples can be used for identifying the limits of microaberration detection. Reads of such samples are binned into equal size bins according to read start. We used bin size of 20kb, although bin size of 50kb was reported to be used in previous study[6].

Artificial data sets mimicking aberrated samples were created from data sets belonging to healthy samples by multiplication of bins corresponding to the pathogenic regions. The multiplication coefficients were guided by a target fetal fraction. Specifically, for a target fetal fraction ff, all bins between the start and the end of the simulated pathogenic region were multiplied with $(1-ff/2)$ for simulation of a chromosomal microdeletion or with $(1+ff/2)$ for a chromosomal microduplication.

We created an artificially aberrated data set for each combination of NIPT data set and pathogenic region (identified in previous chapter) with either varying fetal fraction ratio in [0.05, 0.075, 0.1, 0.125, 0.15, 0.175, 0.2] or varying read count from 5M to 20M with a step of 1M. All artificial data sets were evaluated by the same algorithm described below.

Identification of microaberrations

To identify microaberrations, we employed an approach similar to that described by Zhao et al.[6]. Briefly, we normalized the bin counts and then used the circular binary segmentation algorithm on these bin counts to identify consistent segments of same coverage.

Our approach introduced few steps in normalization routine and bin filtering to improve the accuracy and consistency of prediction. Identified segments were then evaluated using an in-house rule to determine significance. Significant deviations are visualized for individual chromosomes (Figure 1) and for the target syndromes.

Figure 1.

Normalization and filtering

Firstly, we normalized the bins by a two step procedure:

1. LOESS based GC correction for 20kb bins similar to the one described by Alkan et al.[8]
2. PCA normalization to remove higher-order population artifacts on autosomal chromosomes

We used a set of 341 samples for training of PCA normalization. All of the samples were downsampled to 10M reads, binned to 20kb bins, and were considered genetically healthy. Sample bin counts were transformed into principal space and first 15 principal components were stored. The first principal components represent common noise in euploid samples, so to normalize a sample, bin counts corresponding to these 15 first principal components were removed.

Then, only bins that have healthy characteristics were retained – not very low mean and not very high variance. If the read coverage would be uniform, there would be around 7 reads per bin when the sample is normalized to 1M reads. We filtered out bins that had mean read count less than 3.0 (less than half of the ideal mean) or variance higher than 1.5. These numbers were selected by hand to ensure that problematic bins would be filtered out, but we still keep at least 90% of bins.

Finally, a per-bin mean read count was subtracted to obtain data normalized around zero. The mean read counts were trained on a cohort of 1779 healthy samples that have at least 7M reads. Normalization and filtering step is independent of a target syndrome.

Segment identification and CNV calling

We used circular binary segmentation (CBS) algorithm provided by the R package "DNAcopy"[9] to identify same-coverage segments. CBS partitions a chromosome into regions with equal copy numbers, therefore it can detect the change point quite precisely. However, this algorithm overly partitions a chromosome. Thus, we used a simple rule to determine the significance of a segment. In an ideal case, the deletion/duplication of fetal chromosome will mean a decrease/increase by a factor $mb*ff/2$, where ff is the fetal fraction and mb is mean bin count. Since this is a crude simplification, we mark as significant all segments that overstep 60% of this theoretical increase/decrease. This percentage can be varied and represents a trade-off between sensitivity and specificity of prediction. The segment was categorized as maternal aberration if it oversteped 60% of maternal level $mb*(1-ff)/2$. When the fetal fraction is close to 50%, we can not determine is we are dealing with the maternal or fetal aberration, but these fetal fractions are extremely rare. Furthermore, this approach is in theory able to distinguish between maternal and fetomaternal variants, but the distinction is very unreliable due to focus on fetal variants. Thus, in further text we label both fetomaternal and maternal aberrations as simply "maternal". The minimal length of segment categorized as significant was set to 400,000 bases for maternal and 600,000 bases for fetal detections.

Preparation of control DNA sample mixes

The control DNA sample mixes were prepared as a mixup of healthy female plasma DNA and affected male DNA with confirmed microdeletion syndrome with different ratios to simulate different fetal

fraction. This mixup was then sequenced and analysed. In more details:

Genomic DNA from clinically affected male probands with confirmed microdeletion syndrome were acquired from Coriell Repository biobank (Supplementary Table 1). Additional two anonymized male DiGeorge samples were donated from the Clinic of Genetics at the University Hospital in Bratislava. In each relevant case written informed consents consistent with the Helsinki declaration were obtained. In addition, genomic male DNA was fragmented with dsDNA shearase according to manufacturer's protocol in order to reach fragments less than 500bp of size. Plasma DNA was received from non-pregnant female volunteers to use as "maternal" part of the artificial mixup. Sequencing libraries were then prepared and quantified both from non-pregnant plasma and sheared genomic DNA individually, with a PCR-free modification of our previously described method[10]. Libraries were prepared using the TruSeq Nano DNA kit (Illumina).

Affected male genomic libraries were then mixed with female healthy plasma libraries to create artificial libraries aiming for different fetal fractions between 5% and 20%. Massively parallel sequencing on NextSeq (Illumina) platform was then performed targeted to 20M uniquely mapped pair-end reads (2x35bp) per sample. This number of reads (instead of more often used 10M) was selected to improve sensitivity, while keeping sequencing costs reasonable. The fetal fraction was measured according to reads mapped on Y chromosomes. Figure 8 and Supplementary Table 1 summarize the control DNA sample mixes.

Results

<u>Identification of critical regions of chromosomal microdeletion associated syndromes</u>

For the five selected syndromes we identified 570 records corresponding to samples with pathogenic phenotypes and 245 records for benign phenotypes in the ISCA database. These records were used for determination of critical regions. For further analyses, we filtered out outliers and kept 533 regions corresponding to the five selected syndromes. Coverage plots, results of determination of sizes, and

positions of all microdeletions associated with syndromes of interest are summarized in Table 2 and visualized in Figures 2-3 and Supplementary Figures 1-3. Comparison of studied syndromes in ISCA and DECIPHER databases[14] at the time of our study is available in Supplementary Table 2.

Figure 2.

Figure 3.

Table 2.

Analyses of *in-silico* prepared artificial data

To test the sensitivity of detection, we tested the algorithm on 200 different NIPT data sets and on 533 pathogenic regions from the ISCA database. In the first analysis, we kept the read count fixed to 20 millions (20M) and the fetal fraction varied from 5% to 20% with a step of 2.5%. The second analysis fixed the fetal fraction to 10% and varied the read count from 5M to 20M with a step of 1M.

Detection accuracy for different fetal fractions

Fetal fraction and size of the deletion were previously reported to be the most crucial factors in detection of chromosomal microdeletions[6]. Sensitivity calculations for different fetal fractions and sizes of microdeletions at fixed read count to 20M reads per sample were performed and estimated sensitivity can be seen in Figure 4. Figures for particular syndromes and for 10M reads can be found in the Supplementary material.

Out of the 746,200 (533x200x7) carried out simulations, the simulated syndrome was correctly predicted in 571,255 cases (sensitivity = 76.6%). Furthermore, this sensitivity increased to 99.6% if the fetal fraction was at least 10% and the size of the deletion was at least 3Mb. Very poor sensitivity in the

0M-1M range is caused by our strict filtering of detections with small sizes (more info in Materials and Methods), which, as a trade-off, increases specificity.

In Supplementary Figures 4-9, we report sensitivity for a case, when critical regions are enlarged by 2Mb on both sides. However, there is no significant increase of sensitivity.

Figure 4.

Figure 5.

Detection accuracy for different read counts

Next analysis focused on estimation of sensitivity of microdeletion detection at different levels of read counts per sample. Fetal fraction was fixed to 10% in this case. Results from calculations for read counts ranging from 5M to 20M of reads per sample are presented in Figure 6 and 7.

Out of the 1,705,600 (533x200x16) carried out simulations, the simulated syndrome was correctly predicted in 937,335 cases (sensitivity = 55.0%). Furthermore, this sensitivity increased to 97.1% if the read count was at least 15M and the size of the deletion was at least 3Mb. Figures for particular syndromes can be found in the Supplementary material.

Figure 6.

Figure 7.

Validation on control samples

Out of 29 tested DNA samples, created by mixing-up of DNA samples from physiological pregnancies and microdeletion positive control samples, we were able to correctly identify 24 (Figure 8 and

Supplementary Table 1). All 5 unidentified samples had DiGeorge syndrome, with fetal fraction below 10%, and size of the deletion below 3Mb. Surprisingly, the read count seems to have minimal effect on prediction accuracy, however, we cannot rule out the possibility that limitations of data points skewed this finding.

Figure 8.

Discussion

Following confirmation of the presence of cffDNA in maternal blood and its use for fetal sex determination[15] first NIPT applications for common aneuploidies were introduced[16] and quickly becoming widely used in prenatal care worldwide[17]. According to the updated statement of the American College of Medical Genetics and Genomics[11] there is strong evidence, that NIPT can replace conventional screening for Down, Edwards, and Patau syndrome as it can be performed from 9th gestational week. Moreover, the use of whole genome sequencing based tests, in principle, allows detection of a wider range of chromosomal aberrations. In line with this, studies from 2015 using SNP based whole genome scans[5] or low coverage whole genome sequencing (0.2x coverage)[6], suggested high sensitivities for detection of the five most frequent microdeletion syndromes, the same set that was used in our work too. These studies had, however, limited means to validate their performance, since real samples with invasively or postpartum confirmed results of NIPT detected microaberrations are available in very limited numbers. Therefore, mostly artificially prepared data or DNA sample mix-ups are generally used for proof of principle pilot studies. Both scenarios were tested in our study yielding comparable results. However, it should be noted that some characteristics of NIPT samples are not realistically represented in sample mixups o in-silico samples, for example fragmentation patterns[18]. We carried out evaluation of performance of our algorithm for detection of microdeletions using low uniquely mapped read count at 10M that is currently considered for standard for reliable detection of

295 Down, Edwards, and Patau syndromes. The motivation for our effort was to define limitations when
296 such low read counts are used. Use of lower read counts is one of key means to reduce substantial
297 sequencing analysis costs per sample. When required read counts drop to a reasonable level around
298 10M reads, use of middle throughput massively parallel sequencing platforms, such as Illumina
299 NextSeq 500/550, becomes an option, creating thus a potential to a wider adoption of testing across the
300 world.
301
302 Critical region determination
303 Similarly to our study, Zhao et al.[6] used clinical database information (DECIPHER database[14]) to
304 define a critical region specific for each of the syndromes. We instead used the ISCA database to
305 manually identify the critical region for every syndrome. The main advantage of our approach is the
306 possibility to define critical regions of microdeletion syndromes more specifically, for example
307 DiGeorge syndrome is previously described in literature as 22q11.21 deletion[19], but the real critical
308 region is not the whole band, only its 3Mb middle part. On the other hand, the drawback is that some
309 genes with clinical significance might be hypothetically missed, as some of the deletions have
310 overhangs from the critical regions possibly encompassing genes too. Our study brings also new
311 information that could be used in the further specification of the size as well as localization of the
312 tested microdeletions as both these parameters were found to be between the four most critical ones[6,12],
313 since there are only very few pathologic detections that overlap the critical regions both from ISCA and
314 DECIPHER databases (Supplementary Table 2).
315
316 Fetal fraction, position, and size of microdeletion
317 As fetal fraction and deletion size were found to be the most critical parameters in NIPT, different
318 combinations of them were tested. Based on a real range of fetal fraction in routine NIPT testing[5,6], we
319 tested fetal fractions from 5% to 20%. Lower fetal fractions than 5% are problematic due to increased

number of false negative detections. 533 simulated cases were evaluated for 200 different samples. Our approach achieved accuracy of 79.3% for 10% fetal fraction with 20M read count, which further increased to 98.4% if we searched only for deletions longer than 3Mb.

To support our *in silico* findings, we designed an artificial laboratory sample evaluation test. To do that, we used artificial mixtures for all studied syndromes using control DNA samples with precise information about the microdeletion size and position. The only undetected samples were those with DiGeorge syndrome shorter than 3Mb and with fetal fraction lower than 10% (Figure 8). Thus, the results are in accordance to those of simulated data.

Read count importance

Later on, as we tested the influence of read count, the fetal fraction was fixed to 10%, the percentage corresponding to average fetal fraction in pregnant women in the most relevant weeks of pregnancy for NIPT (between 10th and 13th week of pregnancy)[13]. From Figure 6 (and Supplementary Figures 17-21) we concluded that the influence of the read count is significant and the increase of prediction accuracy does not stop at 10M reads. Higher number of reads allows for further increasing of accuracy. Based on our results, we recommend to use approximately 16M-17M (appr. 0.35x genome coverage for 2x35bp reads) reads for analyses, due to fact that the detection rate reaches a plateau for 10% fetal fraction and >3Mb deletion size around this point (Figure 6). Using even more reads could still be beneficial, especially for small deletions and low fetal fractions, but it does not add to the prediction accuracy in most of the tested cases, raising thus unnecessarily the costs of the analysis. On the other hand, if the fetal fraction is higher (>12.5%) and we focus only on moderate deletion sizes (>3Mb), even coverage as low as 10M reads was shown to be sufficient, suggesting that this test can (and should) be included as part of a basic NIPT even with low coverage. It should be mentioned, however, that deletions causing DiGeorge syndrome (the most abundant syndrome from our list) are usually shorter than 3Mb (Figure 8). Moreover, the genomic location for this syndrome contains a small unmappable region in

the middle, which further decreases prediction accuracy. For this syndrome, we recommend at least 20M reads to be used.

As one of the proof of the applicability of our approach in a routine practice, so far, we detected one microdeletion causing Cri-Du-Chat syndrome in a sample from routine NIPT. This finding was subsequently confirmed using conventional methods after amniocentesis.

Conclusions

Our results suggest that it is possible to incorporate microaberration detection into whole-genome based NIPT as part of the offered screening/diagnostics procedure, with no or only slight increase in read depths. This is, on the other hand, highly dependent on the specific parameters of the used test as well as on the aims of testing. Final decisions on the use and evaluation of the test results, together with specific test parameters, should be, however, a compromise between the cost of the test, the specific aims of testing and the required sensitivity and specificity. Limitations of this approach should always be kept in mind, while professional judgement of a skilled and properly trained evaluator is still in place. Moreover, using this approach, it is possible to distinguish between mother and fetus derived microdeletions, which is based on expected gain or loss of read counts per bin according to determined fetal fraction. This distinction is available for almost the whole range of fetal fractions observed in clinical practice, except of data with fetal fraction around 50%. These would be indistinguishable due to similar ratio of maternal and fetal DNA. The expected values can be seen on Figure 1 as dashed lines.

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372

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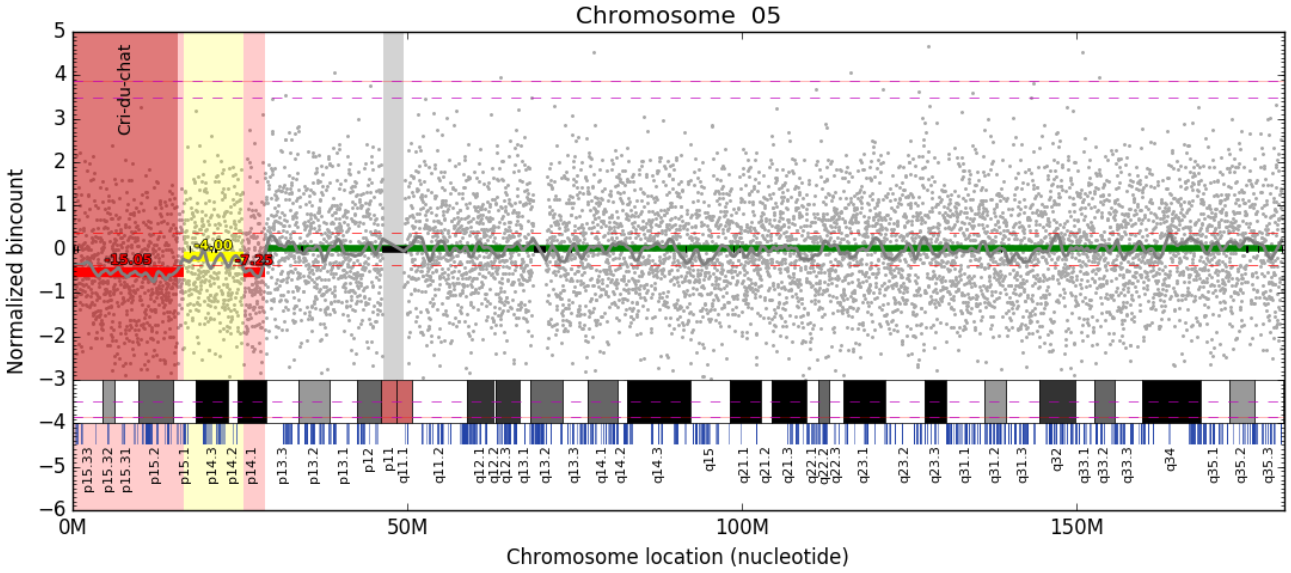


Figure 1.: *Visualization of segment significance. Bin read counts are depicted as gray dots, segments and their significance as colored horizontal lines. Here, we can see an approximately 16.2M long fetal deletion covering the whole Cri-Du-Chat region and a small deletion in the p14.1 region, both depicted in red. However, a possibly fetal deletion depicted by yellow between them may suggest that we are dealing with only one long deletion. Light gray vertical band depicts unmappable region around the centromere, black bands signify bins that did not pass filtration for healthy characteristics (see Normalisation and filtering in Materials and methods section) and are excluded from the analysis. Light red band emphasizes the detected deletion and pathogenic region. The approximated Z-score of the deletion is displayed over the reported segments. Estimated level of aberration based on the fetal fraction of this sample (12.6%) is visualized as red dashed line for fetal aberration and magenta dashed line for maternal aberration.*

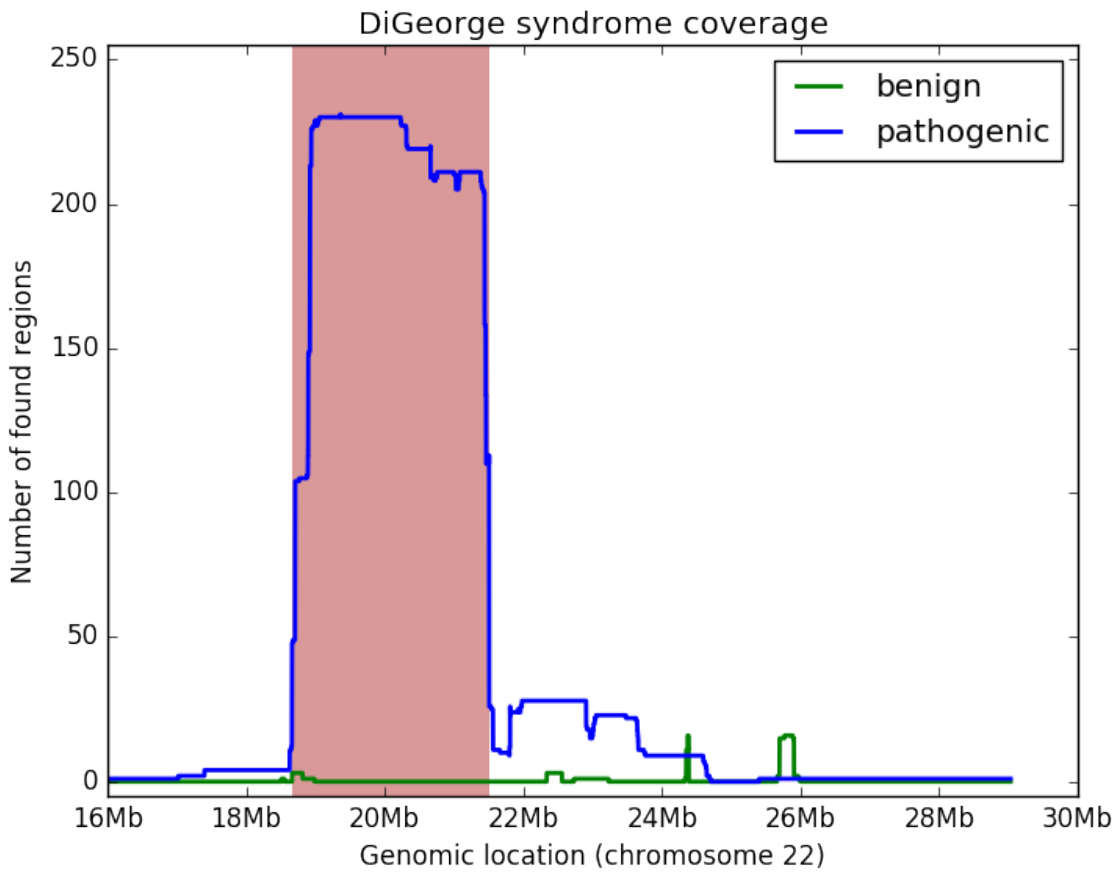


Figure 2.: *Coverage plot representing genomic position of critical region of DiGeorge syndrome. Coverage of pathogenic and likely pathogenic deletions is denoted by blue, while coverage of benign and likely benign microdeletions is in green. The critical region is visualized in red.*

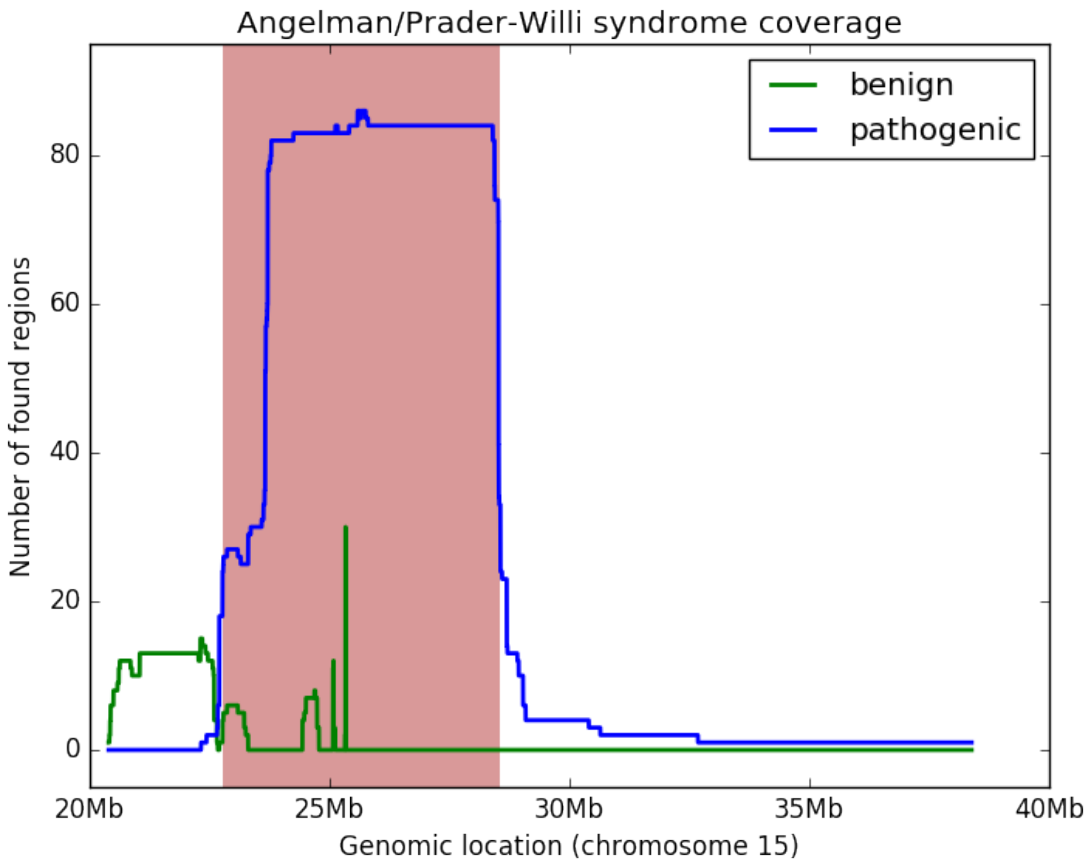


Figure 3.: *Coverage plot representing genomic position of critical region of Prader-Willi / Angelman syndromes. Coverage of pathogenic and likely pathogenic microdeletions is denoted by blue, while coverage of benign and likely benign microdeletions is in green. The critical region is visualized in red.*

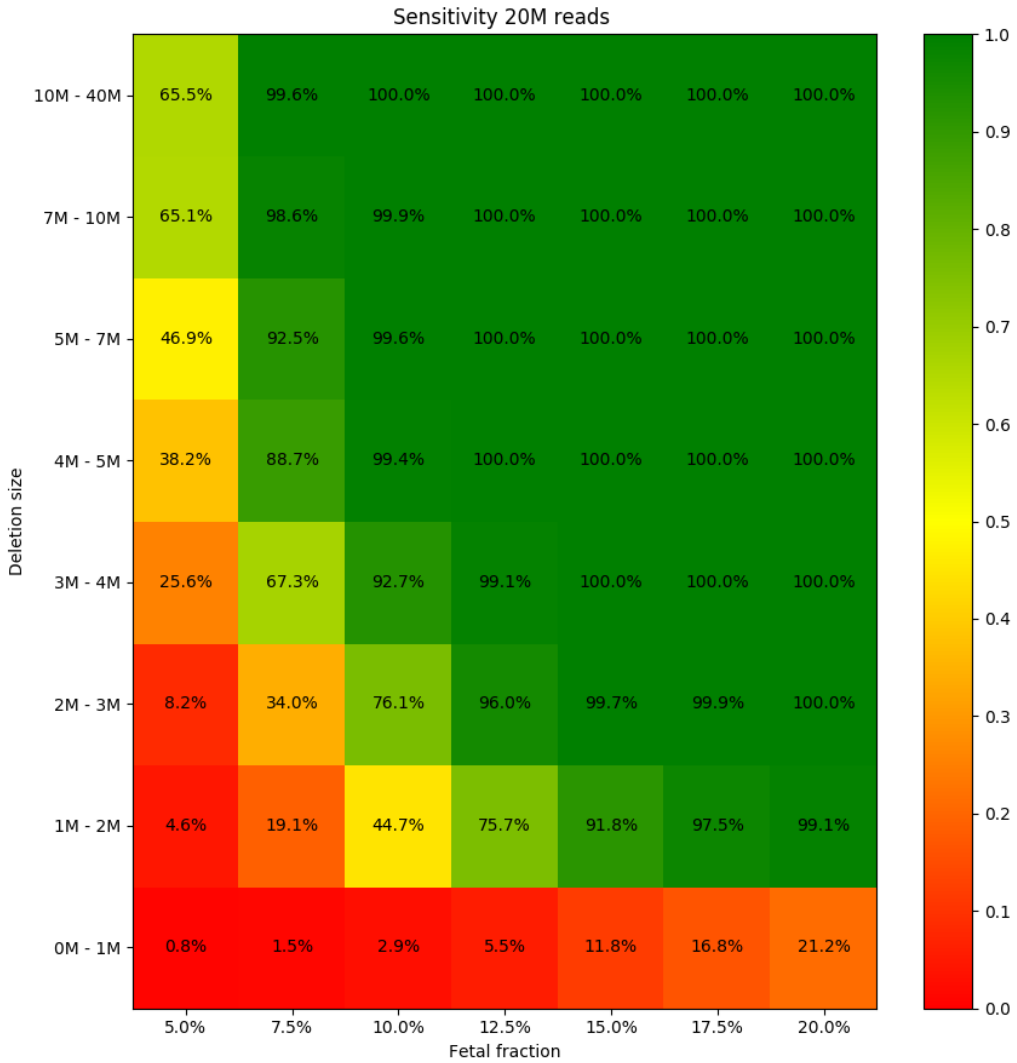
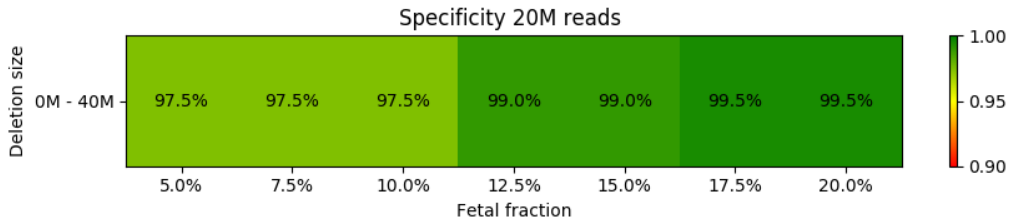


Figure 4.: *Sensitivity of the prediction for different fetal fraction and microdeletion size. Read count was set to 20M in each sample.*



449 ***Figure 5.:*** *Specificity of the prediction for different fetal fraction and microdeletion size. Read count*
450 *was set to 20M in each sample.*

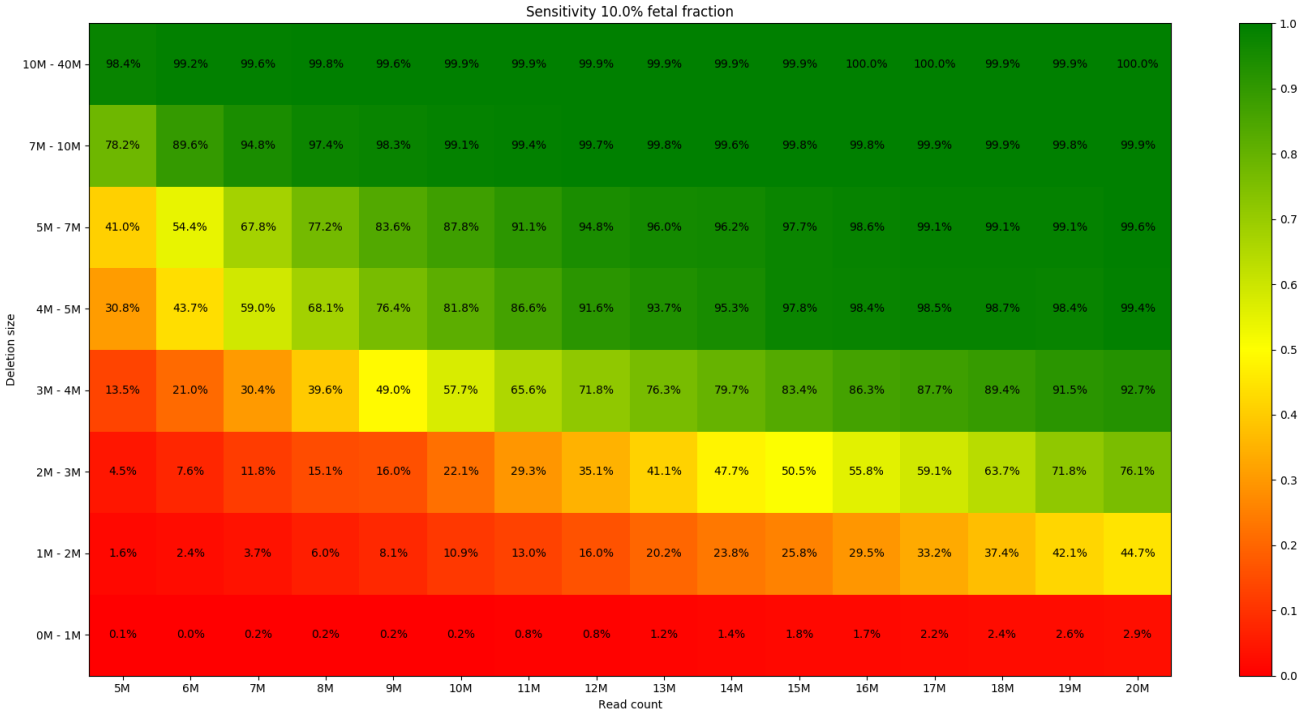


Figure 6.: *Sensitivity of the prediction for different read count and microdeletion size. Fetal fraction was set to 10% for all samples.*

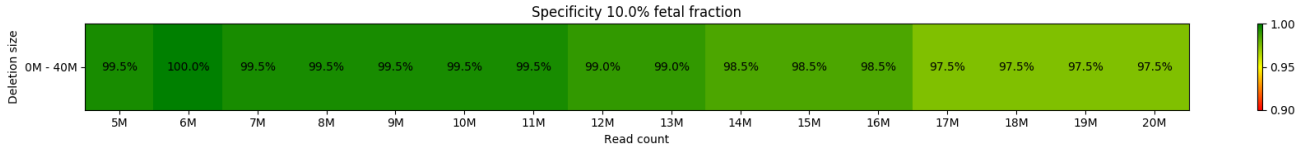


Figure 7.: *Specificity of the prediction for different read count and microdeletion size. Fetal fraction was set to 10% for all samples.*

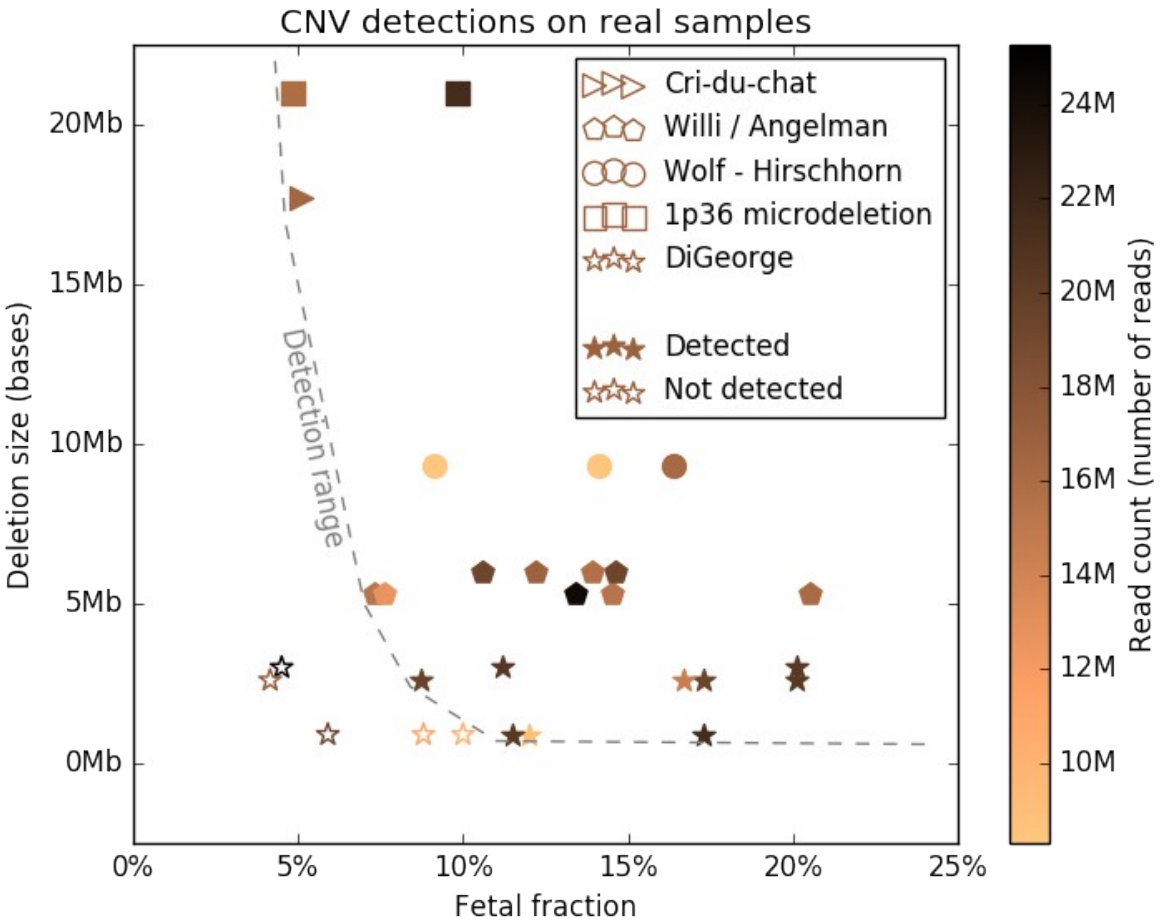


Figure 8.: *Control samples and their detection accuracy. Y axis shows the deletion sizes, X axis the fetal fraction, the color of the point determines its read count. Different syndromes are plotted with different shapes. Detection range was plotted based on all control sample mixups. More detailed data is in the Supplementary Table 1.*

Syndrome	Deletion size						
	0--1Mb	1--2Mb	2--3Mb	3--4Mb	4–5Mb	5–10Mb	10–40Mb
1p36 deletion	8	20	15	13	12	27	5
Wolf-Hirschhorn	0	5	5	12	5	11	10
Cri-du-chat	0	2	2	3	4	8	31
Angelman / Prader-Willi	6	0	0	1	48	34	1
DiGeorge	12	22	205	6	0	0	0

Table 1: *Counts of the microdeletions from the ISCA database.*

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Syndrome	Chromosome	Start of the region	End of the region
DiGeorge	22	18,661,724	21,505,417
Wolf-Hirschhorn	4	85,040	2,010,761
Angelman / Prader-Willi	15	22,779,922	28,559,437
Cri-du-chat	5	1	15,678,560
1p36 deletion	1	564,424	21,598,492

469

470 ***Table 2.:*** *Size and positions of the pathogenic regions for target syndromes (hg19).*