Output from the ”RH-MLPA-Analysis” program **version: 5.21 - 9 March 2010**

Analysis and print of results exemplified by the ”SALSA MLPA P095 Aneuploidy” kit

On the following pages a detailed description of the output of the program and notes regarding the computer analysis methods are exemplified by analysis of prenatal samples sample by the P095 kit. More details regarding the computational methods of the P095 kit can be found on:

Automatic analysis of multiplex ligation-dependent probe amplification products (exemplified by a commercial kit for prenatal aneuploidy detection).
Gerdes T, Kirchhoff M, Bryndorf T. - Electrophoresis. 2005, 26, 4327-4332

The analysis program ”RH-MLPA-Analysis.mdb” requires Microsoft Access 2000 or higher, and it is currently able to analyse MLPA electrophoresis data that are exported from an ABI after fragment sizes and peaks are determined by one of the below programs:.

1) GeneScan 3.7 (for the ABI 3100 we have made software that can export the analysis data automatically).
2) GeneMapper (by version 5.21 the program is streamlined to work on GeneMapper data)
3) Peak Scanner

The printed output is illustrated at the end of this document (for a 47,XY,+21 case analysed by the MLPA kit P095-A2 (lot 0109)):

1) Example 1a shows a simple data sheet where the peaks are sorted by increasing fragment size.
2) Example 1b shows the resulting analysis sheet with putative diagnosis and advanced computations for groups of peaks (i.e. the groups that are characteristic for the actual MLPA probe set).
   The automatic diagnosis and quality evaluation produced by the software is only intended to assist in making the final MLPA diagnosis, i.e. we cannot warrant for its usefulness.

Normally it is sufficient to let the program print out the resulting analysis sheet. It contains quality assessments, and statistics regarding the groups of probes that are characteristic for the actual probe set (for P095 it is chromosome 13, 18, 21, X and Y).
But from time to time it might be handy to look at the simple data sheet where the peaks are sorted in order of increasing fragment lenghts, and therefore also in increasing normalization group order (A, B, C, D). This sheet does also show all peaks that appear between the MLPA probe peaks.

As a kind of output the program also saves many of the computed data per sample in an internal log-table. A button in the main menu allows the user easily to see the most essential data of the log, and to delete log results.

Capillary Electrophoresis

Capillary electrophoresis on the ABI separates the probes of the MLPA kit in such a way that the amplification products are represented by peaks that are detected by increasing fragment length.

Exclusion of tiny peaks

Electroferogram peaks less than 90 RFU (Relative fluorescent units) in height are excluded (except peaks representing Y chromosome). (Our ABI is set to exclude all peaks lower than 50 RFU).
(The user can change limit value).

Normalization of peak areas

As the ligated probe peak areas often decrease with increasing fragment length, each peak area is normalized in relation to a group of neighbouring peaks. The kit chromosome specific peaks of P095-A2 are divided into four normalization groups according to fragment length: The first 10 chromosome specific probes define the first group A, followed by three groups B, C and D with 10, 8 and 8 probes, respectively. Each group contains two peaks from each of the chromosomes 13, 18, 21 and X. There

are only four Y chromosome specific peaks, two in the first group and two in the second. Each peak area is normalized by dividing it with the mean peak area of the group.
(The 9 control fragments of the P095-A2 kit are normalized this way: The four ligation independent DNA Quantity fragments (Q fragments) are normalized separately as a fifth group, whereas the three DNA denaturation control fragments (D-fragments) at 88-92-96 nt and the control X- and Y-fragments at 100 nt and 105 nt, respectively, are normalized by dividing their peak areas with the mean peak area of the A group).
The group letters A, B, C and D can be seen in the column ”Peak Label” on the result sheet.
If a Y-probe peak appears for a female case then its group letter is written with lowercase (a or b for P095-A2) to illustrate that it does not contribute to the normalization, likewise the normalization group of the 9 control fragments of P095-A2 is written with lowercase a.

Identifikation of peaks
The fragment lengt of the sample peaks are compared to mean fragment lengths known from a reference of corresponding normal cases (Instructions for training the program by normal samples can be found on www.chromosomelab.dk). Seldomly a “bad” Rox Standard peak makes local shifts in the resulting peak sizes detected by GeneScan, GeneMapper or Peak Scanner. RH-MLPA-Analysis tries to detect such shifts and adjusts the reference data accordingly. After this, each peak of the sample is assigned to the closed reference peak size by a method that gives large peaks higher weight when looking for the closest reference. Hereby small unspecific amplification products are avoided.
Please note about ROX and LIZ Size Standards:
1) If you have excluded the 250bp ROX or LIZ peak from being used for size determination of the peaks, then it should be excluded for both the samples that you use as normal reference, and for your test samples, because the analysis program often cannot detect the resulting large shifts in the resulting peak sizes arround 250bp, and thus it skips the peaks from analysis. (Exclusion of this peak is recommended by Applied Biosystems.)
2) It might also be a good idea to exclude the 35bp peak for both reference and test samples. Because if you normally have included the 35bp peak then the resulting size locations for the MLPA DQ-control fragments peaks in the 64bp to 82bp range shift much when you e.g. have to exclude the 35bp Standard peak due to e.g. primer dimers near 35bp.

Computation of ratio values
For each normalized peak area it is computed how large it is compared to the mean value of the corresponding peak of the set of normal samples (separate statistics are made for female samples and for male samples). If the Y-peaks are larger than a small limit value then the sample is compared to the male reference. If the mean Y-level appears to be essential, but lower than 25% of the normal Y-level of males then two reports are printed: One where all peaks are compared to normal male peaks and one where the peaks are compared to normal female peaks. This might happen when the sample is contaminated by maternal blood.
For the P095 probeset and similar probesets where many probes at the same time turn out to be deletions or gains, each peak ratio is finally divided by the median ratio value of all peaks. Otherwise all normal probe ratios of e.g. a trisomy 21 case will appear to have ratios below 1 after normalization, and the trisomy 21 ratio becomes much lower than the expected 1.5.
For chromosome 13, 18, 21, and female X the expected ratios for disomy, trisomy, and monosomy probes are 1.0, 1.5, and 0.5, respectively. For normal male X- and Y-chromosomes the expected ratio is 1.0, but 2.0 for male disomy X chromosomes and male disomy Y-chromosomes. Triploid samples are normalized as being diploid so the fragment areas appear as being scaled down by an additional factor 2/3. Therefore the expected ratios for chromosomes 13, 18, 21, X, and Y in a 69,XXY sample are 1.0, 1.0, 1.0, 1.33 and 0.66, respectively. For mosaic samples the ratio reflects the level of mosaicism, e.g. ratio 1.25 for a 50% trisomy 18 mosaicism.

Computation of weighted mean ratio values for chromosome 13, 18, 21, X and Y
For each chromosome 13, 18, 21, X and Y the mean ratio value and corresponding standard deviation (SD) are computed. As it appears that some probes are more reliable than others, each probe is assigned a weight according to its reliability during the mean ratio computations. The weights are

shown on the detailed report. (The "Ref.Weight" is 1/CV (CV="coefficient of variance"=SD/mean), normalized so that the sum of "Ref.Weights" for each chromosome 13, 18, 21 respectively, becomes 1.0.)
The weighted mean ratio and the corresponding weighted coefficient of variation (CV) are shown with bold type on the output sheets. (We use CV (= SD/mean) as this is suitable for later quality assessment, because SD normally is large for large mean ratios and small for small mean ratios.

For each mean ratio of chromosome 13, 18, 21, X and Y it is tested by significace whether the mean ratio is placed outside a reference interval around ratio 1.0

The used reference intervals are based on statistics of normal samples, and they are set so that the chromosome ratios for about 99% of all normal samples are inside the normal references:

- Referenceinterval for chromosome13, 18 and 21   1.0 ± 0,1 (0,90 to 1,10)
- Referenceinterval for female X   1,0 ± 0,1 (0,9 0 to 1,10)
- Referenceinterval for male X   1,0 ± 0,13 (0,87 to 1,13)
- Referenceinterval for male Y   1,0 ± 0,24 (0,76 to 1,24).

The significance is expressed by the P-level that gives the probability for a measured mean ratio to be placed further avay from the reference interval than what was actually measured. See figure 1.

Figure 1. Illustration of an 8 probe mean ratio value that is significantly higher than ratio 1.1

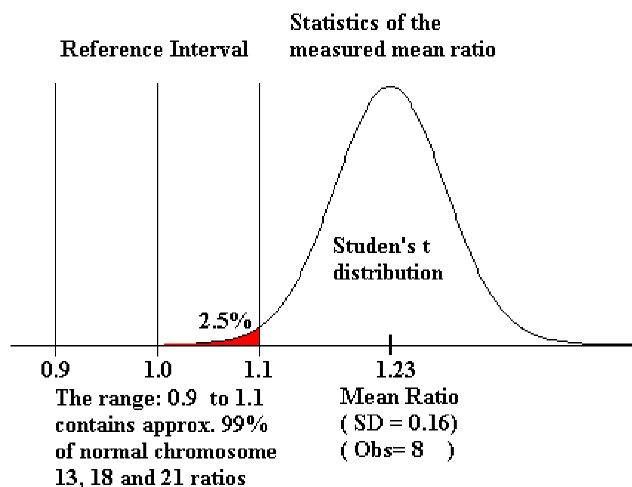

Fig. 1 shows a mean ratio 1.23 compared to ratio 1.1. With the assumption that the individual 8 probe ratio values are normally distributed, the probability for observing a similar ratio value and SD at a higher ratio than 1.23 is 2,5%.
One could also say that the probability is 2,5% for getting a mean ratio below 1.1 for repeated MLPA analysis of the actual sample.
This probability is the one-tailed P-levels shown on the result sheet.

If the P-level is ≤ 5% we (for the time being) consider the sample to be abnormal.
For 1< P ≤ 5% the text ”Low significance P= n,nn%” appears on the result sheet.
For P ≤ 1% the text ”High significance P= n,nn%” appears on the result sheet.

Example showing part of the outcome of a trisomy 13 sample:

Peak			Ref.	Size	Peak			Normalized Peak Area							
no.	Label	Size	Ref. Size	Size diff.	Heigh	width	Area	Peak Area	Ref. Mean	Ref. SD	Ref. Weight	Position p-tel band	Ratio	Dist. in SD	low 1.0 high
22	13 A	146.04	145.92	0.12	2193	9.8	21569	1.681	1.167	0.051	1.48	13q32.1	1.44	10.1 *	·IIII
26	13 A	177.33	177.35	-0.02	1565	10.0	15580	1.214	0.852	0.062	0.89	13q13.3	1.43	5.9 *	·IIII
30	13 B	218.89	218.82	0.07	2116	10.4	21960	1.529	1.077	0.050	1.40	13q14.2	1.42	9.1 *	·IIII
34	13 B	262.51	262.45	0.06	1550	10.9	16937	1.179	0.777	0.057	0.88	13q21.33	1.52	7.0 *	·IIIII
38	13 C	309.98	309.93	0.05	1422	11.4	16252	1.611	1.125	0.067	1.09	13q34	1.43	7.3 *	·IIII
42	13 C	355.22	355.19	0.03	1169	13.1	15327	1.519	1.017	0.073	0.90	13q13.1	1.49	6.9 *	·IIII
47	13 D	398.53	398.39	0.14	1422	12.6	17980	1.709	1.094	0.120	0.59	13q14.2	1.56	5.1 *	·IIIII
51	13 D	442.70	442.59	0.11	1056	13.7	14417	1.370	0.855	0.074	0.75	13q34	1.60	6.9 *	·IIIII
Chromosome 13				Mean	1562	11.5	17503	1.477	0.995	0.069	1.00	(CV: **0.04**)	**1.47**		**P= 0.000%**

High significance P= 0.000% **Female Reference**

Trisomy 13

Ratio 1.47 is found. Thereotically 'Trisomy 13' has ratio 1.5

Fig. 2 illustrates how nice the measured and the thereoetical ratio for a trisomy 13 can appear. Note the extreme high significanse. Without the generel ratio correction mentioned in "Computation of ratio values" the actual trisomy 13 ratio would have been be measured to 1.33.

Example showing part of the outcome of a mosaic DNA sample 45,X[20]/46,XX[10]:

Peak			Ref.	Size	Peak			Normalized Peak Area							
no.	Label	Size	Ref. Size	Size diff.	Heigh	width	Area	Peak Area	Ref. Mean	Ref. SD	Ref. Weight	Position p-tel band	Ratio	Dist. in SD	low 1.0 high
17	X A	152.90	152.82	0.08	2754	9.4	25915	0.785	1.088	0.059	0.94	Xq12	0.72	-5.1 *	III·
24	X A	183.67	183.64	0.03	2292	9.6	21993	0.667	0.824	0.042	1.00	Xq23	0.81	-3.7	II·
30	X B	228.62	228.59	0.03	3056	9.8	29966	0.882	1.066	0.048	1.14	Xp21.3	0.83	-3.8	II·
34	X B	271.66	271.66	0.00	2852	10.0	28523	0.840	1.068	0.040	1.36	Xp11.4	0.79	-5.7 *	II·
39	X C	317.67	317.78	-0.11	1587	11.2	17718	0.790	1.060	0.081	0.66	Xq28	0.74	-3.3	III·
43	X C	362.62	362.49	0.13	2216	10.9	24128	1.075	1.320	0.070	0.97	Xp22.12	0.81	-3.5	II·
47	X D	407.86	407.84	0.02	1615	11.8	19015	0.815	1.029	0.062	0.85	Xq25	0.79	-3.5	II·
51	X D	451.54	451.42	0.12	1268	11.8	14962	0.642	0.829	0.039	1.09	Xp21.1	0.77	-4.8 *	II·
Chromosome X				Mean	2205	10.6	22778	0.812	1.035	0.055	1.00	(CV: **0.04**)	**0.79**		**P= 0.002%**

High significance P= 0.002% **Female Reference**

Monosomy X

Ratio 0.79 is found. Thereotically 'Monosomy X' has ratio 0.5

Fig. 3 illustrates that for the MLPA kit P095 the program is designed to report the most often abnormalities when the found ratio is outside the referenceinterval. Here a monosomy X is reported because the ratio is < 1.0, i.e. the user have to look at the mean ratio value and check that it fits to the thereotical ratio.

(These examples are generated by an older version of the program. For P095-A2 the probes are listed in p- to q-arm order, instead of the shown fragment size order. This makes detection of partial deletions/gains easier.)

Partial deletion or gain

Individual peaks having the normalized area placed more than 4 standard deviations (SD) from the corresponding reference ratio suggests a possible partial deletion or gain.

The "distance in SD" reflects the individual probe reliability because peaks having a large SD needs to be placed far away from the reference ratio than peaks having a small SD to get a high "distance in SD" value. (For normally distributed data the probability for observing an area outside ± 3 SD is less than 1%).

On the output sheet this is called "Dist. in SD", and peaks having "Dist. in SD" less than -4 or greater than 4 are marked by an "*" to the right for the "Dist. in SD" value.

The help to catch "significant" partial deletions or gains for samples where none of the chromosomes 13, 18, 21, X eller Y appear to have a whole chromosome gain or deletion, it is made so that the program prints the names for probes where the probe ratio is ouside a 0.65– 1.30 "normal range", and where the probe at the same time is marked by an "*" (due to being more than 4 SD away from the corresponding reference area). If only one "abnormal" probe is detected then no probename is printed. (The 0.65 and 1.3 limits are also used for other probesets like the P036 and P069 in this way: "Individual peaks having normalized area > 4.0 SD from the ref. mean and ratio < 0.65 or > 1.3 indicate 'abnormal' probe area.")

Example showing part of the outcome of a partiel chromosome 18 deletion:

------ Peak Data ------									------ Normalized Peak Area ------								
No.	**Label**	**Size**	Ref. size	Size diff.	MRC size	**Height**	**Width**	**Area**	Peak Area	Ref. Mean	Ref. SD	Ref. Weigh	Position p-tel	Position band	**Ratio**	**Dist. in SD**	**1.0 low high**
17	18 A	140.09	140.13	-0.04	142	3231	9.4	30347	1.032	1.189	0.048	1.17		18q21.1	0.87	-3.3	I·
21	18 A	170.65	170.79	-0.14	172	2347	9.9	23148	0.787	0.918	0.058	0.75		18q21.32	0.86	-2.3	I·
25	18 B	209.88	209.91	-0.03	211	3058	9.9	30345	0.937	0.986	0.036	1.29		18q11.2	0.95	-1.3	·
29	18 B	252.83	252.87	-0.04	256	3910	10.1	39680	1.226	1.177	0.043	1.29		18q23	1.04	1.1	·
33	18 C	298.88	298.95	-0.07	301	972	10.9	10597	0.527	1.104	0.054	0.97		18p11.32	0.48	-10.7 *	IIIII·
37	18 C	346.15	346.19	-0.04	346	1376	11.3	15525	0.773	0.700	0.045	0.74		18q21.33	1.10	1.6	·I
42	18 D	390.09	390.06	0.03	391	2403	11.5	27633	1.365	1.261	0.060	1.00		18q11.2	1.08	1.7	·I
46	18 D	433.90	433.96	-0.06	436	912	11.7	10705	0.529	1.047	0.064	0.78		18p11.21	0.50	-8.1 *	IIIII·
Chromosome 18					Mean	2276	10.6	23498	0.897	1.048	0.051	1.00		(CV: **0.27**)	**0.87**		

Quality assessment	**Quality limits**	**Quality**
Mean A-group area / mean Q-frag. area	>0.65 (1.50)	4.14
Mean height of first probes AB	> 450 (800)	3051
Mean height of last probes CD	> 280 (500)	1727
Ratio of mean heights AB/CD ('slope')	<3.00 (2.50)	1.77
Mean group CV of weighted ratio	<0.20 (0.15)	0.13
2 unidentified peak areas / 33 peak areas	< (0.02)	0.00

The weighted mean ratios are tested for being outside ratio
1 ± 0.10 for chromosome 13, 18, 21 and female X
1 ± 0.13 for male X and 1 ± 0.24 for Y.
(One-tailed significance is high for p<=1%, and low for p<=5%)

Female Reference

Normal 13, 18, 21, XX

Check: 18p11.32 18p11.21

Fig. 4 illustrates a partial deletion of chromosome 18. The standard deviation and thus CV for the found mean ratio is large, because not all the 8 probes for chromosome 18 agree on a deletion.

(The example is generated by an older version of the program. For P095-A2 the probes are listed in p- to q-arm order, instead of the shown fragment size order. This makes detection of partial deletions/gains easier.)

Essential quality assessments

The sample quality is assessed and commented at the left side at the bottom of the resulting analysis sheet, and in case of quality problems a summary is printed at the right side as either a "Poor Quality!" note, or a note telling how many of the below quality warnings there are for the case.
(Note: all the build in quality figures are set by experience. You can modify them to suit your needs.)

Peak area of ligation dependent probes in relation to DNA quality control fragments (Q-fragments)
If the mean area of the ligated probes (that are used for normalization of the group A peaks) is small in relation to the mean area of the ligation independent control probes (Q-fragments) then a mild or a severe warning appears. A too small value sugests that less than 20 ng test DNA is present in the analysed sample. (Compared to the published article (see page 1), the program is now able to handle all 4 ligation independent Q-fragments of the MLPA kit.)
Mean A-group / mean Q-fragment area:
At quality ≤ 1.50 the quality is "low"   At quality ≤ 0.65 the quality is "Too low!"
(For good quality samples the peaks of the Q-fragments might be too low to be detected. If less than 2 control fragments are detected, then a "?" is written instead of a quality figure.)

Peak area of CpG control fragments (D-fragments) in relation to ligation dependent probes
The mean area of the two CpG probes is compared to the mean area of the probes that are used for normalization in normalization group A. When this ratio is small then the denaturation of the sample DNA may have been incomplete. Currently we don't have enought statistics to set these limits for P095-A2, so they are set low.
Mean CpG-area / mean A-group area:
At quality ≤ 0.65 the quality is "low"   At quality ≤ 0.30 the quality is "Too low!"
(The program currently also checks whether the D-fragment probe 2p14 likewise is low as this might indicate an incomplete hybridization. In case of a too low value this note will be added to the result sheet "The small 2q14 area indicates poor hybridization!".)

Peak heights of ligation dependent probes in relation to the ABI peak height level
Applied Biosystems has told us thet the peak heights should be in the range 200 to 4000 RFU.
If the mean peak height of the first two normalizations groups is low a mild or a severe warning appears. The same happens for the last two normalization groups.

Mean height of the first "half"of peaks (normalization group A, B):
At quality ≤ 800 the quality is "low"   At quality ≤ 450 the quality is "Too low!"

Mean height of the last "half" of peaks (normalization group C, D):
At quality ≤ 500 the quality is "low"   At quality ≤ 280 the quality is "Too low!"

Slope of peaks (Ratio of mean heights AB/CD ('slope'))
If the slope of peak heights sorted by increasing fragment lengths is large then the sample doesn't fit to the samples that were used for training the reference sets. So if the mean height of the first "half" of peaks (group A, B) is low in relation to the mean height of the second "half" of peaks (group C, D) a mild or a severe warning appears.
At quality ≥ 2.50 the quality is "low"   At quality ≥ 3.00 the quality is "Too low!"

The mean of the peak area variations inside a chromosome group should be low
For each chromosome group (13, 18, 21, X, Y) the "CV" ("coefficient of variance" = SD/mean) tells how equal the same kind of probe ratios look. The mean of these CV's should not be too high if you are going to evaluate the mean ratio of each group (partial deletions/gains spoil this quality measure).
At quality ≥ 0.15 the quality is "low"   At quality ≥ 0.20 the quality is "Too low!"
(We use CV (= SD/mean) as this is more suitable for quality assessment than SD, because SD normally is large for large mean ratios and small for small mean ratios.)

The number of unidentified peaks in relation to the identified ones should not be too high
If too many unidentified peaks appear among the ligation dependent peaks a mild warning is made.
At quality ≥ 0.02 the quality is "low"   (here there is no "To low")

Quality assessments and warnings that preced the putative diagnosis

<u>If more than 10 peaks are missing, the diagnosis is replaced by:</u>
"BAD: Too few peaks for analysis!"

<u>If the slope is higher than the max limit, the diagnosis is replaced by:</u>
"BAD: Too high slope!"

<u>If the peaks generally are too low, the diagnosis is replaced by:</u>
”BAD: Too low peaks!” and a comment "Too low peaks for analysis!
(This happens when the mean height is less than half of the min. limit of “Mean height of 'first half' of probes”).

<u>If the mean peak area is too low in relation to the DQ-control fragments, the diagnosis is replaced by:</u>
BAD: Too low DNA!   (It might be the ligation that did not work, but we blame the DNA contents).

Other less essential quality assessments and warnings

<u>The peaks should be fond at the right fragment length</u>
If an ”identified” peak is more than 0.5 bp from the normal fragment lenght then this is marked by an ”*” in the column ”Size diff.”

<u>Peak heights should be below a limit about 7000 RFU</u>
The column ”Peak Height” is marked by an ”*” if the peak height is > 7000 RFU (optional).
On the ABI 3100 the maximum fluorescence intensity is 8100 RFU, but as the background fluorescence has been subtracted before the peaks are exported, a limit about 7000 RFU is fine. If all peaks of e.g. a trisomy 21 are marked by the height “*” for an otherwise qood quality sample, the mean trisomy 21 ratio becomes smaller than it would otherwise have been if all peaks were smaller. This is because some of the probe 21 signals are truncated because they have passed the 8100 RFU limit.

<u>The peak width normally increase when the fragment length increase</u>
Peaks being unexpectedly wide or narrow (i.e. not being close to predicted values based on linear regression of all peak widths of the sample) are marked by an “*” at the “Peak width” column.
Too narrow peaks might bee false “spike” peaks that often are detected by all the colours of the fragment analysis system.

<u>If some few peaks are missing this kind of warnings appear:</u>
E.g. "3 peaks are missing! "

<u>If less than 4 peaks are higher than 1000 RFU this kind of warnings appear:</u>
E.g. " Only 2 peaks are higher than 1000!"

<u>If more than 4 peaks are higher than 7000 RFU (optional) this kind of warnings appear:</u>
“Note that 6 peaks are higher than 7000”

<u>Y-peaks for when female reference data is used:</u>
" Note the Y peaks!”

Other things

<u>The effect of normalization</u>
A simple measure of how much the normalization has decreased the peak area variation can be seen by the comparing ”Coef. of variance” (the bottom of the ”Peak Area” column) to the SD of the normalized Peak Area (bottom of the normalized “Peak Area” column). The difference is a simple measure for the effect of the normalization.

The different kind of putative diagnoses generated when analysing P095 samples

The **putative diagnoseses** for chromosome 13, 18, 21, X and Y depend on the reference data sex, the P-level and whether the ratio is below or abowe ratio 1.0.
(The program reports what it measures according to the statistics, even when the result is absurd. Eg. there are no mosaicism regarding male without an X.)

- If the P-level is higher than 15% the result is assumed to normal, but be avare of partial deletions or gains.
- If the P-level is higher than 5% and less than 15% neither “Normal” nor “Abnormal” is reported. Instead notes like “Increased ratio of chromosome Y" for P less than 10%
  and like “Increased ratio of chromosome Y?" for P between 10% and 15%
- If the P-level is ≤ 5% the putative diagnosis reports abnormal findings.
  - For 1< P ≤ 5% the text ”Low significance P= n,nn%” appears on the result sheet.
  - For P ≤ 1% the text ”High significance P= n,nn%” appears on the result sheet.

All "abnormal" results are accompanied by a **diagnosis note** telling the value of the theoretically expected ratio value, and it is up to the user to decide whether large differnces between an actual and an expected ratio is caused by mosaicism or another copy number than reported.
The diagnosis note should always be read even though some of the obvious large differences automatically add "(mosaicism?)" to the tentative diagnosis. Warnings of potential mosaicism are added for significant ratios less than 1.3 when a ratio of 1.5 is expected, higher than 0.7 when a ratio of 0.5 is expected, or less than 1.8 when a ratio of 2.0 is expected.

Result of computations	**Putative Diagnosis**
P > 15% and female ref. data	Normal 13, 18, 21, XX
P > 15% and male ref. data	Normal 13, 18, 21, XY
P-level is between 10% and 15% and ratio >1	Increased ratio of chromosome 13|18|21|X|Y?
P-level is between 10% and 15% and ratio <1	Decreased ratio of chromosome 13|18|21|X|Y?
P-level is between 5% and 10% and ratio >1	Increased ratio of chromosome 13|18|21|X|Y
P-level is between 5% and 10% and ratio <1	Decreased ratio of chromosome 13|18|21|X|Y
P ≤ 5%, ratio >1 and chromosome 13|18|21	Trisomy 13|18|21
(mosaicism is suggested if the ratio is < 1.3)	Trisomy 13|18|21 (mosaicism?)
P ≤ 5%, ratio >1, female ref. data and chromosome X	Trisomy X
(mosaicism is suggested if the ratio is < 1.3)	Trisomy X (mosaicism?)
P ≤ 5%, ratio <1 and chromosome 13|18|21	Monosomy 13|18|21
(mosaicism is suggested if the ratio is > 0.7)	Monosomy 13|18|21 (mosaicism?)
P ≤ 5%, ratio <1, female ref. data and chromosome X	Monosomy X
(mosaicism is suggested if the ratio is > 0.7)	Monosomy X (mosaicism?)
P ≤ 5%, ratio >1, male ref. data and chromosome X|Y	Male with extra X|Y
(mosaicism is suggested if the ratio is < 1.8)	Male with extra X|Y (mosaicism?)
P ≤ 5%, ratio <1, male ref. data and chromosome X|Y	Male without X|Y (mosaicism?)
Low X-ratio having P ≤ 5% and high Y having P≤15% or Low X-ratio having P≤10% and high Y having P ≤ 5%	69,XYY?
Low Y-ratio having P ≤ 5% and high X having P≤10% or Low Y-ratio having P≤15% and high X having P ≤ 5%	Contamination by mat. DNA or 69,XXY?

Examples of additional diagnosis notes for "abnormal" results:

Trisomy 21:	”Ratio 1.39 is found. Theoretically 'Trisomy 21' has ratio 1.5”
Monosomy X:	”Ratio 0.60 is found. Theoretically 'Monosomy X' has ratio 0.5”
Trisomy X:	”Ratio 1.45 is found. Theoretically 'Trisomy X' has ratio 1.5”
Male with extra X :	”Ratio 1.51 is found. Theoretically 'Male with extra X' has ratio 2.0” Here the putative diagnosis is " Male with extra X (mosaicism?)"
Male with extra Y:	”Ratio 2.05 is found. Theoretically 'Male with extra Y' has ratio 2.0”
69,XYY?:	"Theoretically X and Y ratios of 69,XYY are 0.66 and 1.33"
69,XXY or contam.?:	"Theoretically X and Y ratios of 69,XXY are 1.33 and 0.66"

Case: P095-A2-trisomy-21.txt   Folder: P095-A2-vs01-examples   Date: **10-03-2010**

P095-A2-vs01 Aneuploidy MLPA kit (lot 0109)   Male Reference: P095-A2-vs01-t05   Ver.: 5.21

Peak no.	Peak Label	MRC Size	Peak Size	Size corr.	Ref. Size	Size diff.	Peak Height	Peak Area	Peak Width	Width diff	Normalized Peak Area: Peak Area	Normalized Peak Area: Ref. mean	Normalized Peak Area: Ref. Std	Normalized Peak Area: Ratio	Low 1.0 High
1			0.00				74	775	10.5						
2			2.51				106	598	5.6						
3			6.58				574	4815	8.4						
4			8.73				238	2502	10.5						
5			9.81				187	1401	7.5						
6			12.44				581	3449	5.9						
7			15.43				537	6655	12.4						
8			16.39				577	6935	12.0						
9			18.30				180	1561	8.7						
10			22.25				143	1118	7.8						
11			27.51				208	817	3.9						
12			30.26				365	3426	9.4						
13			37.92				660	4169	6.3						
14			38.88				548	3888	7.1						
15			39.95				146	808	5.5						
16			41.51				82	1006	12.3						
17			50.59				2512	16235	6.5						
18			54.74				762	8676	11.4						
19	64 -	64	60.45	-0.19	60.91	-0.46	1143	12553	11.0	5.40	1.358	1.185	0.13	1.146	·|
20	70 -	70	66.68	-0.17	66.95	-0.27	683	8038	11.8	6.15	0.869	0.816	0.14	1.066	·|
21			69.32				75	399	5.3						
22			71.13				74	427	5.8						
23	76 -	76	72.94	-0.17	73.13	-0.19	781	8522	10.9	5.25	0.922	1.034	0.12	0.891	|·
24	82 -	82	79.31	-0.16	79.41	-0.10	625	7870	12.6	6.89	0.851	0.962	0.13	0.885	|·
25	6 a	88	85.69	-0.14	85.90	-0.21	4413	40645	9.2	3.46	2.113	2.027	1.25	1.042	·
26	2 a	92	91.08	-0.15	91.30	-0.22	2785	17391	6.2	0.46	0.904	0.956	0.14	0.946	|·
27	1 a	96	97.19	-0.17	97.34	-0.15	1909	11609	6.1	0.25	0.603	0.961	0.36	0.628	||||·
28	X a	100	100.91	-0.15	101.15	-0.24	1855	10713	5.8	-0.08	0.557	0.629	0.07	0.885	|·
29	Y a	105	105.45	-0.12	105.67	-0.22	2902	17805	6.1	0.25	0.925	0.987	0.10	0.938	|·
30			125.18				67	599	8.9						
31			127.29				53	651	12.3						
32	21 A	136	134.12	-0.11	134.22	-0.10	5835	36071	6.2	0.11	1.875	1.443	0.06	1.300	·|||
33	18 A	142	141.10	-0.07	141.15	-0.05	4082	25166	6.2	0.04	1.308	1.408	0.07	0.929	|·
34	13 A	148	147.06	-0.06	147.13	-0.07	3678	22765	6.2	0.03	1.183	1.273	0.06	0.929	|·
35	X A	154	154.03	-0.05	154.05	-0.02	2072	12868	6.2	0.00	0.669	0.630	0.04	1.061	·|
36	Y A	160	159.43	-0.07	159.59	-0.16	2679	17142	6.4	0.15	0.891	0.893	0.08	0.997	·
37	21 A	166	165.58	-0.09	165.62	-0.04	4696	29860	6.4	0.07	1.552	1.139	0.09	1.362	·||||
38	18 A	172	171.36	-0.11	171.52	-0.16	3901	24920	6.4	0.06	1.295	1.302	0.05	0.995	·
39	13 A	178	178.23	-0.10	178.41	-0.18	2913	18339	6.3	-0.08	0.953	1.059	0.05	0.900	|·
40			181.93				59	321	5.4						
41	X A	184	184.61	-0.10	184.77	-0.16	1343	8616	6.4	0.00	0.448	0.426	0.05	1.052	·|
42			188.40				51	350	6.9						
43	Y A	193	192.72	-0.09	192.83	-0.11	1394	8759	6.3	-0.19	0.455	0.427	0.06	1.067	·|
44	21 B	202	202.28	-0.06	202.31	-0.03	5238	33188	6.3	-0.20	1.820	1.290	0.08	1.411	·||||
45	18 B	211	211.18	-0.03	211.24	-0.06	3393	21749	6.4	-0.18	1.193	1.163	0.07	1.026	·
46	13 B	220	219.76	-0.01	219.76	0.00	3519	22442	6.4	-0.27	1.231	1.283	0.07	0.959	·
47	X B	229	229.74	-0.01	229.70	0.04	1569	10649	6.8	0.07	0.584	0.681	0.05	0.857	|·
48	Y B	238	239.07	0.02	239.11	-0.04	1395	9339	6.7	-0.09	0.512	0.508	0.04	1.009	·
49	21 B	247	246.23	0.01	246.23	0.00	5591	37056	6.6	-0.20	2.032	1.485	0.08	1.368	·||||
50	18 B	256	254.15	0.01	254.03	0.12	2766	18794	6.8	-0.09	1.031	1.207	0.09	0.854	|·
51	13 B	265	263.69	0.01	263.72	-0.03	1902	13013	6.8	-0.11	0.714	0.899	0.06	0.794	||·
52	X B	274	272.04	0.01	272.05	-0.01	1664	11372	6.8	-0.17	0.624	0.657	0.05	0.949	|·
53	Y B	283	281.03	-0.01	281.03	0.00	2386	16238	6.8	-0.26	0.890	0.827	0.05	1.076	·|
54	21 C	292	289.49	-0.03	289.48	0.01	2847	19265	6.8	-0.35	1.484	1.127	0.07	1.317	·|||
55	18 C	301	299.35	-0.05	299.41	-0.06	2170	15350	7.1	-0.11	1.182	1.261	0.07	0.938	|·
56	13 C	310	310.22	-0.06	310.35	-0.13	1924	13437	7.0	-0.28	1.035	1.130	0.07	0.916	|·
57	X C	319	318.06	-0.08	318.19	-0.13	824	6151	7.5	0.15	0.474	0.622	0.05	0.761	||·
58	21 C	337	336.89	-0.06	336.97	-0.08	2594	18986	7.3	-0.12	1.462	1.026	0.06	1.424	·||||
59	18 C	346	346.42	-0.04	346.50	-0.08	1490	10833	7.3	-0.23	0.834	0.845	0.06	0.987	·
60	13 C	355	356.69	-0.01	356.65	0.04	1984	16315	8.2	0.65	1.256	1.214	0.06	1.035	·
61	X C	364	363.20	0.01	363.20	0.00	1360	10089	7.4	-0.20	0.777	0.775	0.06	1.003	·
62	21 D	382	381.88	0.05	381.85	0.03	2396	18564	7.7	0.01	1.223	0.954	0.05	1.281	·|||
63	18 D	391	390.60	0.05	390.52	0.08	2551	20142	7.9	0.09	1.327	1.376	0.06	0.964	·
64	13 D	400	398.61	0.06	398.45	0.16	2514	19680	7.8	-0.03	1.296	1.287	0.10	1.008	·
65	X D	409	407.68	0.05	407.65	0.03	1022	7989	7.8	-0.10	0.526	0.573	0.04	0.919	|·
66	21 D	427	425.37	0.05	425.28	0.09	2360	20216	8.6	0.53	1.332	0.973	0.06	1.368	·||||
67	18 D	436	435.29	0.04	435.32	-0.03	2109	17247	8.2	0.08	1.136	1.117	0.07	1.017	·
68	13 D	445	443.86	0.04	443.79	0.07	2189	18075	8.3	0.10	1.191	1.243	0.08	0.958	·
69	X D	454	452.85	0.03	452.74	0.11	851	7182	8.4	0.22	0.473	0.477	0.04	0.992	·

Norm. method 2

"Size corr. " shows the corrections this sample makes temporarily to the "Ref. Size" values during peak classification.

"Width diff." shows the difference between the actual peak width and the width that is estimated by linear regression of the peak width of all peaks. I.e. the program makes a model describing how the peak widths of this sample increase by increasing fragment lengths, and the difference of the actual width to the predicted is shown here.

"Size diff." marked by ----- means that the peak was missing, but the peak and 0 value for area etc values is added to be able to make statistics.

Example 1a

Case: P095-A2-trisomy-21 Folder: P095-A2-vs01-examples Date: 10-03-2010

P095-A2-vs01 Aneuploidy MLPA kit (lot 0109) Male Reference: P095-A2-vs01-t05 Ver.: 5.21

No.	Label	Size	Ref. size	Size diff.	MRC size	Height	Width	Area	Peak Area	Ref. Mean	Ref. SD	Ref. Weigh	Position p-tel band	Ratio	Dist. in SD	1.0 low high
		Peak Data							**Normalized Peak Area**							
19	64 -	60.45	60.91	-0.46	64	1143	11.0	12553	1.358	1.185	0.134	1.13	64 nt	1.15	1.3	·|
20	70 -	66.68	66.95	-0.27	70	683	11.8	8038	0.869	0.816	0.139	0.75	70 nt	1.07	0.4	·|
23	76 -	72.94	73.13	-0.19	76	781	10.9	8522	0.922	1.034	0.115	1.15	76 nt	0.89	-1.0	|·
24	82 -	79.31	79.41	-0.10	82	625	12.6	7870	0.851	0.962	0.126	0.97	82 nt	0.89	-0.9	|·
Ctrl: Q-fragments					Mean	808	11.6	9246	1.000	0.999	0.129	1.00	(CV: **0.13**)	**0.99**		
25	6 a	85.69	85.90	-0.21	88	4413	9.2*	40645	2.113	2.027	1.250	0.43	6p21.3 CpG isl.	1.04	0.1	·
26	2 a	91.08	91.30	-0.22	92	2785	6.2	17391	0.904	0.956	0.138	1.85	2q14 synt.	0.95	-0.4	|·
27	1 a	97.19	97.34	-0.15	96	1909	6.1	11609	0.603	0.961	0.360	0.72	1p36.3 CpG isl.	0.63	-1.0	||||·
Ctrl: D-fragments					Mean	3036	7.2	23215	1.207	1.314	0.583	1.00	(CV: **0.20**)	**0.88**		
28	X a	100.91	101.15	-0.24	100	1855	5.8	10713	0.557	0.629	0.067	0.97	Xq23	0.88	-1.1	|·
29	Y a	105.45	105.67	-0.22	105	2902	6.1	17805	0.925	0.987	0.099	1.03	Yq11.21	0.94	-0.6	|·
Ctrl: X- & Y-fragments					Mean	2379	6.0	14259	0.741	0.808	0.083	1.00	(CV: **0.04**)	**0.91**		
60	13 C	356.69	356.65	0.04	355	1984	8.2	16315	1.256	1.214	0.060	1.13	13q13.1	1.04	0.7	·
39	13 A	178.23	178.41	-0.18	178	2913	6.3	18339	0.953	1.059	0.045	1.31	13q13.3	0.90	-2.3	|·
46	13 B	219.76	219.76	0.00	220	3519	6.4	22442	1.231	1.283	0.065	1.10	13q14.2	0.96	-0.8	·
64	13 D	398.61	398.45	0.16	400	2514	7.8	19680	1.296	1.287	0.098	0.74	13q14.2	1.01	0.1	·
51	13 B	263.69	263.72	-0.03	265	1902	6.8	13013	0.714	0.899	0.063	0.80	13q21.33	0.79	-3.0	||·
34	13 A	147.06	147.13	-0.07	148	3678	6.2	22765	1.183	1.273	0.063	1.13	13q32.1	0.93	-1.4	|·
68	13 D	443.86	443.79	0.07	445	2189	8.3	18075	1.191	1.243	0.083	0.84	13q34	0.96	-0.6	·
56	13 C	310.22	310.35	-0.13	310	1924	7.0	13437	1.035	1.130	0.067	0.95	13q34	0.92	-1.4	|·
Chromosome 13					Mean	2578	7.1	18008	1.107	1.173	0.068	1.00	(CV: **0.07**)	**0.94**		
55	18 C	299.35	299.41	-0.06	301	2170	7.1	15350	1.182	1.261	0.073	0.94	18p11.32	0.94	-1.1	|·
67	18 D	435.29	435.32	-0.03	436	2109	8.2	17247	1.136	1.117	0.069	0.89	18p11.21	1.02	0.3	·
45	18 B	211.18	211.24	-0.06	211	3393	6.4	21749	1.193	1.163	0.068	0.93	18q11.2	1.03	0.4	·
63	18 D	390.60	390.52	0.08	391	2551	7.9	20142	1.327	1.376	0.058	1.29	18q11.2	0.96	-0.8	·
33	18 A	141.10	141.15	-0.05	142	4082	6.2	25166	1.308	1.408	0.067	1.14	18q21.1	0.93	-1.5	|·
38	18 A	171.36	171.52	-0.16	172	3901	6.4	24920	1.295	1.302	0.053	1.35	18q21.32	0.99	-0.1	·
59	18 C	346.42	346.50	-0.08	346	1490	7.3	10833	0.834	0.845	0.064	0.73	18q21.33	0.99	-0.2	·
50	18 B	254.15	254.03	0.12	256	2766	6.8	18794	1.031	1.207	0.090	0.73	18q23	0.85	-2.0	|·
Chromosome 18					Mean	2808	7.0	19275	1.163	1.210	0.068	1.00	(CV: **0.05**)	**0.97**		
49	21 B	246.23	246.23	0.00	247	5591	6.6	37056	2.032	1.485	0.080	1.06	21q11.2	1.37	6.9*	·||||
44	21 B	202.28	202.31	-0.03	202	5238	6.3	33188	1.820	1.290	0.082	0.90	21q21.1	1.41	6.5*	·||||
37	21 A	165.58	165.62	-0.04	166	4696	6.4	29860	1.552	1.139	0.089	0.73	21q21.1	1.36	4.7*	·||||
58	21 C	336.89	336.97	-0.08	337	2594	7.3	18986	1.462	1.026	0.060	0.97	21q21.3	1.42	7.2*	·||||
66	21 D	425.37	425.28	0.09	427	2360	8.6	20216	1.332	0.973	0.055	1.00	21q22.11	1.37	6.5*	·||||
54	21 C	289.49	289.48	0.01	292	2847	6.8	19265	1.484	1.127	0.071	0.90	21q22.11	1.32	5.0*	·|||
32	21 A	134.12	134.22	-0.10	136	5835	6.2	36071	1.875	1.443	0.060	1.36	21q22.13	1.30	7.2*	·|||
62	21 D	381.88	381.85	0.03	382	2396	7.7	18564	1.223	0.954	0.050	1.09	21q22.3	1.28	5.4*	·|||
Chromosome 21					Mean	3945	7.0	26651	1.597	1.180	0.068	1.00	(CV: **0.04**)	**1.35**		**P= 0.000%**
61	X C	363.20	363.20	0.00	364	1360	7.4	10089	0.777	0.775	0.057	1.05	Xp22.12	1.00	0.0	·
47	X B	229.74	229.70	0.04	229	1569	6.8	10649	0.584	0.681	0.052	1.02	Xp21.3	0.86	-1.9	|·
69	X D	452.85	452.74	0.11	454	851	8.4	7182	0.473	0.477	0.039	0.95	Xp21.1	0.99	-0.1	·
52	X B	272.04	272.05	-0.01	274	1664	6.8	11372	0.624	0.657	0.055	0.93	Xp11.4**	0.95	-0.6	|·
35	X A	154.03	154.05	-0.02	154	2072	6.2	12868	0.669	0.630	0.037	1.31	Xq12	1.06	1.0	·|
41	X A	184.61	184.77	-0.16	184	1343	6.4	8616	0.448	0.426	0.045	0.73	Xq23**	1.05	0.5	·|
65	X D	407.68	407.65	0.03	409	1022	7.8	7989	0.526	0.573	0.043	1.04	Xq25	0.92	-1.1	|·
57	X C	318.06	318.19	-0.13	319	824	7.5	6151	0.474	0.622	0.050	0.96	Xq28	0.76	-3.0	||·
Chromosome X					Mean	1338	7.2	9365	0.572	0.605	0.047	1.00	(CV: **0.11**)	**0.95**		
36	Y A	159.43	159.59	-0.16	160	2679	6.4	17142	0.891	0.893	0.085	0.92	Yp11.31	1.00	0.0	·
43	Y A	192.72	192.83	-0.11	193	1394	6.3	8759	0.455	0.427	0.058	0.65	Yp11.31	1.07	0.5	·|
53	Y B	281.03	281.03	0.00	283	2386	6.8	16238	0.890	0.827	0.052	1.39	Yp11.3	1.08	1.2	·|
48	Y B	239.07	239.11	-0.04	238	1395	6.7	9339	0.512	0.508	0.042	1.05	Yq11.21	1.01	0.1	·
Chromosome Y					Mean	1964	6.5	12870	0.687	0.664	0.059	1.00	(CV: **0.04**)	**1.04**		
Mean values				-0.03		2589	7.0	17719	**1.063**	1.000	0.062	**4**		1.05	Total of all except	
Standard deviations				0.08	(Coef. of variance:			0.441)	0.419	0.314				0.18	Ctrl and '?' peaks	

Quality assessment	**Quality limits**	**Quality**
Mean A-group area / mean Q-frag. area	>0.65 (1.50)	2.08
Mean CpG-area / mean A-group area	>0.30 (0.65)	1.36
Mean height of first probes AB	> 450 (800)	3101
Mean height of last probes CD	> 280 (500)	1949
Ratio of mean heights AB/CD ('slope')	<3.00 (2.50)	1.59
Mean group CV of weighted ratio	<0.20 (0.15)	0.07
4 uinidentified peak areas / 41 peak areas	< (0.02)	0.00

The weighted mean ratios are tested for being outside ratio
1 ± 0.10 for chromosome 13, 18, 21 and female X
1 ± 0.13 for male X and 1 ± 0.24 for Y.
(One-tailed significance is high for p<=1%, and low for p<=5%)

High significance P= 0.000% **Male Reference**

Trisomy 21

An "*" marks: Size Diff.>0.5, Peak Height>7000, unexpected peak width, and "Dist. in SD">4.0.
Ratio group mean and coefficient of variance (CV) are weighted by the ref. weights
Labels A,B,... define normalization groups; a,b,... labeled probes do not contribute to normalization.
Mean Rox height is 335 (14 peaks). CV of ROX heights for peaks above 100 nt is: 0.08

Ratio 1.35 is found. Theoretically 'Trisomy 21' has ratio 1.5

Example 1b