Bionano Solve Theory of Operation: Bionano EnFocus^{TM} Fragile X Analysis

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Revision History

Revision	Notes
A	Initial document release

Introduction

Expansions and contractions of simple sequence repeats are associated with more than 40 diseases including Fragile X Syndrome, Huntington's disease, myotonic dystrophy, facioscapulohumeral muscular dystrophy (FSHD) and Friedreich's ataxia. Expanded repeats are unstable and may expand during intergenerational transfer. The associated disorders tend to increase in severity with each successive generation. Phenotype severity is often correlated with the amount of pathogenic expansion or contraction. Thus, accurate sizing of the repeats is crucial. Southern blotting is the primary method for analyzing pathogenic repeats. The repetitive and polymorphic nature of these regions presents difficulties for both polymerase chain reaction (PCR), where the polymerase is unable to traverse through long repeats, and sequencing based methods, which face limitations in read lengths.

Fragile X syndrome is associated with cognitive impairment and is the most common heritable cause of intellectual disability. Nearly all cases of Fragile X syndrome are caused by an expansion of a CGG triplet repeat region in the *FMR1* gene. Fragile X repeat sizes are characterized in the following ranges:

- Normal: 5 – 40 repeats
- Pre-mutation: 55 - 200 repeats
- Full mutation: > 200 repeats

Bionano Genomics has developed a targeted analysis workflow for Fragile X Syndrome based on optical genome mapping (OGM) with the Bionano Genomics Saphyr platform. The Saphyr platform can detect germline SVs >500 bp in size including repeat expansions/contractions and provides a high-resolution analysis of the Fragile X repeat region.

Briefly, the workflow selects the subset of molecules that align to regions of interest and then assembles that subset into consensus maps. The workflow then uses the resulting consensus maps as input to the Bionano EnFocus^{TM} Fragile X Analysis. This analysis first determines the number of alleles in the *FMR1* repeat region on chromosome X and then sizes these regions. The analysis then calculates the probability that the size of the repeat region on each allele exceeds 200 copies of the CGG triplet. The analysis results can be imported into Bionano Access, a graphical user interface tool for visualization and curation. Access can generate a summary of the results in pdf and in JSON format.

Note that the method described cannot detect single-nucleotide variants that do not impact enzyme sequence motif sites and may miss small variants with potential functional impacts. The pipeline only supports Bionano's DLE-1 labeling enzyme.

Analysis workflow

Local assembly of regions of interest

The Fragile X analysis pipeline first analyzes intervals of interest by selecting molecules that align to the *FMR1* locus and performing a local assembly using only those molecules. The pipeline then analyzes the repeat content of those resulting genome maps that align to the CGG repeat region in the *FMR1* gene on chromosome X. The pipeline also assembles and analyzes other selected regions of the genome as part of a quality-control process (discussed in a subsequent section; Table 1).

The local assembly workflow is similar to the standard Bionano de novo whole-genome assembly workflow, but the key difference here is that this workflow uses a reference as a guide and only assembles targeted regions. This workflow performs local assembly of those targeted regions using parameters specifically optimized for effective assembly of the *FMR1* repeat regions. This workflow significantly reduces the assembly time and is effective in assembling the targeted regions despite such regions' typical complexity.

Identification of intervals encoding the CGG repeat array

We identified the coordinates of CGG repeat array (chrX: 147,912,037-147,912,111bp) in the *FMR1* gene in the hg38 reference genome using the UCSC Genome Browser. The interval of interest refers to the narrowest interval containing the CGG repeat array in an assembled map. The workflow uses the labels at chrX 147,910,189 bp and 147,918,814 bp as the endpoints of the reference interval. An interval aligning to the reference is the observed interval (O) which the workflow uses to the infer the number of repeats in a sample.

Repeat count estimation

The workflow uses Bayesian probability to compute the repeat count in a sample. Since the DLE-1 enzyme does not directly label the bounds of the CGG repeat array in the *FMR1* gene, the interval of interest consists of the CGG repeat array (R) and flanking region (C) in which R varies among samples and C is a constant value. C is calculated by [reference interval – reference CGG repeat array size].

The pipeline uses the posterior probability to compute the number of CGG repeat units (R) in a query sample:

$$P(R+C|O) = \frac{P(O|R+C)\cdot P(R+C)}{P(O)}$$

$$P(O) = \sum_{R\in[R_L,R_U]} P(O|R_i+C)P(R_i+C)$$

where

- $P(R+C)$, the prior probability, is the probability of the total size of the interval (R + C). Currently, we assume that prior probability follows a uniform distribution.

- $P(O|R+C)$ is the likelihood function for a Gaussian distribution $N(\mu, \sigma^2)$, in which μ and σ^2 were pre-determined at the assembly step of the pipeline.
- $P(R+C|O)$ is the posterior probability.

In the final report, R is quantified by:

- a repeat number with the maximum posterior probability (MAP)
- a 99% credible interval (CI) giving the upper and lower bounds of the repeat number estimation
- the probability that R >= 200 units

Assessment of molecule support

The pipeline analyzes the molecule-to-map alignments to assess the amount of molecule support for a given map. The number of molecules spanning across the repeat start and end sites provide supporting evidence for the repeat count estimation. This pipeline provides this information in the final report.

Quality control

Inferred sex of sample

The analysis pipeline includes running the whole genome copy number pipeline and can infer sex information for the sample. The pipeline infers the sex to be male if non-trivial coverage of chrY is found, otherwise it determines the sex to be female. The pipeline does not handle more complex sex chromosome configurations. If external data is available, one could compare the inferred sex with the external data and check for consistency.

Assessment of molecule quality

The pipeline collects data on molecule alignment quality to the reference. To ensure that the molecule quality is sufficient for downstream analyses, mapping rate should be at least 70%, the effective coverage should be at least 75X, and the molecule N50 should be at least 200 kbp.

Selection and assessment of stable regions

To assess consensus map level quality, the pipeline analyzes regions of the genome that are deemed stable (Table 1) based on the hg38 reference.

Selection of stable regions

We selected one region per autosome (for a total of 22 regions) based on analyses of 58 *de novo* assemblies of Bionano human control samples. We analyzed the consensus map-to-reference alignment for these controls. For each reference interval and for each sample, we computed the absolute percent difference between the interval length of the reference and that of a given map. We then computed the mean absolute percent difference across controls for each interval. We then sorted these, and after excluding regions with insufficient data or too many alignments, we selected the regions with the lowest mean absolute percent differences, which we assume to be the most stable.

Assessment of stable regions

When analyzing a sample of interest, the Fragile X pipeline assembles molecules from the stable regions in Table 1. We analyze the resulting consensus maps and consensus map-to-reference alignment in a similar fashion. The pipeline expects the consensus maps to be consistent with the reference for the selected regions. Based on expected sizing errors, the absolute percent differences between the map and the reference should not exceed 1.2%. The pipeline requires that at least 90% of the regions be under this threshold.

Chr	Coordinates		Chr	Coordinates
1	222,324,492 - 222,349,194		**12**	25,901,387 - 25,914,482
2	203,598,419 - 203,624,005		**13**	26,774,581 - 26,796,446
3	31,786,552 - 31,805,963		**14**	49,469,153 - 49,487,044
4	159,395,859 - 159,416,605		**15**	60,014,272 - 60,041,969
5	37,318,756 - 37,335,731		**16**	77,498,326 - 77,517,842
6	53,262,654 - 53,282,806		**17**	1,377,309 - 1,389,336
7	26,961,779 - 26,970,051		**18**	12,367,665 - 12,396,092
8	121,580,578 - 121,595,557		**19**	13,335,300 - 13,361,530
9	116,981,783 - 117,012,768		**20**	47,394,428 - 47,417,351
10	62,135,760 - 62,157,297		**21**	37,268,614 - 37,282,468
11	78,075,503 - 78,100,220		**22**	38,154,243 - 38,163,301

Table 1. List of stable regions based on hg38 coordinates included in the quality control assessment.

Data summary

The pipeline compiles all the data necessary for Bionano Access to visualize the maps, to highlight the repeat regions and to generate the final Fragile X analysis report. The key data files are compressed into a zip file, and results are summarized in a JSON file, both of which are automatically transferred into Bionano Access.

The JSON file is also available for download and direct import into the customer’s reporting tool for parsing and presentation. See Bionano EnFocus™ Fragile X Analysis JSON File Format Specification (PN 30458) for detailed information on the content and format of the JSON file.

Performance summary

To evaluate the capability of detecting disease causing repeat expansions, we analyzed the *FMR1* repeats relevant to Fragile X syndrome using Coriell cell lines and deidentified patient samples with known repeat sizes as well as unaffected control samples assumed to be negative for the Fragile X repeat expansion. In addition, 10 female samples were simulated by combining one male with a normal repeat and one with the full mutation expansion. We observed the expected expansion alleles with sizes consistent with annotation, with the largest expansion being almost 1000 copies.

Sample validation

We analyzed a total of 75 samples, in which 37 carried the *FMR1* mutation (repeat >=200) and 38 had a repeat number < 200. The categories of validation set can be found in Table 2.

Sex	R < 100	R in [100, 200]	R >= 200	Negative control (assumed R < 100)
Female	4	5	18	11
Male	6	3	19	9

Table 2. Overview of validation samples. R refers to the independently determined repeat size.

The Fragile X analysis produces a probability that a sample's repeat number (R) is greater than 200. We used the reported probability (P) that R >= 200 to assess the test's analysis performance as follows:

- True Positives (TP): Samples annotated with R > 200 where at least one allele is called with P > 99%
- True Negatives (TN): Samples annotated with R < 200 where all alleles are called with P < 50%
- False Positives (FP): Samples annotated with R < 200 with at least one allele called with P > 99% and
- False Negatives (FN): Samples annotated with R > 200 with all alleles called with P < 99%

Overall, the validation identified one false negative, relative to the available southern blot results, which was annotated with 200 repeat units. The analysis reported that the false negative has 0.54%, 53.96% and 64.28% chance to carry *FMR1* mutation at 80X, 175X and 300X coverage, respectively. From the analysis of 75 samples, the EnFocus™ Fragile X Analysis pipeline was assessed to have 97% sensitivity and 100% PPV (Table 3).

		Prediction	
		+	-
Annotation	+	36	1
	-	0	38

Table 3. Confusion matrix showing diagnostic performance of 75 samples

Performance at different coverage levels

We assessed the accuracy at different coverage levels using a random down-sampling approach. We evaluated samples at three coverage tiers: 400 Gb (80X), 800 Gb (175X) and 1.2 Tb (>=200X). Results in Table 4.

Dataset	**400 Gb (80X)**		**800 Gb (175X)**		**1.2 Tb (> 200X)**	
Deidentified samples	44/45	98%	38/39	97%	36/37	97%
Males	28/28	100%	22/22	100%	20/20	100%
Females	16/17	94%	16/17	94%	16/17	94%
Simulated samples	10/10	100%	*data not available*			
TOTAL	54/55	**98%**	38/39	**97%**	36/37	**97%**

Table 4. Summary of accuracy by coverage tier

Definition of confidence ranges

The uncertainty of analysis performance tends to occur in the marginal cases which possess repeat number close to 200 repeat units. Overall, if 99% CI upper bound < 195 or CI lower bound > 215, the pipeline can achieve 100% accuracy (Table 5).

Coverage	**CI < 195**			**CI [195, 215]**			**CI > 215**				**Coverage level summary**				
	TN	***FN***	***Acc.***	***TN***	***FN***	***Acc.***	***TP***	***FN***	***FP***	***Acc.***	***Sens.***	***Spec.***	***PPV***	***NPV***	***Acc.***
400 Gb	33	0	100%	5	1	83%	36	0	0	100%	97%	100%	100%	97%	98%
800 Gb	13	0	100%	3	1	75%	22	0	0	100%	96%	100%	100%	94%	97%
1.2 Tb	12	0	100%	3	1	75%	21	0	0	100%	95%	100%	100%	94%	97%
CI level Summary			100%			79%				100%					

Table 5. Test produces 100% PPV with no false positives at all coverage levels. Assessments were 100% accurate when upper bound of CI < 195 and lower bound > 215.

Repeatability

We selected a subset of 4 samples to perform reproducibility analysis by running three technical replicates of each sample. In all cases, results show analytical consistency with identical analysis results. Results for representative female and male samples are in Table 6 and Table 7.

data	mol. N50 (kbp)	mapping rate	effective cov.	allele 1				allele 2			
				repeats	*mutation prob.*	*CI*	*repeat coverage*	*repeats*	*mutation prob.*	*CI*	*repeat coverage*
400 Gb	332.4	0.972	105.4	741	> 99.9 %	669 – 816	34	82	< 0.01 %	24 – 143	50
	313.2	0.967	103.4	587	> 99.9 %	518 – 658	32	57	< 0.01 %	3 – 114	46
	309.1	0.962	102.6	626	> 99.9 %	556 – 698	30	93	< 0.01 %	34 – 154	54
800 Gb	332.5	0.972	210.8	656	> 99.9 %	586 – 729	29	79	< 0.01 %	20 – 139	51
	313.3	0.967	206.8	660	> 99.9 %	590 – 733	28	88	< 0.01 %	29 – 149	54
	309.0	0.962	205.2	613	> 99.9 %	544 – 685	26	79	< 0.01 %	20 – 139	61
1.2 Tb	381.3	0.980	223.7	658	> 99.9 %	588 – 731	29	62	< 0.01 %	6 – 120	41
	364.4	0.976	222.7	583	> 99.9 %	515 – 655	30	79	< 0.01 %	20 – 139	58
	360.0	0.973	222.3	609	> 99.9 %	540 – 681	32	76	< 0.01 %	18 – 137	59

Table 6. Repeatability analysis for female sample 95645_3 annotated as 250-750 repeats

data	mol. N50 (kbp)	mapping rate	effective cov.	*repeats*	*mutation prob.*	*CI*	*repeat coverage*
400 Gb	332.4	0.973	106.8	133	0.32 %	73 – 194	27
	282.1	0.963	101.4	122	0.08 %	62 – 183	39
	279.6	0.959	101.6	106	0.01 %	47 – 167	30
800 Gb	332.5	0.972	213.7	122	0.08 %	63 – 184	50
	282.2	0.963	203.0	108	0.01 %	49 – 169	54
	279.6	0.959	203.2	91	< 0.01 %	33 – 153	37
1.2 Tb	378.5	0.979	223.6	118	0.05 %	59 – 180	51
	328.6	0.968	219.9	106	0.01 %	46 – 167	54
	336.8	0.968	218.6	120	0.06 %	61 – 182	54

Table 7. Repeatability analysis male sample annotated as 88 repeats

Analysis of low-quality samples

Sample treatment

We analyzed samples with degraded quality to define minimum input quality requirements. Three female samples with a range of annotated repeat sizes (101, 160 and 250-750) were treated to reduce quality. DNA from samples was sheared 12X to reduce molecule N50 and the amount DLE-1 labeling enzyme was reduced to lower mapping rates and effective coverage. 800 Gb of data was generated for all replicates. Sample data were downsampled for additional analysis at 400 Gb. All samples had data generated with 12X shearing alone, with 85% of DLE-1 and with both shearing and reduced enzyme. Additionally, one sample was repeatedly treated with 70%, 50% and 30% less DLE-1. Samples and treatment replicates are summarized in Table 8.

Treatment	Expected effect	# analyses
12 X shearing	Reduce molecule N50	3
85% DLE-1	Reduce mapping rate and coverage	3
12X shearing + 85% DLE-1	Both	3
70% DLE-1	Reduce mapping rate and coverage	1
50% DLE-1	Reduce mapping rate and coverage	1
30% DLE-1	Reduce mapping rate and coverage	1
12X shearing + 50% DLE-1	Both	1
12X shearing + 70% DLE-1	Both	1
TOTAL		**14**

Table 8. Overview of low-quality sample treatments

Treated samples yielded mapping rates from 43 – 96% at both the 400 and 800 Gb coverage level. Molecule N50 ranged from 189 kb – 325 kb. Effective coverage ranged from 39x to 109x at the 400 Gb coverage level and

78x to 217x at the 800 Gb level. (Table 9)

effective coverage	400 Gb analyses	800 Gb analyses
35 – 75x	3	0
75 – 90x	2	1
90 – 110x	9	1
> 110x	0	12
mapping rate	**400 Gb analyses**	**800 Gb analyses**
40 – 70%	2	2
70 – 85%	3	3
85 – 90%	3	3
> 90%	6	6
molecule N50	**400 Gb analyses**	**800 Gb analyses**
< 190 kb	1	1
190 – 210 kb	4	4
> 210 kb	9	9

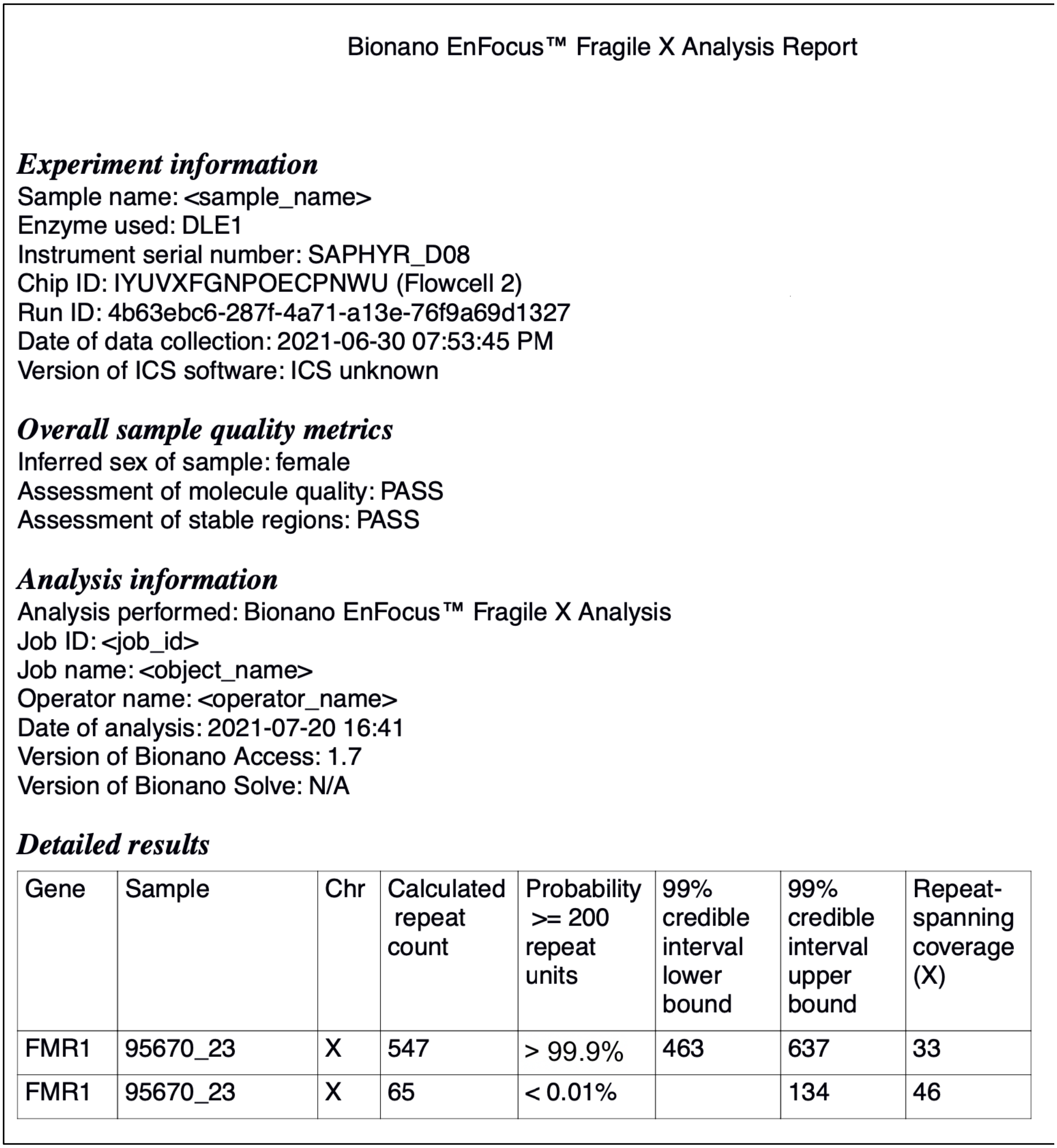
Table 9. Distribution of samples across quality metrics

All samples were called correctly at the 800 Gb range. One down-sampled replicate annotated as 89 repeats was called incorrectly as an expansion at the 400 Gb range. Downsampling was repeated 20 times and the correct result was reported in all other iterations.

EnFocus^{TM} Fragile X Analysis Report

Bionano Access generates a PDF summary report that contains the key results from the Fragile X analysis pipeline. The first page of the report is a summary page that contains basic information about the sample being analyzed and the main findings on the Fragile X repeat region. An example of this summary page is shown in Figure 1.

The summary page is followed by detailed results pages where each page shows an assembled Bionano map that contains the Fragile X repeat. The maps shown correspond to entries in the results table on the first page. The molecules that support the assembled maps can be shown below the maps as supportive evidence. An example of a heterozygous full mutation repeat expansion alleles (i.e. one allele greater than 200 repeats and one less than 200) is shown in Figure 2 and Figure 3.



Bionano EnFocus™ Fragile X Analysis Report

Experiment information

Sample name: <sample_name>
Enzyme used: DLE1
Instrument serial number: SAPHYR_D08
Chip ID: IYUVXFGNPOECPNWU (Flowcell 2)
Run ID: 4b63ebc6-287f-4a71-a13e-76f9a69d1327
Date of data collection: 2021-06-30 07:53:45 PM
Version of ICS software: ICS unknown

Overall sample quality metrics

Inferred sex of sample: female
Assessment of molecule quality: PASS
Assessment of stable regions: PASS

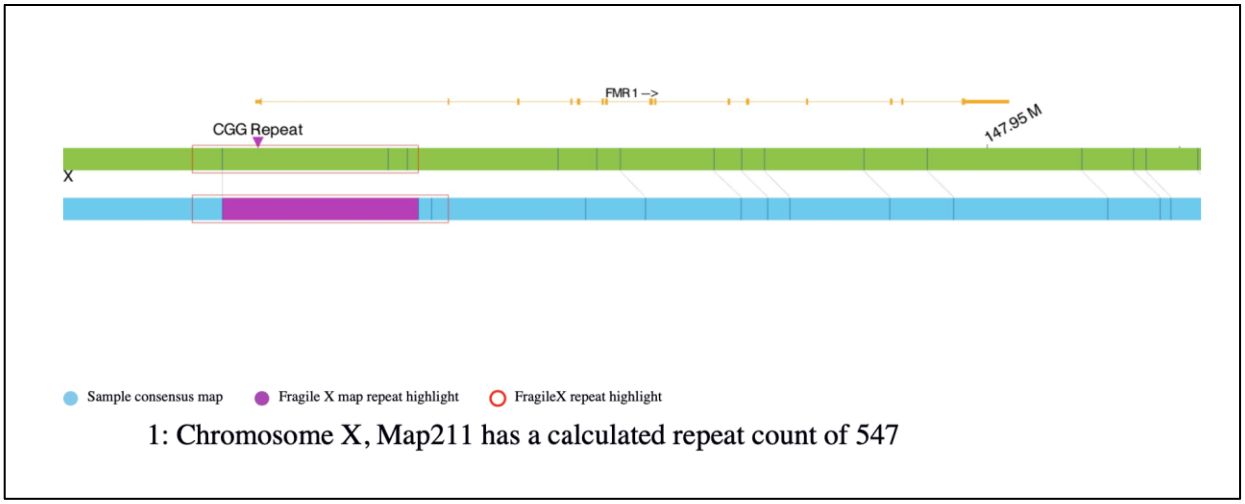
Analysis information

Analysis performed: Bionano EnFocus™ Fragile X Analysis
Job ID: <job_id>
Job name: <object_name>
Operator name: <operator_name>
Date of analysis: 2021-07-20 16:41
Version of Bionano Access: 1.7
Version of Bionano Solve: N/A

Detailed results

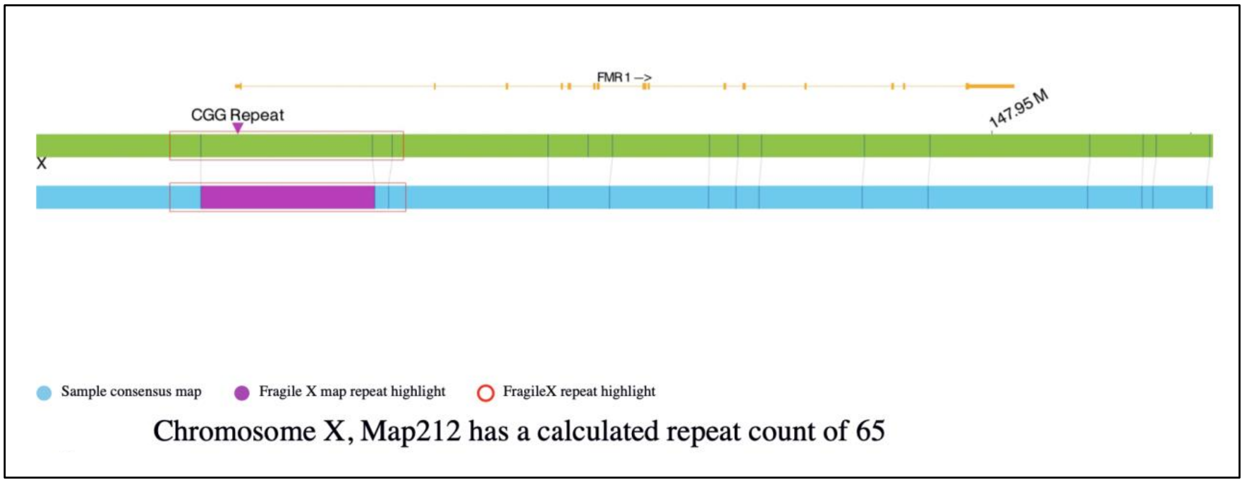
Gene	Sample	Chr	Calculated repeat count	Probability >= 200 repeat units	99% credible interval lower bound	99% credible interval upper bound	Repeat-spanning coverage (X)
FMR1	95670_23	X	547	> 99.9%	463	637	33
FMR1	95670_23	X	65	< 0.01%		134	46

Figure 1. Example summary page from EnFocus^{TM} Fragile X analysis report.



1: Chromosome X, Map211 has a calculated repeat count of 547

Figure 2. Visualization of allele 1 with 547 repeats



Chromosome X, Map212 has a calculated repeat count of 65

Figure 3. Visualization of allele 2 with 65 repeats

FAQs

1. How does data quality impact Fragile X analysis results?

As discussed in the quality control section, the pipeline looks at three specific criteria at the molecule quality level (map rate, molecule N50 > 150 kbp, and effective coverage) and also checks the consensus map quality. Having sufficiently long molecules and sufficient coverage ensures that the repeats can be fully spanned, and that haplotypes can be assigned. It also helps ensure that the map-level errors are low.

2. How does de-duplication work?

If the sample is a female or an aneuploid male, the pipeline outputs all *FMR1* relevant maps. For a diploid male, if there are maps with the same repeat counts, the pipeline picks one representative one (with the longest length).

3. What is the performance to detect mosaic repeat expansion alleles?

The current Fragile X pipeline has been validated for germline samples without significant mosaicism.

4. How do you interpret the probability score (P >= 200)?

P >= 200 refers to the probability that the sample has the *FMR1* full mutation of >= 200 repeat units. The probability has no indication for the *FMR1* pre-mutation range.

5. Why is the estimated repeat number zero?

In some cases, unresolved close labels (distance <= 1kbp) can occur at the boundary of the interval of interest. When one-to-multiple label alignment happens, the irrelevant region needs to be corrected by adding an extra space. Overcorrection of an irrelevant region can cause underestimation of the repeat number. Thus, an estimated repeat count of zero indicates the sample possesses a small number of repeats. However, overcorrection events won’t impact diagnostic performance of the pipeline

6. How much time does it take to run the EnFocus Fragile X analysis?

For the recommended coverage of 400 Gbp, the analysis takes1.5 hours on a Saphyr Compute and 3.5 hours on the Bionano Access Server.

Technical Assistance

For technical assistance, contact Bionano Genomics Technical Support.

You can retrieve documentation on Bionano products, SDS's, certificates of analysis, frequently asked questions, and other related documents from the Support website or by request through e-mail and telephone.

Type	Contact
Email	**support@bionanogenomics.com**
Phone	**Hours of Operation:** **Monday through Friday, 9:00 a.m. to 5:00 p.m., PST** **US: +1 (858) 888-7663**
Website	**www.bionanogenomics.com/support**

Bionano Genomics, Inc.
9540 Towne Centre Drive, Suite 100
San Diego, CA 92121