

# Detection of copy number variations from NGS data using read depth information: a diagnostic performance evaluation

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1 **Detection of copy number variations from NGS data using read depth information: a**  
2 **diagnostic performance evaluation**

3 **Running title : Evaluation of a CANOES-centered workflow**

4

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27 **ABSTRACT**

28 The detection of Copy Number Variations (CNVs) from NGS data is under-exploited as chip-based  
29 or targeted techniques are still commonly used. We assessed the performances of a workflow  
30 centered on CANOES, a bioinformatics tool based on read depth information.

31 We applied our workflow to gene panel (GP) and Whole Exome Sequencing (WES) data, and  
32 compared CNV calls to Quantitative Multiplex PCR of Short Fluorescent fragments (QMSPF) or  
33 array Comparative Genomic Hybridization (aCGH) results.

34 From GP data of 3,776 samples, we reached an overall Positive Predictive Value (PPV) of 87.8%.

35 This dataset included a complete comprehensive QMPSF comparison of 4 genes (60 exons) on  
36 which we obtained 100% sensitivity and specificity.

37 From WES data, we first compared 137 samples to aCGH and filtered comparable events (exonic  
38 CNVs encompassing enough aCGH probes) and obtained an 87.25% sensitivity. The overall PPV  
39 was 86.4% following the targeted confirmation of candidate CNVs from 1,056 additional WES.

40 In addition, our CANOES-centered workflow on WES data allowed the detection of CNVs of any  
41 size that were missed by aCGH. Overall, switching to a NGS-only approach should be cost-  
42 effective as it allows a reduction in overall costs together with likely stable diagnostic yields. Our  
43 bioinformatics pipeline is available at : [https://gitlab.bioinfo-diag.fr/nc4gpm/canoes-centered-](https://gitlab.bioinfo-diag.fr/nc4gpm/canoes-centered-workflow)  
44 workflow.

45

46

47

48 **KEYWORDS**

49 Exome, panel, CANOES, CNV detection, bioinformatics, sensitivity

## 50 INTRODUCTION

51 Copy-number variations (CNVs) are a major cause of Mendelian disorders (1) as well as risk  
52 factors for common diseases (2). With the advent of next-generation sequencing (NGS), a number  
53 of software tools have been developed to detect CNVs. Whole genome sequencing (WGS) is often  
54 presented as an almost universal technique allowing the assessment of almost any type of variation,  
55 including CNVs and other structural variations. WGS may eventually be used as a first-tier  
56 diagnostics tool in the context of genetically highly heterogeneous disorders. However, the  
57 detection of structural variations from data generated using the technology of short read sequencing  
58 is still associated with a number of false positives. Such events can be detected using a plethora of  
59 bioinformatics tools based on different principles, including Depth Of Coverage (DOC)  
60 information, relative position of paired reads, split reads and DeNovo Assembly (3). Besides the  
61 development of WGS, targeted sequencing of gene panels and whole exome sequencing (WES)  
62 remain of primary use in many diagnostics and research laboratories. They are indeed still  
63 considered as more affordable and of easier access as they can be processed using usual informatics  
64 facilities accessible to most laboratories. Moreover, the input of WGS is questioning in disorders  
65 with low genetic heterogeneity and high phenotypic specificity. Hence, gene panels and WES  
66 remain largely used .

67 The detection of CNVs from exonic capture-based targeted sequencing solutions primarily relies on  
68 DOC information (4,5). Tools based on DOC information compare one sample to a reference, and  
69 predict deletions or duplications depending on the increase or decrease of the DOC as compared to  
70 the reference (figure 1). As each tool was set up and trained on a specific dataset, one of the main  
71 challenges is to evaluate the specificity and sensitivity of a given software tool on large datasets.  
72 Studies evaluating the diagnostic performances of CNV detection pipelines are scarce although they  
73 appear to be critical for their use in routine procedures.

74 In order to optimize CNV detection from NGS data, a classical approach consists in running  
75 multiple tools in parallel and then aggregate the results to keep a CNV as candidate only if multiple  
76 tools called it (6). As it is more effective to do so with tools using different types of bioinformatics  
77 methods (DOC, split reads, etc.), this combinatory approach is most adapted when working on  
78 WGS, or at least if most of the intergenic or intronic regions – where breakends are more frequently  
79 found – are captured. Here, we decided to focus on one tool using the DOC approach as it still  
80 remains the most adapted one for exonic capture. In a *precision workflow* approach, we developed a  
81 workflow based on the already existing software tool CANOES (7). Briefly, CANOES adopts a  
82 pooling strategy to build its reference model, and uses a Hidden Markov Model to represent the  
83 DOC of this model. Lastly, it confronts the samples to the reference in order to call candidate  
84 deletions or duplications.

85 We performed a diagnostic performance evaluation of this workflow regarding gene panel and WES  
86 data, in two steps. First, we compared CNV calls with a reference technique, namely a  
87 comprehensive assessment by Quantitative Multiplex PCR of Short Fluorescent fragments  
88 (QMPSF) (8) or array comparative genomic hybridization (aCGH), regarding targeted gene panel  
89 and WES data, respectively. Second, we implemented our workflow in our routine procedures and  
90 performed an additional evaluation of the positive predictive value of our CANOES-centered  
91 workflow using targeted confirmation of CNVs using an independent targeted technique.

92

93

## 94 **MATERIAL AND METHODS**

### 95 **Gene panel sequencing**

96 In order to evaluate our workflow, we analyzed data from three gene panels (for detailed  
97 information, see supplementary table 1). Patients provided informed written consent for genetic  
98 analyses in a diagnostics setting.

99 Panel 1 was set up to focus on genes involved in predisposition to colorectal cancer and digestive  
100 polyposis or Li-Fraumeni syndrome (9). This panel was implemented in two successive versions.  
101 V1 was used to sequence 11 genes in 2,771 samples. V2 was used to sequence 15 genes (same 11  
102 genes plus 4) in 549 samples. In both versions and for all genes, exons and introns outside repeated  
103 sequences were captured.

104 Panel 2 also has two successive versions and was designed to focus on two clinical indications: (i)  
105 hydrocephaly (3 genes) and (ii) Cornelia de Lange syndrome and differential diagnoses (24 genes in  
106 v1, 30 in v2). In total, 320 samples were sequenced using this panel (240 with v1, 80 with v2). For  
107 this panel, introns outside repeated sequences were captured only for two genes, namely *LICAM*  
108 and *NIPBL*.

109 Panel 3 was designed to focus on genes involved in non-specific Intellectual Disability. It has been  
110 used to analyses 220 samples and is composed of 48 genes (coding regions only). The list of genes  
111 is available upon request.

112

### 113 **Assessment of CNV calls from gene panel data: step 1**

114 For the comparison to a reference technique, we used data obtained from samples for which both  
115 NGS (panel 1, v1) and comprehensive QMPSF screening data were available (n=465). This  
116 QMSPF assessment included all 60 exons of 4 genes from this panel (*APC*, *MSH2*, *MSH6*, *MLH1*)  
117 and was applied to all 465 samples.

118

## 119 **Assessment of CNV calls from gene panel data: step 2**

120 Following step 1, we implemented our CANOES-centered workflow in our routine diagnostics  
121 procedures on NGS data from all three panels (n=3,311 additional samples in total). We performed  
122 confirmations of candidate CNVs using QMPSF or Multiplex Ligation-dependent Probe  
123 Amplification (MLPA) only in samples with a CANOES call. Primers used for QMPSF screening  
124 and validation are available upon request.

125

## 126 **Whole-exome sequencing**

127 Patients provided informed written consent for genetic analyses either in a diagnostics or in a  
128 research setting, following the approval by our ethics committee.

129 Whole exomes were sequenced in the context of diverse research and diagnostics purposes  
130 (supplementary table 1). Exomes were captured using Agilent SureSelect Human All Exon kits (V1,  
131 V2 V4+UTR, V5, V5+UTR and V6) (Agilent technologies, Santa Clara, CA, USA). Final libraries  
132 were sequenced on an Illumina Genome Analyser GAIIX (corresponding to exomes captured with  
133 the V1, V2 or V4UTR kit, n=10), or on an Illumina HiSeq2000, 2500 or 4000 with paired ends, 76  
134 or 100bp reads (Illumina, San Diego, Ca, USA). Exome sequencing was performed in 3 sequencing  
135 centers: Integragen (Evry, France) (n=6), the French National Center of Human Genomics Research  
136 (CNRGH, Evry, France) (n=1,065) and the Genome Quebec Innovation Center (Montreal, Canada)  
137 (n=128) (10). Exomes were all processed through the same bioinformatics pipeline following the  
138 Broad Institute Best Practices recommendations (11). Reads were mapped to the 1000 Genomes  
139 GRCh37 build using BWA 0.7.5a.(12). Picard Tools 1.101 (<http://broadinstitute.github.io/picard/>)  
140 was used to flag duplicate reads. We applied GATK (13) for short insertion and deletions (indel)  
141 realignment and base quality score recalibration. All quality checks were processed as previously  
142 described (10).

143

144 **Assessment of CNV calls from whole exome sequencing data: step 1**

145 For the comparison to a reference technique, we analyzed data from 147 unrelated individuals with  
146 both WES and aCGH data available.

147 Array CGH Analysis. Oligonucleotide aCGH was performed as previously described (14). Briefly,  
148 high-resolution aCGH analysis was performed using the 1x1M Human High-Resolution Discovery  
149 Microarray Kit or the 4x180K SurePrint G3 Human CGH Microarray kit (Agilent Technologies,  
150 Santa Clara, California, USA), using standard recommended protocols. An in-house and sex-  
151 matched genomic DNA pool of at least 10 control individuals was used as reference sample.  
152 Hybridization results were analyzed with the Agilent's DNA-Analytics software (version 4.0.81,  
153 Agilent Technologies) or the Agilent Genomic Workbench (version 7.0, Agilent Technologies). Data  
154 were processed using the ADM-2 algorithm, with threshold set at 6.0 SD or 5.0 SD. CNVs of at  
155 least five or three consecutive probes were retained for analysis, respectively for the 1M and the  
156 180K arrays.

157 WES/aCGH comparison. Array CGH enables the detection of genome-wide rearrangements thanks  
158 to the measurement of the deviation of the fluorescent signal of the patient as compared to a control  
159 DNA. The number of probes depends of the type of chip that is used (here, Agilent 1M or 180K).  
160 The threshold to consider a deletion or a duplication was set to the deviation of 5 or 3 consecutive  
161 probes respectively. This restricts the detection to CNVs of 8kb or for 20kb Agilent 1M and  
162 Agilent180K chips, respectively, on average. On the contrary, as CANOES analysis is based on  
163 WES data, it is strictly restricted to CNVs covering exonic sequences, but it can detect CNVs as  
164 small as one single exon.

165 In order to combine these approaches to evaluate the sensitivity of our workflow, we filtered out  
166 CNVs located in intronic and intergenic regions exclusively from the aCGH data (and on X and Y  
167 chromosomes for the samples processed without gonosome CNV calling). Moreover, as CANOES



168 analysis is based on the calculation of a mean and variance of coverage on a given genomic region,  
169 the detection of polymorphic rearrangements is very uncertain. For that reason, we also filtered out  
170 all polymorphic CNVs from aCGH data. We defined as polymorphic a CNV that overlaps at least at  
171 70% with CNVs reported in the Gold Standard section of the Database of Genomic Variants with a  
172 frequency superior to 1% (15).

173 Regarding the evaluation of the positive predictive value of our workflow, we restricted our analysis  
174 to candidate non-polymorphic CNVs detected from WES data (i) that are theoretically detectable by  
175 aCGH as they encompass at least 3 or 5 probes, depending on the chip used and (ii) that do not  
176 overlap with segmental duplication regions among >50% of the CANOES target regions.

177 As most aCGH data were processed using the hg18 genome as reference, we used the liftover tool  
178 from UCSC (<https://genome.ucsc.edu/cgi-bin/hgLiftOver>) to establish the correspondence to hg19.  
179 If there were no lift over possibility, we manually checked genes encompassing CNVs.

180

## 181 **Assessment of CNV calls from whole exome sequencing data: step 2**

182 Following step 1, we implemented our workflow in our routine procedures. From additional 1056  
183 WES (supplementary table 1), we performed targeted confirmations following the detection of  
184 candidate CNVs by CANOES using QMPSF or ddPCR (16). We focused our confirmations on a list  
185 of 350 genes that belong to the so-called A $\beta$  network (17), as all the samples used at this step were  
186 sequenced in the context of Alzheimer disease research. This list of genes was built thanks to  
187 literature curation on Alzheimer pathophysiology, independently of any genomic information.  
188 Candidate CNVs were selected for targeted confirmation if (i) they encompassed genes belonging  
189 to this network, and (ii) they were not polymorphic i.e. with a frequency below 1% in our dataset.

190 Primers used for QMPSF or ddPCR validation are available upon request.

191

## 192 **CNV calling from NGS data using CANOES**

193 The CANOES software tool implements an algorithm dedicated to the detection of quantitative  
194 genomic variations based on DOC information. Basically, CANOES requires DOC data for each  
195 target of the capture kit used for each of the sample that are analyzed together. It also integrates the  
196 GC content information of each target to reduce the background variability observed in high-  
197 throughput sequencing data (18). The read depth was calculated using Bedtools (19), and the GC  
198 content was determined using the GATK suite.

199 CANOES builds its statistical reference model from a subset of the samples included in the same  
200 analysis (at least 30 samples are recommended). To obtain the best possible fit, CANOES selects  
201 the samples that are the most correlated to the currently analyzed sample. This allows the detection  
202 of small CNVs, but also reduces the detection susceptibility of recurrent events. CANOES uses a  
203 Hidden Markov Model to represent the variability of the DOC distribution built from the selected  
204 samples. Then, it uses the Viterbi algorithm to assign deletions, duplications or normal regions.  
205 After the calling step, a 'Not Applicable' (NA) score is attributed to all CNVs from samples carrying  
206 more than 50 rearrangements. Such samples are usually characterized by higher or lower average  
207 read depth and cannot be compared to the reference model. All CNVs assigned with an NA score  
208 were thus removed from further analyses. As CANOES used the capture kit definition to detect  
209 CNVs, boundaries of events were defined by the start position of the first target and the end position  
210 of the last target detected as deviated in comparison with the model.

211

### 212 **A CANOES-centered workflow**

213 To optimize CANOES performances, we focused on two different approaches, a methodological  
214 approach in sample selection and a bioinformatics approach (Figure 2).

215 As previously described, CANOES defines a statistical model for a particular sample from a  
216 judicious selection of other samples included in the analysis. The first step of our workflow  
217 consisted in the implementation of rules to select the samples that should better be analyzed

218 together. In order to get enough material to build an efficient statistical model and following the  
219 CANOES recommendations, we always worked with at least 30 samples. Importantly, we analyzed  
220 samples with the less technical variability from each other. Practically, this consists in analyzing  
221 samples from the same run, and not to merge multiple runs if not necessary. When merging multiple  
222 runs was inevitable (e.g., sequencing of less than 30 samples per run), we combined sequencing  
223 runs from the same platform and processed using the same technical conditions, including the same  
224 number of samples per lane in order to reduce read depth variability from each sample. Of note,  
225 CANOES is not originally set up for the analysis of CNVs on gonosomes, but we implemented  
226 modifications in the original script in order to include gonosomes in our analyses. Hence, we ran  
227 our workflow after gathering either  $n \geq 30$  males or  $N \geq 30$  females for the analysis of gene panels 2  
228 and 3 that contain X-linked genes and of WES data.

229

### 230 **Bioinformatics optimization**

231 The first step consisted in the modification of the target definition from the capture kit information.  
232 We decided to merge close targets (less than 30 pb) if they covered the same exon. Concerning gene  
233 panels that include introns, we decided to split large targets that include both intronic and exonic  
234 regions.

235 In order to gain flexibility in our analysis and to be able to add or remove samples easily, we  
236 implemented a two-step strategy consisting in (i) performing the read count step for each sample  
237 separately, and then (ii) aggregating selected samples before running CANOES. Doing so allowed,  
238 for example, intra-familial analyses including patient-parent trio approaches, where cases can be  
239 analyzed without taking related samples into account, preventing biasing the statistical model.  
240 Finally, we removed non-informative regions from our analyses. We considered a region as non-  
241 informative if more than 90% of the samples each had less than 10 reads on the target. Then, we

242 called the CNVs using CANOES, and annotated the results using AnnotSV (20) in order to get  
243 additional information about the possible effect and populations frequencies.

244

#### 245 **Nextflow integration**

246 In order to complete our optimization of processing and analysis time, we integrated our  
247 bioinformatics pipeline into Nextflow, a data-driven workflow manager (21). This software tool  
248 allows a quick deployment of new pipelines on different kind of computational environments, from  
249 local computers to a cloud environment. Another interest of Nextflow is to increase the performance  
250 by distributing the different steps of the workflow in regards to the computational resources  
251 available. The complete workflow, including the specific adaption of CANOES to analyze  
252 gonosomes, is available on <https://gitlab.bioinfo-diag.fr/nc4gpm/canoes-centered-workflow>.

253

## 254 **RESULTS**

255 After building a workflow centered on the CANOES tool, we assessed its performances in the  
256 context of (i) gene panel NGS data and (ii) WES data, both generated following capture and  
257 Illumina short read sequencing.

258

#### 259 **Gene panel sequencing data**

260 We first evaluated the performances of the CANOES tool using targeted sequencing data of a panel  
261 of 11 genes (panel 1, n=465 samples). In parallel, all samples were assessed using custom  
262 comprehensive QMPSF assessing the presence or absence of a CNV encompassing any of the 60  
263 coding exons of 4 of these genes. We identified 14 CNVs by QMPSF (12 deletions, 2 duplications,  
264 size range: [1,556pb – 97Kpb]). All of them were accurately detected by our CANOES-based  
265 workflow from NGS data (Table 1). In addition, no additional CNV was called by CANOES,

266 allowing us to obtain a sensitivity and a specificity of 100% (95%CI:[73.24-100]) for those 4 genes.  
267 (see supplementary table 2).  
268 To further assess the Positive Predictive Value (PPV) of our workflow in the identification of CNVs  
269 from gene panels, we applied it to additional NGS data obtained from 3 gene panels (2,222 samples  
270 from panel 1, 320 samples from panel 2, and 220 samples from panel 3). We detected 101 candidate  
271 CNVs in 98 samples and assessed their presence using either QMPSF or MLPA (Table 2). We  
272 validated 87/101 CNVs (86.13%, 95%CI:[77.50-91.94], false positive rate: 13.9%). Overall, the  
273 PPV of our workflow applied to gene panel sequencing data was 87.83% (95%CI:[80.01-92.94]).  
274 True positive calls of our workflow were 73 deletions (size range: [391pb – 1.06Mpb]) and 16  
275 duplications (size range: [360pb – 39.4Kpb]) (see supplementary table 3). False positives were  
276 mainly deletions (10/14) and 5 of them were monoexonic.

277

#### 278 **Whole exome sequencing data**

279 We then evaluated the performances of our workflow for the detection of CNVs from WES data.  
280 We first applied our workflow to the data obtained from 147 samples with both WES (average  
281 depth of coverage = 110x) and aCGH data available (50 samples assessed with the Agilent 1M chip  
282 and 97 samples with the Agilent 180k chip). Overall, 10 samples were removed due to a high or low  
283 number of rearrangements detected by aCGH or exome, mostly due to low DNA quality or low  
284 coverage in WES.

285 From aCGH data, we detected 1,873 CNVs over the 137 samples remaining, of which 102 were  
286 non-polymorphic exonic CNVs. Our workflow accurately detected 89 (87.2%) of them (Table 1,  
287 supplementary table 4). Among the CNVs that were missed by our workflow, 7 were large (from 14  
288 to 80kb) CNVs that encompassed only one (n=5) or two (n=2) targets defined by the capture kit  
289 (see figure 3).

290 In order to determine the PPV of our workflow from WES data, we selected 223 CNVs called by  
291 our workflow and (i) theoretically detectable by aCGH as encompassing at least 3 (180 k chips) or 5  
292 (1M chips) probes and (ii) which did not overlap with segmental duplication regions for more than  
293 50% of the CANOES targets. Of them, 190 (85.2%) CNVs were confirmed as true positives  
294 following aCGH data assessment (Table 1, supplementary table 5).

295 Of note, an additional set of 519 candidate CNVs were detected by our CANOES-based workflow  
296 that overlapped less than 50% of segmental duplication regions but encompassed less than 3 (180 k  
297 chips) or 5 aCGH probes (1M chips). Hence, they were not reported by the CGH analysis tool and  
298 would then have been overlooked following classical aCGH data analysis. We did not perform  
299 targeted confirmation of all these candidate CNVs. Instead, with the aim to further assess the PPV  
300 of our workflow regarding exonic non-polymorphic CNVs of any size, we applied it to 1,056  
301 additional WES performed in the context of Alzheimer disease research (with no corresponding  
302 aCGH data). We selected non-polymorphic CNVs targeting 355 genes belonging to the A $\beta$  network  
303 involved in the pathophysiology of Alzheimer disease (17), whatever their size. We validated  
304 108/122 candidate CNVs (88.5%, false positive rate: 11.5%) by QMPSF (22) or ddPCR (Table 2,  
305 supplementary table 6). True positive calls of our workflow were 39 deletions (size range: [165pb –  
306 24,2Mpb]) and 69 duplications (size range [166pb – 5,9Mpb]). Interestingly, among the 122  
307 candidate CNVs obtained from our workflow, 75 were considered to be theoretically detectable by  
308 aCGH 1M, and 47 were considered as not detectable by aCGH 1M. Among the ones theoretically  
309 detectable by aCGH, 71 were true positives (94.6%). Among the theoretically not detectable ones,  
310 37 were true positives (78.7%).

311 Overall, the PPV of our CANOES-based workflow was 86.3% from WES data after taking into  
312 account results from step 1 and step 2 altogether.

313

314 **DISCUSSION**

315 Multiple tools have been developed to detect CNVs from NGS data. As long as such tools are being  
316 implemented in diagnostic laboratories, there is a critical need to evaluate their performances.  
317 Previous studies showed a large diversity of performances, while a number was performed using  
318 simulated datasets (23). After having defined a CANOES-centered workflow, we applied it to three  
319 different gene panels and WES data. Overall, we reached very high detection performances  
320 following the comparison with independent techniques.

321 From gene panel data, we obtained a 100% sensitivity among a set of 4 genes, the copy number of  
322 all coding exons of which having been assessed prior to NGS in 465 samples. In addition, we  
323 obtained a 90.3% PPV among all genes with a CANOES call. Such high performances have  
324 previously been reported for other tools applied to small NGS panels (24). Among 14 false  
325 positives, we observed recurrent events, which can be easily reported as so and be ignored in further  
326 analyses. We also observed false positive CNVs in regions homologous to pseudogenes. In that  
327 case, it is possible to reduce false positive calls by improving the design of the capture to reduce the  
328 chance that probes target the homologous regions, or by optimizing the alignment.

329 Of note, for all genes of Panel 1 and two genes of Panel 2, introns were captured in addition to  
330 exons. This might have increased the chances to detect CNVs that can be considered as small from  
331 an exon-only point of view but that can actually be much larger at the genomic level. An advantage  
332 of capturing introns might indeed be a gain in statistical power for the normalization process:  
333 increasing the number of targets may increase the robustness of the model. Among 101 CNVs  
334 detected from NGS data from all 3 panels, 75 CNVs encompassed one of these genes with intronic-  
335 plus-exonic capture. Interestingly, only 18 of these 75 CNVs encompassed a single coding exon.  
336 Such a frequency of monoexonic CNVs is not unexpected regarding mutation screens in MMR  
337 genes (monoexonic deletions accounting for 26.92 to 46.27% of all pathogenic deletions (25–27), or  
338 other rare diseases (28–31), for example. We hypothesize that all other CNVs, encompassing  
339 multiple targets, would probably have been easily detected, had the introns been excluded from the

340 capture design. Further analyses may be required to better assess the performances of our workflow  
341 from single exon CNVs and the effect of including introns or not in the capture design. The  
342 observed higher rate of false positives in CNV calls encompassing genes without introns captured  
343 (22.22%) may also require further assessments,

344 We used here a *precision workflow* approach, focusing on the optimization of one tool based on  
345 DOC. Interestingly, as some of our genes included non-coding sequences in gene panels, these  
346 specific exonic-plus-intronic captures could provide us the possibility to apply complementary tools  
347 using different approaches, like the ones developed for WGS. This can indeed increase both  
348 detection performances of CNVs and the spectrum of structural variants that can be detectable in  
349 these data.

350 Of note, all our panels included multiple genes. We do not expect that a design including a single  
351 gene, even with its intronic sequences, would reach the sufficient number of targets for CANOES to  
352 build a robust model.

353 We also applied our workflow to multiple WES datasets and reached an overall PPV of 86.38 %  
354 (95%CI:[82.19 – 89.72]). As for gene panel CNV detection, a confirmation by an independent  
355 technique is hence still required following the detection of a candidate CNV from WES data,  
356 although this high value allows a limited number of molecular confirmations. One of the major  
357 features usually required to apply a new technique in a diagnostic workflow is a high sensitivity as  
358 compared to a reference technique. Here, we reached a sensitivity of 87.25% (95%CI:[78.84 –  
359 82.77]). Although the sensitivity was not 100%, it is important to notice that aCGH is considered as  
360 reference here although the spectrum of events that can be detected is still limited. When comparing  
361 our results to aCGH data, it appeared that we missed fewer events than the potential number of true  
362 positive CNVs that were missed by aCGH itself. Indeed, from aCGH data, we missed 13 CNVs, but  
363 our analyses called 519 candidate CNVs from corresponding WES data and which were  
364 theoretically undetectable by aCGH (i.e. either small CNVs or in regions with no aCGH probes



365 coverage). Our PPVs suggest that the vast majority are eventually true. There is no reason to think  
366 that some of the CNVs detected by CANOES only might not be as or more deleterious than CNVs  
367 detected by both techniques or exclusively by aCGH. Knowing that aCGH misses many CNVs,  
368 even using the high-sensitivity chips such as the Agilent 1M one, and even if other chip designs  
369 might increase aCGH performances on coding regions, switching to a WES-only approach for CNV  
370 detection in a diagnostic setting should not reduce the overall diagnostic yield while allowing a  
371 significant drop of costs.

372 As compared to aCGH, CANOES allowed the identification of CNVs of any size in regions not  
373 covered by probes but also for small CNVs including few exons. In addition, it is important to  
374 notice that the majority of CANOES false negatives were also CNVs with only few exons, which  
375 implies few targets for CANOES although non-coding probes may help detect some of them by  
376 aCGH. This decreased rate of detection of CNVs encompassing few targets has already been shown  
377 in other datasets (32,33) and appears as a limitation inherent to DOC comparison methods.

378 Of note, it is possible to increase the detection of small events or events in complex regions by  
379 using the “GenotypeCNV” function of CANOES. The aim of this function is to look precisely at  
380 specific regions and call the genotype of the sample for these specific regions, however it is  
381 associated with an increase in false positive calls (29), as well as an increase in time and  
382 computational resources needed. In particular cases, when known core genes have already been  
383 identified in a given disorder, it is possible to combine our approach to call CNVs at the exome  
384 level and focus on specific genes using the GenotypeCNV function applied to every exon of these  
385 genes to increase the detection performances in core genes at the same time.

386 Of note, beyond the above-mentioned limitations of CNV detection tools from NGS data, somatic  
387 CNVs remain a challenge, both for array-based technologies and for NGS-based tools (34). Among  
388 the CNVs detected by our workflow, at least one was considered as likely somatic, as suggested by  
389 QMPSF data. However, the sensitivity of DOC tools might remain low in this context (34).

390 In conclusion, we performed an evaluation of the performances of a CNV detection workflow based  
391 on read depth comparison from capture-prepared NGS data, one of the most popular methods for  
392 NGS in research and diagnostic settings. We highlight very high sensitivity and positive predictive  
393 value, for both NGS gene panel and whole exome sequencing. Although the sensitivity was not  
394 perfect for WES data as compared to aCGH, a number of additional true calls were not detected by  
395 the so-called reference technique. This highlights the absence of a genuine gold standard up to now.  
396 Overall, we consider that switching to a NGS-only approach is cost-effective as it allows a  
397 reduction in overall costs together with likely stable diagnostic yields.

398

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405

#### 406 **CONFLICTS OF INTEREST**

407 None

408

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508

509 **FIGURE LEGENDS**

510 **Figure 1. Principles of Depth Of Coverage (DOC) comparison.** Schematic distribution of reads  
511 among three different samples over 5 sequenced exons. **(A)** absence of any CNV. **(B)** Duplication of  
512 two exons (2 and 3). **(C)** Deletion of exon 4. In order to call those CNVs, software tools have to  
513 establish a reference. Some tools compare paired data from the same patient, *e.g.* tumor tissue  
514 against germline, while others build their reference from a pool of samples and then compare a  
515 given sample to this reference, as the CANOES tool used in our workflow.

516

517 **Figure 2. CANOES-centered workflow.** File (square) with their format in parenthesis, and  
518 process (rounded) constituting the workflow. From the original capture kit definition, we merge  
519 closed target from the same exon, then do in parallel the DOC and the GC content estimation. We  
520 regroup DOC individual files depending on the project, sequencing batch, unrelated samples, and  
521 remove non-informative regions. The last steps consist in CNV calling using CANOES and  
522 annotation with annotSV.

523

524 **Figure 3. Example of a CNV detected by aCGH but missed by the CANOES-centered**  
525 **workflow.**

526 A CNV (highlight region) detected by a-CGH encompassing multiple CGH probes (1M probes  
527 array, in gray) but only one target from the SureSelect V5 capture kit. Of note, this deletion would  
528 have been missed by using a 180k probes array CGH (in black).

529

530 **Figure 4. Example of CNVs detected by the CANOES-centered workflow from WES data but**  
531 **missed by aCGH.**

532 A. The highlighted region represents the CNV called by the CANOES-centered workflow,  
533 encompassing one exon of *RHCE*.

534 B. View of the same region from DNA-Analytics (aCGH data 1M) in the same patient. This deletion  
535 was not called following aCGH data analysis as the number of deviated probes did not reach the  
536 threshold for calling. However, as 3 probes (in white) were deviated, this allows the confirmation of  
537 the deletion of the region.

538

539

## 540 **Appendix. Collaborators**

### 541 **The FREX Consortium**

542

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