# Study of Visual Review and Sanger Confirmation Outcomes in Exome Sequencing Testing

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## Introduction

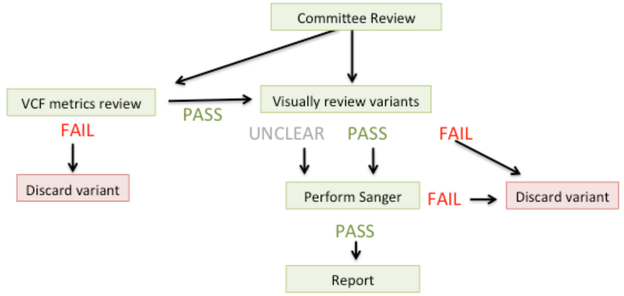
Next-generation sequencing (NGS) is widely used in Exome Clinical Diagnostic Testing. However, the main current standard of care for reporting germline small variants such as Small Nucleotide Changes (SNVs) and indels remains orthogonal confirmation through capillary sequencing.

We investigated the necessity of capillary confirmation to achieve clinical-grade quality and accuracy in the presence of a rigorous visual review process. The ultimate goal has been to determine on whether an orthogonal confirmation Policy Change was clinically sound from a Quality Assurance perspective.

## Methods

### Brief Overview of Variant Selection and Confirmation Processes at Personalis

Based on a consolidated Small Variants Orthogonal Confirmation Policy (implemented since ACE Clinical Exome testing offerings inception), all NGS-identified and clinically reported variants have been subjected to a two-steps confirmation: 1) Visual Inspection (interchangeably called visual review in this presentation) of pileup reads; 2) Sanger (Capillary) sequencing confirmation. Briefly, after NGS-identified variants filtering and phenotypic prioritization has occurred, a scientific-medical "Committee Review" discussion of variants step occurs, thus determining the set of variants to be further classified and clinically reported. In preparation and conjunction with this step, a "Visual Review" step is performed consisting of a technical analyst manually inspecting pileup reads in JBrowse according to the guidelines as documented in a dedicated Procedure (Standard Operating Procedure or SOP). Key to this process is, and not limited to, assessing on whether a) the variant presentation is in concordance with predicted zygosity, b) supporting reads do not show significant mismatches/soft-clipping, c) the variant is present on both strands and d) the variant is not always positioned at the end of the read. The visual review step also includes the analysis of metrics from the vcf as documented in the SOP, such as read depth, genotype quality scores, quality filters and allelic frequency. As part of the Sanger variant confirmation process, gene variant specificity is assured by *in silico* approaches not limited to BLAST of the amplicon to the reference genome to confirm that the amplicon is unique to that locus (gene) and to rule out the possibility of reporting variants located in paralogous regions and / or pseudo-genes. A picture depicting the above process is presented below:




### Brief Overview of this Study Methodological Approach

In order to ascertain the necessity of clinically performing or not Sanger sequencing confirmation for all variants, we sought to assess the specificity and sensitivity of Visual Inspection of pileup reads as a means to confirm SNVs using Sanger-confirmed variants as a "gold standard" validated set. We further aimed to use this study to improve metrics to guide Visual Inspection processes.

To this aim, we reviewed the concordance of outcomes from Visual Inspection with capillary sequencing results for SNVs in our clinical laboratory over a time-defined reporting window. The outcomes of Visual Inspection were classified into the following categories:
1) PASS (both SOP-defined passing QC metrics, plus presence and zygosity of the variant is confirmed), permitting the variant to progress to Classification and Sanger confirmation prior clinical reporting;
2) UNCLEAR, because the variant only partially meets SOP-defined Quality Metrics and passing criteria, thereby requiring Sanger confirmation prior to progress to Classification;
3) FAIL, because the variant overtly and clearly does not meet SOP-defined Quality Metrics and passing criteria

In summary, variants that clearly passed or were deemed "unclear" at the Visual Inspection step – unless dismissed for other non-technical reasons - would progress to Sanger confirmation.

## Results

Over this study time-defined observation window, visual review was performed on 178 clinical reporting candidate SNVs, 161 of which passed visual review. Of the variants that did not progress to clinical reporting, 15 failures were technical, based on the variants not meeting the SOP-defined JBrowse and vcf Quality Metrics. Additionally, the status of 2 variants was unclear after visual review and had to be clarified by Sanger confirmation.
Results are summarized below:

| Review Outcome | Number of Variants | Passing or failing fraction |
|---|---|---|
| Visual Review = PASS | 161 | 0.91 |
| Visual Review = FAIL (Technical) | 15 | 0.08 |
| Visual Review = UNCLEAR (Technical) | 2 | 0.01 |
| **Total** | **178** | |

Next, out of the total 163 variants that met PASS or UNCLEAR criteria, only 120 variants were selected for clinical reporting based on molecular genetics clinical reporting criteria (e.g. relevance of the variant to the proposed disease) and selected for final Sanger confirmation prior clinical report inclusion. The outcome of Sanger confirmation results are reported below:

| Variant Visual Review status | Sanger confirmation = PASS | Sanger confirmation = FAIL |
|---|---|---|
| PASS | 118 | 0 |
| UNCLEAR | 1 | 1 |

In summary, we found that all (100%) SNVs that unambiguously passed Visual Inspection steps according to approved (SOPs) were confirmed by capillary sequencing. Only two variants were described and flagged as 'unclear' or 'ambiguous' based on visual inspection; the first variant was confirmed being present by capillary sequencing, while the second was confirmed negative for the allele (i.e. being absent upon Sanger sequencing traces analysis).

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## Conclusions

Our results demonstrate that, if appropriately applied, visual review quality steps are highly robust in predicting SNVs that will pass capillary confirmation, and conservative in flagging those that might fail. None of the variants that passed visual review were revealed as false positive upon Sanger confirmation. However, Sanger confirmation of all SNV variants that remain unclear at visual review is still warranted and performed by the Personalis Clinical Laboratory.

It is the current practice (i.e. the Small Variants Confirmatory Policy at Personalis) of performing Sanger confirmation for all indels, *de novo* (when suggested by NGS proband-parents testing), and mosaic variants, and for SNVs with unclear Visual Inspection outcomes. Variants confirmation is also required for all variant types when mandated by agencies specific regulatory requirements such as New York State patient specimens.

In addition to Visual Review steps performed by directly inspecting JBrowse pileups, additional *in silico* steps are implemented to ascertain on whether a gene variant falls into a highly homologous region or a gene with known paralogous sequences or pseudo-genes. To this purpose, routine *in silico* approaches are adopted in parallel with Visual Inspection steps such as e.g. usage of the Variation Viewer web browser (centered on the gene and the gene variant chromosomal coordinates), and BLAST of the chromosomal sequences surrounding the variant of interest.

Taken together, our data demonstrate that clinical-grade quality and accuracy of Exome Sequencing Testing can be achieved without capillary sequencing confirmatory steps in reporting SNVs if proper visual review of next-gen sequencing traces is implemented in conjunction with additional *in silico* complimentary approaches. Additional data correlating visual review with capillary sequencing confirmation steps are currently gathered, in particular in relation to other variation types (such as indels, ambiguous quality variants, mosaic variants and variants in "at risk" regions), since concerns of false-positives for insertions and/or deletions warrant further investigation and visual review pass *versus* fail quality metrics definition.

Additional Quality Assurance steps are in progress to monitor the proper implementation of the above described Small Variants Confirmatory Policy and to take proper corrective actions as / if applicable.

## References

1) Variation Viewer - https://www.ncbi.nlm.nih.gov/variation/view/
2) BLAST - https://blast.ncbi.nlm.nih.gov/
3) CAP Molecular Pathology Checklist - MOL.35850 NGS Confirmatory Testing
4) NYS DOH – Guidelines for Validation Submissions of Next Generation Sequencing (NGS) assays under the NYS Testing Category of Genetic Testing –Molecular

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