ClearSeq Plus Workflow

1. Plan run
   - DQC Calculator
   - Sequencing Calculator

2. Multiplex PCR
   - DNA sample
   - nplexes per single sample

3. Quality Control
   - 2% Agarose
   - Microfluidic-based method

4. Mixing plexes of single sample

5. Purification
   - Amplicon library
   - Purified amplicon library

6. Dilution step & Universal PCR
   - Purified amplicon library
   - Tagged amplicon library

7. Quality Control
   - 2% Agarose
   - Microfluidic-based method
   - GeneScan pattern on ABI capillary sequencer

8. Purification
   - Tagged amplicon library
   - Purified tagged amplicon library

9. Equimolar pooling
   - Purified tagged amplicon library
   - Sequencing sample

10. NGS (Sequencer)

11. Data analysis

Agilent Technologies
<table>
<thead>
<tr>
<th>N°</th>
<th>Information</th>
<th>Turn-around time (* hands-on time)</th>
<th>SFP</th>
</tr>
</thead>
</table>
| 1  |  • Quality Control extracted DNA  
  • **DOC Calculator:** advised for FFPE-derived DNA, based on outcome Agilent QC-Plex  
  • **Sequencing Calculator:** schedule samples to combine in one sequencing run                                                                 | ~30 min*  
  2h 15 min | Yes |
| 2  |  • Prepare Multiplex master mix per plex (n plexes) using ClearSeq kit  
  • Add DNA: 20-50 ng per plex (HMW) or according to QC-Plex output (FFPE)  
  • Run PCR                                                                                                                                  | ~15 min*  
  ~30 min* | |
| 3  |  • Recommended for germline ClearSeq assays only                                                                                                                                                    | ~30 min* | |
| 4  |  • Mix all plexes per sample (predefined mixing scheme) to obtain complete single-tube amplicon library  
  • Amplicon library still contains small residual DNA fragments (dNTPs, primers/primer dimer)                                                | ~15 min* | |
| 5  |  • Add optimized volume of Agencourt AMPure XP magnetic beads to amplicon library  
  • Purify and elute with water                                                                                                              | ~30 min*  
  ~45 min (30 min*)  
  2h 15 min | Yes |
| 6  |  • Prepare one Universal master mix per sample using MID kit  
  • Add diluted purified amplicon library  
  • Run PCR  
  • Tagged amplicon library still contains small residual DNA fragments (dNTPs, primers/primer dimer)                                   | ~15 min*  
  ~30 min*  
  2h 15 min | Yes |
| 7  |  • Recommended for all ClearSeq Plus assays                                                                                                                                                    | ~30 min* | |
| 8  |  • Add Agencourt AMPure XP magnetic beads to tagged amplicon library  
  • Purify and elute with water                                                                                                              | ~30 min*  
  ~45 min (30 min*)  
  2h 15 min | Yes |
| 9  |  • Measure concentration of each purified tagged amplicon library  
  • Note that for assays utilizing Amplification Reagent 3 (AR3) spectrophotometry needs to be used  
  • Dilute libraries in TE and pool equimolarly to obtain single-tube sequencing sample                                                      | ~30 min* | |
| 10 |  • Prepare sequencing template according to NGS system manufacturer’s instructions  
  • Sequence                                                                                                                                  |                  |     |
| 11 |  • Data analysis                                                                                                                                                                                                                                                                    |                  |     |

CNV = Copy Number Variation  
FFPE = Formalin-Fixed Paraffin-Embedded  
HMW = High Molecular Weight  
NGS = Next-Generation Sequencing  
SFP = Safe Freezing Point (conditions between -15°C and -25 °C)  
TE = Tris-EDTA buffer  

U.S. and Canada  
**genomics@agilent.com**  
PR7000-0719  
© Agilent Technologies, Inc. 2017  
Printed in USA, September, 2017  
For Research Use Only. Not for use in diagnostic procedures.  
5991-8366EUC