ClearSeq Workflow

1. Plan run
   - DQC Calculator
   - Sequencing Calculator

2. Multiplex PCR
   - DNA sample
   - n plexes per single sample

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

4. Dilution step & Universal PCR
   - n plexes per sample
   - n tagged plexes per sample

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

6. Mixing plexes of single sample

7. Purification
   - Tagged amplicon library

8. Equimolar pooling
   - Purified tagged amplicon library

9. NGS (Sequencer)
   - Purified tagged amplicon library
   - Sequencing sample

10. Data analysis

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<table>
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<tr>
<th>N°</th>
<th>Information</th>
<th>Turn-around time (* hands-on time)</th>
<th>SFP</th>
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</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 (nplexes) using ClearSeq kit  
• Add DNA: 20-50 ng per plex (HMW) or according to QC-Plex output (FFPE)  
• Run PCR | ~30 min*  
2h 15 min | Yes |
| 3  | • Recommended for germline ClearSeq assays only | ~30 min* | |
| 4  | • Prepare one Universal master mix per sample (nplexes) using MID kit  
• Add diluted n plexes per sample  
• Run PCR | ~30 min*  
~15 min*  
2h 15 min | Yes |
| 5  | • Recommended for all ClearSeq assays | ~30 min* | |
| 6  | • Mix all plexes per sample (predefined mixing scheme) to obtain complete single-tube tagged amplicon library  
• Tagged amplicon library still contains small residual DNA fragments (dNTPs, primers/primer dimer) | ~15 min* | Yes |
| 7  | • Add Agencourt AMPure XP magnetic beads to tagged amplicon library  
• Purify and elute with water | ~45 min  
(30 min*) | Yes |
| 8  | • 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* | |
| 9  | • Prepare sequencing template according to NGS system manufacturer’s instructions  
• **Sequence** | | |
| 10 | • 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**  
**For Research Use Only. Not for use in diagnostic procedures.**