ThunderBolts™ Open Source Guide

Design, development and validation of a custom NGS Assay

RainDance Technologies, Inc.

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The RainDance ThunderBolts System/RainDance Source and ThunderBolts Open Source are for Research Use Only. Not for use in diagnostic procedures.

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1. Introduction to ThunderBolts NGS Sequence Enrichment

ThunderBolts Open Source Guide provides a "how-to" guide for users looking to apply RainDance Technologies' microfluidic-based droplet PCR enrichment chemistry to create a custom ThunderBolts NGS Panel for Illumina sequencing. The ThunderBolts Open Source features a fast and simple workflow that is optimized for the Illumina NGS systems and compatible with FFPE, tissue and plasma sources with as low as 10 ng of amplifiable gDNA. With the ThunderBolts Open Source workflow, researchers can go from sample to NGS data in 2-3 days.

In general, the streamlined ThunderBolts Open Source workflow for customized targeted resequencing panels is the same as currently implemented for pre-validated (fixed) panels released by RainDance (Figure 1).

Figure 1: Generic workflow for a typical ThunderBolts NGS enrichment Assay. Turnaround time (TAT) is dependent on amplicon read length, the sequencer used and the version of the sequencing kit used. TAT from the RainDance sample enrichment start point to raw sequencing data output (fastq) is 48h-51h if running the MiSeq v2 kit (8-samples).
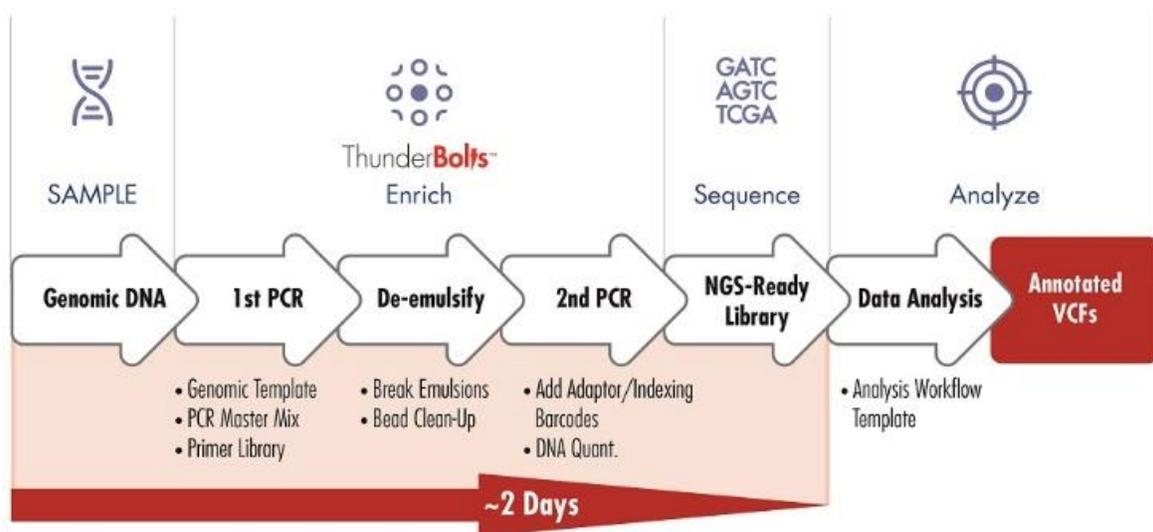
ThunderBolts Wet Lab Workflow Details Time to Process and Possible Stopping Points

Times reference processing of 2 source chips (16 lanes).

- Prepare 1st PCR reactions (30 min) and generate droplets (ThunderBolts System[1], 70 min)
- Cycle 1st PCR (3.5hr) *storage o/n in cycler at 12C
- 55-cycle PCR targets region of interest with targeted primers. Each targeted primer is tailed with RainDance DirectSeq™ universal adapters to allow direct sequencing post 2nd PCR
- Collapse emulsions and remove oil phase (16 min) *storage at -20C
- Paramagnetic bead-based cleanup (35 min) *storage at -20C
- Removes primer dimers
- 2nd PCR (85 min)
- 10-cycle PCR incorporates RainDance DirectSeq "primers" for sample indexing and Illumina platform specific adapters for cluster generation/sequencing
- Paramagnetic bead-based cleanup (35 min) *storage at -20C
- Quantify amplicon yields (90 min) *storage at -20C
- Agilent Bioanalyzer or Tape Station (size/concentration).
- Pool samples for Illumina sequencer run
- Illumina sequencing
- Sequence data analysis

[1] The ThunderBolts System is the same as the RainDance Source instrument.

In all cases, the RainDance DirectSeq method streamlines library generation for Illumina MiSeq sequencing with adaptors and indexes attached via PCR-amplification. ThunderBolts Open Source utilizes DirectSeq methodology, wherein a tailed primer strategy enables directional addition of Illumina NGS adapters along with indexes which allow sample multiplexing during sequencing (Figure).

Figure 2: RainDance's DirectSeq method enables direct NGS-platform specific sequencing. In detail, this tailed primer strategy allows directional addition of the Illumina adaptor and index sequences during a 2-step PCR. [SS-F/R: sequence specific forward/ reverse primer, Tail-F or Ad-F: reverse primer adapter tail, Illumina R Ad: Illumina reverse adapter]
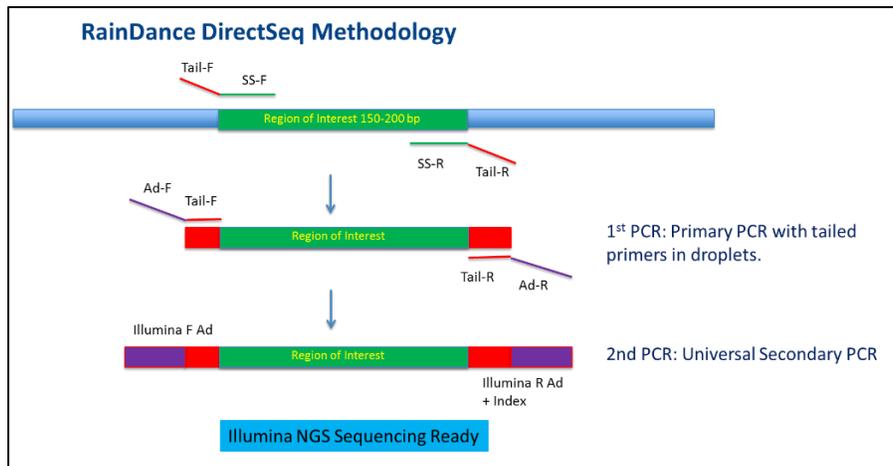
In the first step, primers designed to the region of interest are tailed with directional adapters, which are incorporated during the 1st PCR. In the second step, primers containing sequences complementary to 1st step tails allow for PCR-based directional addition of Illumina platform specific sequencing adapters and sample indexing. Final PCR amplicons contain the target regions of interest, adaptor sequences necessary for Illumina cluster formation, and indexes for sample multiplexing.

While the overall work flow is consistent from panel to panel, targeted amplicon length will affect some processes. This document will address this subject and further detail the DirectSeq methodology, as well as guide the design, development and validation of a custom panel using the ThunderBolts Open Source (48-well or 96-well) Pack.

2. Panel Design

For the design of a custom targeted resequencing NGS panel, the following should be considered:

- DirectSeq Methodology
- Panel Assay Design (1st PCR Primers)
- Sample Indexing (2nd PCR Primers)
- NGS Coverage
- Amplicon Length and PCR Cleanup
- NGS platform specific sequencing requirements

2.1 DirectSeq™ Methodology (1st PCR Adapter Tails and 2nd Round PCR Primers)

When designing PCR assays for custom ThunderBolts Panels it is necessary to understand the RainDance DirectSeq Methodology in detail as DirectSeq adapters (1st PCR) and primers (2nd PCR) affect primer sequence and ultimate amplicon size (Figure). Final PCR products contain 126 bp of sequence additional to targeted region of interest. In detail, final (2nd step) PCR amplicons contain (1) target regions of interest, (2) sequences required for direct Illumina sequencing, and (3) six nucleotide molecular barcodes for sample indexing.

- **1st PCR step** - Primers designed to the region of interest are tailed with directional adapters which are incorporated during 1st PCR. These tailed adapters are each 17 nucleotides in length, adding a total of 34 nucleotides to targeted first step PCR products.
- **2nd PCR step** - Primers containing sequence complementary to 1st step tails allow for PCR-based directional addition of Illumina platform specific sequencing adapters and sample indexes. The forward and reverse 2nd PCR primers are respective 60 and 66 bases in length. The reverse primers alone enable sample indexing. Together, the 2nd PCR forward and reverse primers add an additional 92 bases to the 1st PCR amplicon lengths.

Allowing for both the 1st PCR (34bp) and the 2nd PCR (92bp) targeted sequence adapter additions, final 2nd PCR products are 126bp greater than the target amplicon sequences themselves. Therefore, a targeted library of 150-200bp results with a true amplicon size range of 276-326bp.

Figure 3: RainDance DirectSeq Methodology - A 2-step PCR incorporates molecular sample barcoding and platform-specific sequencing adapters, forming secondary PCR products appropriate for direct Illumina sequencing with no further library preparation steps.
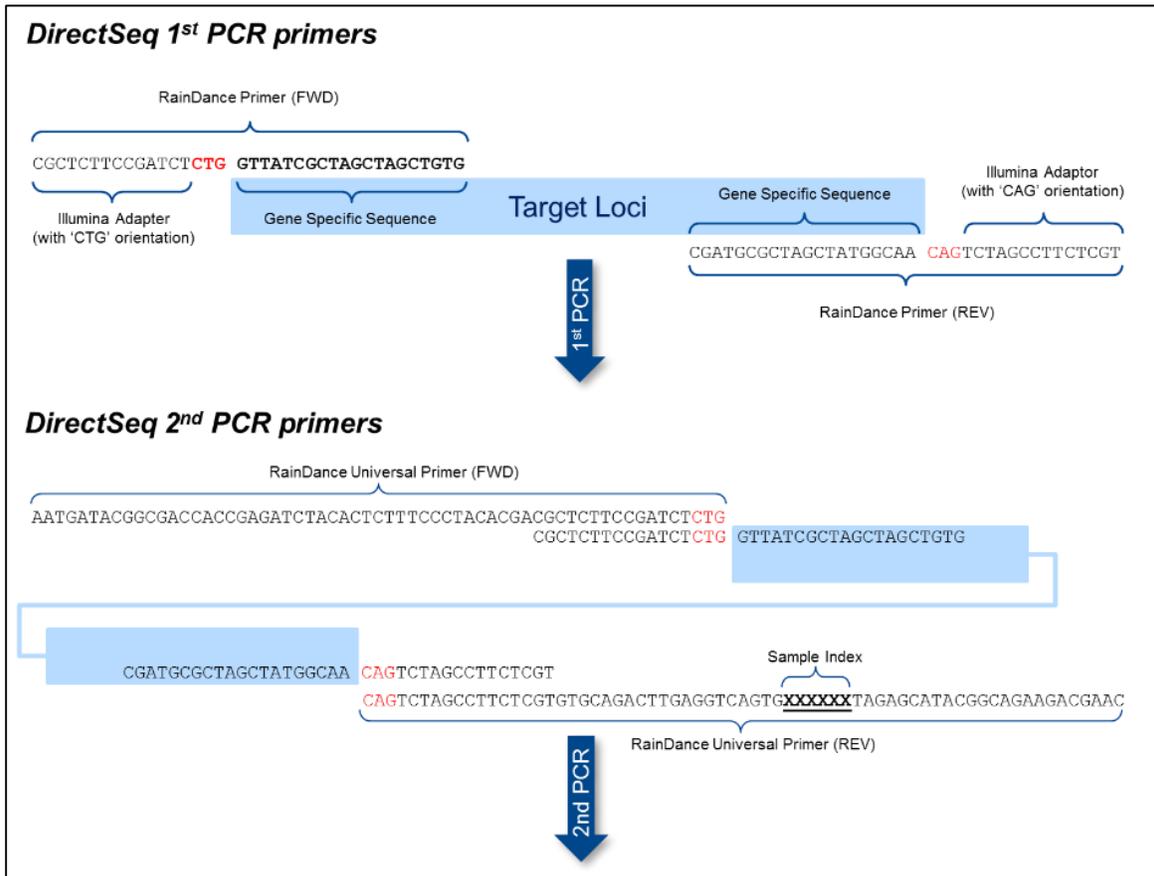
The 3 nucleotide orientation sequences (highlighted red in Figure 4) provide orientation for the 2nd round primers off the 1st round PCR products and thus, ultimately sequence directionality. As illustrated, the orientation sequences (red) sit at the 3' end of the RainDance sequencing primers (

Figure). This placement necessitates the use of RainDance specific sequencing primers when sequence orientation is desired. If sequence orientation is not of interest, the standard Illumina-platform specific sequencing primers may be utilized.

Figure 4: RainDance DirectSeq 2nd PCR amplicon is suitable for direct Illumina platform specific sequencing.
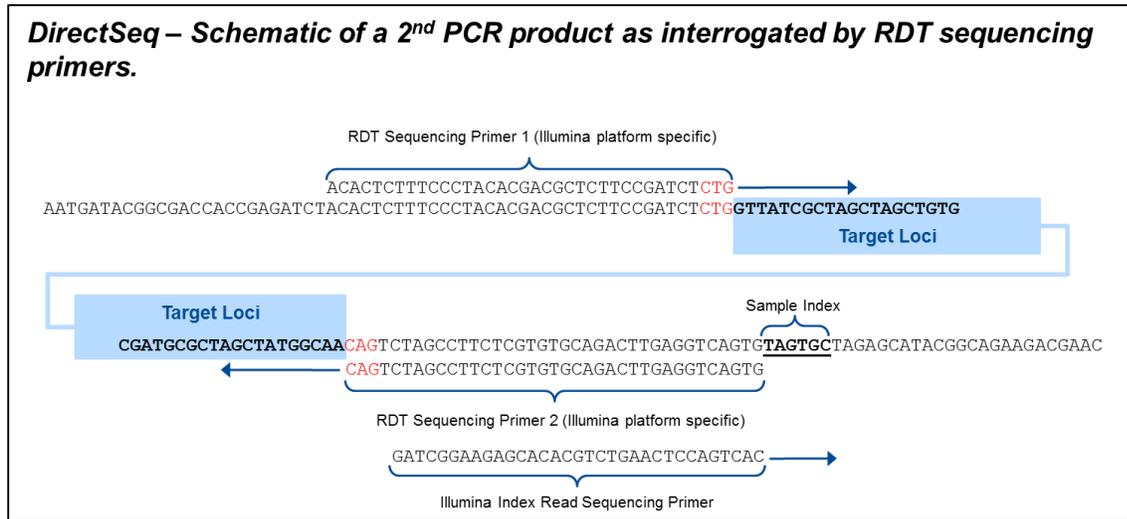
2.2 Panel Assay Design (1st PCR Primers)

Panel assay design (or 1st PCR primer design) can be broken up into two stages.

- 1st PCR primers designed to target region of interest.
- RainDance DirectSeq adapters added in silico to 1st PCR primers.

2.2.1 1st PCR primers designed to target region of interest.

During the panel design phase, a bioinformatics pipeline should be used to design multiplex PCR primers targeting regions of interest and amplicons of similar size. Briefly, RainDance suggests such a bioinformatics pipeline is based on Primer 3.0 conditions, optimally targeting melting temperatures suitable to a 54°C Tm and gene specific primer sequences of 20 nucleotides. For the best result, target amplicons of similar size and avoid hairpins and primer dimers. Furthermore, during the final stage of primer design, each primer pair should be blasted back to the human genome, hg19, to ensure low cross-reactivity. In addition, primer pairs should be blasted to the human dbSNP to limit variant sequence underlying 3' primer ends and resulting amplification skew. A minimum of three iterations of primer sets should be generated *in silico*. Back up designs aid overall panel development turnaround time with backup primer pairs readily available for panel implementation in cases of first round primer design failure.

During the primer design step, it may be necessary to design overlapping amplicons to cover target regions that are longer than the constrained length of the amplicons. If that is the case, overlapping amplicons should be placed in separate reactions to eliminate the possibility of generating short reads that originate from the forward primer of one amplicon and the reverse primer of another amplicon. It is also advantageous to screen primer designs in silico using available multiplexing tools to identify primers that might interact with one another. Primer3 or

http://bioinfo.ut.ee/multiplx/ are suitable for this primer dimer screen. (It is up to the user to verify software licensing.) *In silico* interacting amplicons can be placed in separate reactions to avoid this interaction in vitro.

When designing 1st PCR primers, keep in mind that the PCR cleanup method employed by the ThunderBolts process is recommended only for PCR amplicons ≥150bp (section 2.5). As 1st step PCR tails add 34 nucleotides to targeted region of interest, primer design should target amplicons ≥ 133 bp (150-34).

2.2.2 DirectSeq adapters added in silico to 1st PCR primers

The next step is the *in silico* addition of RainDance DirectSeq adapters or tails to the 5' end of targeted primer designs. The DirectSeq adapters are directional and thus, the two DirectSeq tails must be appropriately applied to the locus specific sense and antisense primer sequence. (See below text box for example). (RainDance to provide an excel worksheet to automate the addition of DirectSeq tails to locus specific primers using the Excel concatenate function).

RainDance Tail	Sense Sequence	Sense Sequence Tailed
CGCTCTTCCGATCTCTG	GACAATAACACCAGCACTCC	CGCTCTTCCGATCTCTGGACAATAACACCAGCACTCC
RainDance Tail	Antisense Sequence	Antisense Sequence Tailed
TGCTCTTCCGATCTGAC	GAAGATGCTTTTTACACACTGG	TGCTCTTCCGATCTGACGAAGATGCTTTTTACACACTGG

2.3 Sample Indexing (2nd PCR Primers)

The forward 2nd PCR primer is completely universal. The reverse 2nd PCR primer is universal other than its incorporation of a 6 nucleotide molecular sample index. Due to the reverse primer mediated indexing, each sample will require a distinct reverse primer. In contrast, the forward RainDance DirectSeq primer is universal and should be used for all secondary PCR reactions. (RainDance provides an excel spreadsheet with 2nd PCR primer sequence information).

Sample indexing in a flow cell lane is enabled by the 6 nucleotide molecular indexes added in the RainDance DirectSeq process (Figure . The specific 6 nucleotide length coupled with the 4 base structure of DNA (A, G, C, T) provides for indexing of up to 4096 samples (4096=4^6). Users should order secondary reverse primers with appropriate level of indexing for intended pooled sequencing runs. (e.g., 16 samples require 16 indexes or 16 secondary reverse PCR primers) In addition, color balance across the two laser detection system used by the Illumina NGS platforms is essential, and therefore the indexes used for sample multiplexing must have the correct mix of A/C and G/T bases. See Illumina for further index requirements.

2.4 NGS Read or Base Coverage

The limit of sample multiplexing is based on the degree of coverage desired coupled with the number of amplicons in a panel, the intended amplicon read length and finally, the sequencer capacity. There are two ways to look at coverage levels, overall bases sequenced and total number of amplicon reads.

For the latter, if you have a 10,000,000 read capacity, a panel with 200 amplicons and desire a 1000x mean read per amplicon level, then you could load: (10,000,000)/(200)/(1000) = (50 samples). Another important consideration is the percentage or reads that map to the target regions out of the total number of reads. This is typically in the 70%-90% range. If the number was 80%, this would reduce the number of reads to 40 samples (50*0.8).

For sequencing runs of the ThunderBolts Cancer or Myeloid Panel, RainDance multiplexes samples on the MiSeq as follows. Variances in sample loading between panels are tied to the combined variance of amplicon number and read length, and for custom panels sample loading should be determined based on panel complexity. Moreover, there is an inverse correlation between the number of amplicons in a panel and the number of samples multiplexed in a sequencing run. Therefore, intended sample multiplexing requirements should be considered during the design phase of custom panels.

- ***ThunderBolts Cancer Panel (50 genes)***
  224 amplicons of approximately 150-200bp gene of interest
  ≥24x samples (assuming 2500x mean read depth/amplicon, and MiSeq v2 kit)

- ***ThunderBolts Myeloid Panel (49 genes)***
  548 amplicons of approximately 186-220bp gene of interest
  8-16x samples (assuming 2500x mean read depth/amplicon; MiSeq v2 or v3 kits) v2=8x; v3=16x

2.5 Amplicon Length and PCR Cleanup

Targeted amplicon size affects the ThunderBolts PCR cleanup process and must be considered prior to primer design. The SPRIselect reagent volumes used in PCR cleanups vary dependent on target amplicon sizes, as well as expected background primer dimer size. If the amplicon targets and expected primer dimer sizes fall outside the conditions tested and provided below by RainDance, users must optimize SPRIselect bead concentrations for their custom panel. According to the supplier (Beckman Coulter), SPRIselect beads are appropriate for amplified products in the range of 150bp – 850bp. In addition, primer dimer size must be considered, and this size will be affected by the length of the sequencing adapter tails. For details see later section on PCR cleanup (sec 3.4).

2.6 NGS Platform Specific Sequencing Requirements

2.6.1 Illumina sequencer compatibility

MiSeq, HiSeq, and NextSeq sequencers are all compatible with ThunderBolts NGS-enrichment. However, the biochemistry of the NextSeq reads the G base as dark, or absence of signal. Thus, a string of 5Gs at the 5' end of the sequence specific region of the primer should be avoided in the primer design phase.

2.6.2 Sequencing primers

RainDance DirectSeq® utilizes a 3-nucleotide orientation sequence at 3' end of 2nd round PCR primers. RainDance specific sequencing primers provide for the utilization of these sequences. Moreover, in the presence of this orientation feature, RainDance sequencing primers enable

balanced representation of all four bases throughout the clusters of the Illumina flow cell. As MiSeq has a single lane, it is appropriate to apply the RainDance DirectSeq Adapters as instructed in the Illumina protocol for custom sequence adapters.

On the HiSeq, all lanes of the Flow Cell will use the same adapter sequence. Users cannot mix RainDance sequencing primers with the standard Illumina sequencing primers (i.e., mixed samples on a flow cell). However, users wishing to run mixed samples on a flow cell may ensure that the non-RainDance library proportion is >25% of the total flow cell content and use standard Illumina platform primers to sequence mixed samples. Alternatively, spike in PhiX control DNA at >10% , and use standard Illumina platform primers to sequence these samples. Both the PhiX spike and the standard Illumina proportion will provide the necessary mixed base population for base calling algorithms.

2.6.3 NGS read length

Bases sequenced are a function of the Illumina chemistry used and may be changed within the range of that sequencing chemistry. Bases sequenced can be set in the Illumina Manifest file to be any read length ≤ the chemistry used (i.e., 2x250 chemistry limits read length to 250 bases). RainDance recommends you set the read length dependent on the longest amplicon insert.

3. Panel Development and Validation

For panel development and validation, the following should be considered:

- Primer order and storage
- Multiplex primer concentration
- PCR efficiency normalization
- PCR cleanup

3.1 Primer Order and Storage

Primer order scale and concentration will vary according to customer specific needs. However, all primers should be ordered as desalted and wet in 10 mM Tris, pH8 suspension. In addition, RainDance typically recommends ordering oligonucleotides on the 50 nmole scale at a 1 mM concentration. This quantity is provided as guidance to ensure primer lots are sufficient to accommodate wet lab processes for determining optimal primer concentration and approximately 3 rounds of primer normalization.

Received oligonucleotide stocks may be stored at 4°C for up to 2 weeks or at -20°C long term. Freeze/thaw cycles should be avoided and no more than 3 freeze/thaw cycles should occur per aliquot. Following custom panel optimization, multiplex primer sets should be vortexed thoroughly, quick spun, and aliquoted at 20x concentration for long term storage at -20°C.

It is suggested that 3 iterations of your multiplex design are developed *in silico*. For wet lab work, RainDance recommends you order only the 1st design iteration, but refer back to the 2nd and 3rd iterations as necessary for primer pairs that drop out during the multiplexing step.

3.2 Multiplex Primer Concentration

The optimal primer concentration may vary between panels and must be determined for each custom panel. RainDance has experienced success with 10x concentrations of 0.4µM and 0.2 µM. These 10x multiplex primer stocks are added to the 1st PCR for respective final 1x concentrations of 0.04 and 0.02µM. With these concentrations in mind, RainDance suggests titrating primer concentrations in the first round of multiplexing. Prepare a titration of 10x multiplex primer stocks and subsequently setup 1st round PCR with constant DNA template inputs and final concentrations of 0.05, 0.04, 0.03, 0.02, and 0.01 µM, each in triplicate. Each primer condition should be carried thru the ThunderBolts work flow to the Agilent Bioanalyzer step. Bioanalyzer runs may then be utilized to pick the optimal primer concentration or the primer molarity that generates the largest amount of correct amplicon product size with limited or no visibility of primer dimer/off target species. PCR reactions of optimal primer concentration can be carried directly into a round of normalization (sec.3.3).

3.3 PCR Efficiency Normalization

Uniform NGS coverage of amplicons correlates with normalized PCR efficiencies. Therefore, the PCR primer of a custom panel must be normalized. This is accomplished by carrying each reaction of optimal primer concentration (see above step) thru the remainder of the ThunderBolts process to an Illumina sequencing run. To assess intra-amplicon performance variation, generate a histogram of the coverage levels for each of the primer pairs (Figure). Intra-amplicon PCR efficiency is then normalized by adjusting relative primer pair concentrations. By splitting the reads per amplicon histogram into 3 sections ("low", "target", "high"), one can add extra primer to the section representing low coverage levels. Spiking in additional primers for low coverage amplicons may be sufficient to normalize amplicon performance, in which case it will not be necessary to assemble fresh primer multiplexes for the 2nd round of normalization. However, you may find outliers with very high coverage levels. If so, it is recommended that multiplex primer sets are prepared fresh such that the previously high coverage amplicons now contain fewer primers than the median optimal primer concentration.

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Figure 5: Enable uniform NGS coverage with normalization of PCR efficiencies.

Normalize PCR efficiencies by adjusting relative primer pair concentrations.

Gene Specific Amplicons
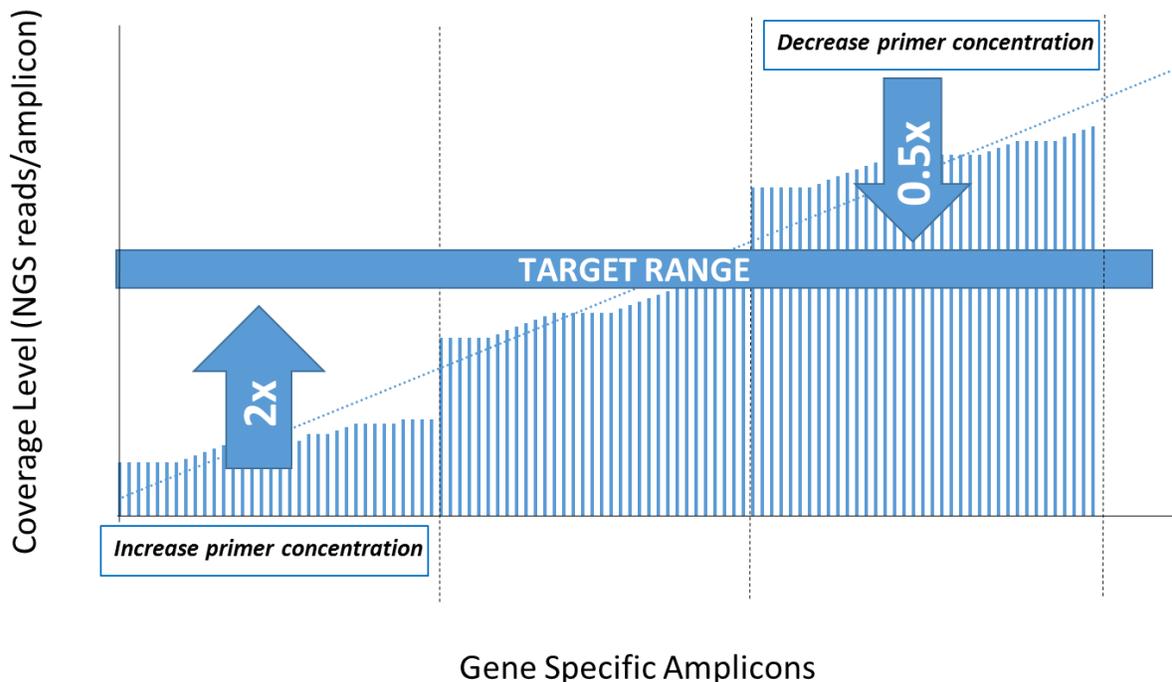
Between 1st and 2nd rounds of normalization, amplicon dropouts should be tested in single-plex bulk PCR reaction to rule out primer synthesis failure, rather than sequencing dropout. For continued dropouts, replacement primers for any amplicon dropouts should be tested for targeted and specific amplification in individual PCR reactions. To this end, primer sets corresponding to amplicon dropouts should be ordered based on your previous 2nd and 3rd iteration in silico designs. Upon receipt, primer pairs for each of the 3 design iterations should be tested in individual bulk PCR reactions and analyzed by gel electrophoresis or Agilent Bioanalyzer for the correct, amplicon yield and absence of a primer dimer band. The primer set producing a specific and targeted amplicon should be spiked into the multiplex primer set in use at the median primer concentration. This round 2 multiplex primer set should be used for a 2nd ThunderBolts run and again, coverage levels should be assessed in an intra-amplicon manner with appropriate primer spikes or adjustments. It is expected that approximately 3 rounds of normalization may be necessary to achieve optimally normalized PCR efficiencies.

3.4 PCR Cleanup

The SPRIselect reagent used in the ThunderBolts workflow for PCR cleanup contains paramagnetic beads which under specific buffer conditions alternately bind or release nucleic acids of differing sizes. Together, targeted amplicon size and primer dimer size determine the optimal volume ratio for SPRIselect reagent to PCR volume. Accordingly, the volume of the SPRIselect reagent added for PCR

cleanups will vary depending on target amplicon sizes, as well as anticipated size of background primer dimers. If the amplicon targets and expected primer dimer sizes fall outside the conditions tested and provided below by RainDance, it is expected that customers will test a subset of SPRIselect bead concentrations for appropriate amplicon purification away from primer dimers. Conditions used for the RainDance-defined ThunderBolts panels were validated as best practices for amplicon and primer dimer sizes specific to the Cancer or Myeloid Panel. Due to differences in anticipated primer dimer length for 1st PCR and 2nd PCR, different ratios are used for each of the PCRs (Figure 6). Conditions listed here are provided as a reference and starting point, but custom panels generating amplicons or primer dimers of varied size will require testing and verification. Excepting the ratio (volume) of SPRIselect reagent added for PCR clean ups, the protocol for SPRIselect bead-based PCR clean ups is standard across ThunderBolts NGS panels.

*Figure 6: SPRIselect reagent ratios as validated in PCR clean ups of the ThunderBolts Cancer and Myeloid Panels.*

Primer length (bp)			Amplicon size: (gene specific sequence)	PCR volume setup	PCR volume post droplet destabilization	SPRI ratio (volume)
Primary PCR	37 bp F/R primers (20 bp gene specific primer plus 17 bp tails)	ThunderBolts Cancer Panel	**184 – 237 bp**: (150-203 bp)	40 µl	33 µl	ratio 1.2 39.6 µl
		ThunderBolts Myeloid Panel	**220 – 254 bp:** (186-220 bp)	40 µl	33 µl	ratio 0.9 29.7 µl
Secondary PCR	60 bp forward primer, 66 bp reverse primer with index	ThunderBolts Cancer Panel	**276 – 329 bp:** (150-203 bp)	25 µl	25 µl	ratio 0.9 22.5 µl
		ThunderBolts Myeloid Panel	**312 – 346 bp:** (186-220 bp)	25 µl	25 µl	ratio 0.8 20 µl

The SPRIselect reagent is appropriate for amplified products in the range of 150bp – 800bp. In fact, the supplier (Beckman-Coulter) defines the SPRIselect beads as "tunable" within this amplicon size range. However, even within this range, cleanup efficacy may be limited by the combination of increasingly long primer dimers with shorter PCR amplicons. The tuning feature of the SPRIselect reagent is explained here. (Consult the Beckman-Coulter website for additional details). The ThunderBolts workflow focuses on what Beckman-Coulter has defined to be left-side size selection. Left-side size selection directly correlates with the respective ratio of SPRIselect reagent volume to PCR volume, and serves to remove primer dimers (of relatively smaller size), while leaving the targeted amplicon product (of larger size) bound to the paramagnetic beads. Illustrated below are two Agilent Bioanalyzer traces (Figure 8), representing the same PCR reaction, before and after bead clean up. The left trace (pre-bead clean up) displays the PCR product in its entirety including primer dimers as well as PCR products, while the right trace (post-bead clean up) displays the same PCR product following primer-dimer removal.

Figure 7: SPRIselect reagent removal of primer-dimers and capture of relatively longer amplified product (figure is from Beckman Coulter web-site)

Left-side size selection
Excludes fragments below target cutoff.

Light blue = Shear   Dark blue = Target region
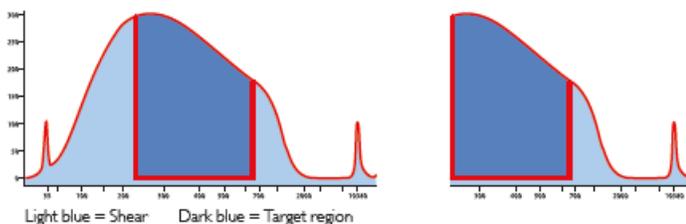
4. Input DNA and QC

This section provides information on starting DNA input requirements. It includes general instructions on upfront processes including DNA shearing and FFPE extraction, as well as QC requirements for all sources of DNA input.

4.1 DNA inputs

The ThunderBolts Open Source is compatible with DNA inputs of cfDNA, gDNA (purified from blood or plasma), gDNA from fresh frozen (FF) tissue, or formalin-fixed paraffin embedded (FFPE) tissue (Figure). The lower limits for the ThunderBolts Cancer and ThunderBolts Myeloid Panels are set at a total of 10ng per sample, or 5ng per each of the two primer subsets in those panels.

Figure 8: Input DNA requirements for ThunderBolts NGS Enrichment System

DNA Source	ThunderBolts Input	A260/A280	Shear DNA	PicoGreen	FFPE DNA QC
High quality genomic DNA	10 ng - 150 ng	1.8–2.0	shear to 500 bp	yes	Not applicable
FFPE genomic DNA	10 ng - 150 ng (*verify copy number)	1.8–2.0	Not applicable	yes	verify amplifiable copy numbers
cfDNA	10 ng - 150 ng	1.8–2.0	Not applicable	yes	Not applicable

Mechanically shear DNA input unless starting material is of FFPE source. For DNA inputs ≤150ng it may prove unnecessary to shear the DNA. However, sheared DNA may produce better results. DNA inputs must pass QC prior to input on the ThunderBolts System. See below for greater detail.

4.2 DNA Shearing

The starting material defines the need for upstream fragmentation of DNA (i.e., plasma or fresh frozen gDNA).

- High molecular weight DNA: DNA of high molecular weight should be mechanically sheared (to 500bp). When DNA inputs are ≤150ng it may prove unnecessary to shear the DNA (even in cases of high molecular weight). However, in the many cases sheared DNA will produce the best results.

- Low molecular weight DNA should never be sheared:
  - o   FFPE DNA
  - o   cfDNA fragments of less than 500bp
  - o   Fragmented Bisulfite converted DNA

RainDance recommends DNA is sheared by the Covaris supplier provided microtube protocol. Use the protocol specific to the target sheer size of 500 bp. While the Covaris protocol is the preferred method, alternate options include the Nebulizer or Qsonica methods. In either case, supplier protocols must be precisely followed and allow for target sheer size of 500bp.

Sheared DNA is next subject to cleanup/concentration via the QIAmini-elute columns or EtOH precipitation. The QIA mini-elute protocol appropriate to the concentration of previously purified samples and thus, relevant here to sheared DNA may be obtained from Qiagen or RainDance. Briefly stated, the sheared DNA sample is supplemented with NaCl at a final concentration of 0.5M prior to addition of PB buffer.

4.3 FFPE DNA Extraction

While multiple kits or techniques exist for the isolation of DNA, particular care must be taken with FFPE templates due to the known DNA degradation that occurs during the formalin fixation procedure. In addition, as FFPE tissue DNA is sensitive to further degradation during processing detailed attention to processing times and handling throughout the FFPE DNA isolation process is imperative. Either the QIAamp DNA FFPE or the ZYMO FFPE kit are recommended for upfront DNA purification

4.4 DNA quantitation

Once DNA has been extracted and/or sheared and purified, final DNA templates must pass QC prior to input on the ThunderBolts System. The Abs260/280 measurement of prepared ThunderBolts input DNA is reflective of sample purity and should be in the 1.8-2.0 range. In addition, it is strongly recommended that a measurement of ThunderBolts input DNA is taken via PicoGreen or other dsDNA binding fluorescence method. This ensures the most accurate quantitation of dsDNA as 260OD readings are affected by both RNA and other contaminants and thus, tend to overestimate DNA concentrations.

4.5 FFPE QC

Working with FFPE DNA requires additional QC. Tissue fixed in FFPE, and not fresh frozen (FF), will be inherently fragmented and must undergo quality assessment prior to input as NGS template. Due to formalin crosslinking of nucleic acids during the FFPE process, DNA isolated from FFPE is most often of lower weight than gDNA from other sources. The degree of fragmentation depends on the type and age of the sample and the conditions used for fixation. In addition, further degradation may occur during the FFPE DNA isolation process. The fragmentation of the recovered FFPE will inherently affect the amplification of your targets. Such degradation may significantly decrease or even negate the

amplification of target amplicons. Accordingly and to ensure optimal NGS results, RainDance recommends the following steps when working with FFPE DNA inputs.

The Abs260/280 measurement of prepared ThunderBolts input DNA is reflective of sample purity and should be in the 1.8-2.0 range. However, the direct measurement of optical density at 260nm is insufficient for determining DNA concentration. Thus, the DNA concentration should be assessed via standard PicoGreen protocols or similar dsDNA binding fluorescent dye assays. In addition to these measurements, the quality of the FFPE DNA must be assessed in an amplification based assay. To this end, RainDance recommends running a qPCR or dPCR assay designed to target PCR amplicons of size greater than the targeted amplicon lengths in your ThunderBolts Custom Panel. For example, if a custom ThunderBolts panel targets gene specific amplicons of 125 bases, one should determine template copy number using a qPCR or dPCR assay designed to amplify target(s) ≥175bp. If desired, one can assay fragmentation of FFPE DNA by running a ladder of PCR assays targeting amplification products of 100, 200, 300 and 400bp. This PCR produced ladder will give insight in to the degree of FFPE DNA fragmentation. The success or failure of this assay with your template, as well as the actual copy number, predicts the success of your template in the ThunderBolts workflow.

Please refer to the following RainDance Application Notes for further suggestions on ThunderBolts input DNA QC:

- Using EvaGreen Dye on the RainDrop Digital PCR System to Quantify NGS Libraries
- RainDrop Digital PCR System as a Pre-test QC and Validation Tool for Sequencing

5. ThunderBolts Open Source Protocol

Please refer to ThunderBolts Cancer Panel Manual (LCN 50-07257) for the full protocol. However, please keep the following steps in mind when designing a ThunderBolts Open Source Panel.

5.1 DNA Inputs
Mechanically shear DNA input unless starting material is of FFPE or cfNA source. For DNA inputs ≤150ng it may not be necessary to shear the DNA. However, sheared DNA may produce better results. DNA inputs must pass QC prior to input on the ThunderBolts System. See the RainDance manual or section 4 above for greater detail.

5.2 Setup Bulk 1st PCR Reactions, Generate Droplets on ThunderBolts System/RainDance Source, and Perform Droplet-based 1st PCR Cycling
See the ThunderBolts System/RainDance Source Operator's Manual (LCN 50-04343) for detailed information regarding the generation of droplets.

For the ThunderBolts Open Source, please use the domed caps that come with the Axygen PCR tubes (Axygen # PCR-0208-C). These tubes/caps must be used for the ThunderBolts Open Source, and these caps are different from those used for the RainDrop Digital PCR System. Please see the ThunderBolts Cancer Panel Assay Protocol in the ThunderBolts Cancer Panel Manual (LCN 50-07257) for more information on how to proceed with the sample emulsions from the ThunderBolts System/RainDance Source instrument.

Figure 9: 1st PCR reaction setup and thermo-cycling conditions:

ThunderBolts Open Source 1st PCR Reaction	1x40 µl rxn	
	ul	final concentration
2x Genotyping Master Mix	20	1x
25x Droplet Stabilizer	1.6	1x
ThunderBolts Open Source Multiplex Primer Set (10x is ~0.2-0.8uM)	4	~0.02 - 0.06 uM
DNA (5 ng – 75 ng)	up to 14.4 µl	5 ng - 75 ng/rxn
H$_2$O	variable	
TOTAL:	40 µl total volume	
WORK COMPLETELY IN PRE-PCR AREA		
ASSEMBLE PREMIX WITH ALL COMPONENTS MINUS DNA		
ADD DNA		

1st PCR Cycling:

ThunderBolts Open Source 1st PCR Thermal Cycling
STEP 1: 94°C/2 min
STEP 2: RAMP 1°C/sec to 94°C
STEP 3: 94°C/30 sec
STEP 4: RAMP 1°C/sec to 54°C
STEP 5: 54°C/30 sec
STEP 6: RAMP 1°C/sec to 68°C
STEP 7: 68°C/1 min
STEP 8: GOTO STEP 2, 54x (TOTAL = 55 cycles)
STEP 9: 68°C/10 min
STEP 10: HOLD AT 12°C

Emulsion PCR necessitates a slow ramp rate of 1°C for droplets as oil temperature conduction is slower than aqueous. Always use a slow ramp rate on emulsion/1st PCR and use the RainDance detailed Genotyping Master Mix with no extra additives (EDTA, Mg, DMSO). The annealing temperature shown here for cycling may be varied dependent on user custom primer melting temperatures. However, the extension temperature of 68°C and the ramp rate/cycling times must remain constant as they are defined functions of the enzyme/master mix and the oil-based droplets. In addition, proper thermal cycling of the emulsion requires a thermal cycler with the specifications detailed in the ThunderBolts Cancer Panel appendix.

5.3 Break Emulsions and Perform Bead-based PCR Cleanup

Break droplet emulsions and remove lower oil phase, the upper aqueous phase contains PCR product. When removing the lower oil phase, leaving behind approximately 5µl of oil is acceptable and will not adversely affect amplicon purification.

In general, perform SPRIselect bead-based purification as stated in the ThunderBolts Cancer Panel Manual (LCN 50-07257), while keeping the previously mentioned constraints in mind regarding the SPRIselect reagent to PCR product ratio. Always prepare EtOH solutions fresh as with time, EtOH absorbs water molecules from air resulting in altered EtOH concentrations.

5.4 2nd PCR (Addition of Adaptor and Index Sequences)

Setup 2nd bulk PCR reactions with appropriate Illumina bar-coded primers. If in the course of 1st PCR setup, sample DNA inputs were split into 2-wells of a chip for two distinct PCR multiplexes, ensure that the same Illumina barcoded 2nd PCR primers (i.e., same indices) are used per matched pair of 1st PCR template DNA samples.

Figure 3: 2nd PCR reaction setup and cycling conditions.

ThunderBolts Open Source 2nd PCR Reactions	1x25 µl rxn	
	ul	final concentration
10x Platinum Taq Pol HiFi Buffer	3.25	1.3x
50 mM MgSO4	0.875	1.75 mM
dNTP mix (10 mM.ea.)	1.125	0.45 mM ea
4M Betaine	2.5	0.4 M
DMSO	1.25	
Platinum Taq Pol HiFi (5 units/µl)	0.5	0.1 units/ul
10x Barcoded Illumina primers (5 µM)	2.5	0.5 uM
1st PCR Template DNA (post-bead clean up)	13	13 µl/rxn
TOTAL:	**25 ul total volume**	
IN PRE-PCR AREA, ASSEMBLE PREMIX WITH ALL COMPONENTS MINUS PRIMERS AND DNA		
ADD PRIMERS IN PRE-PCR WORK SPACE		
MOVE TO POST-PCR WORK SPACE, AND ADD DNA		

Thermal cycling using the following parameters:

ThunderBolts Cancer Panel 2nd PCR Thermal Cycling
STEP 1:   94°C/2 min
STEP 2:   RAMP 1°C/sec to 94°C STEP 3:   94°C/30 sec STEP 4 : RAMP 1°C/sec to 56°C STEP 5:   56°C/30 sec STEP 6:   RAMP 1°C/sec to 68°C STEP 7:   68°C/1 min STEP 8:   GOTO STEP 2, 9x (TOTAL = 10 cycles)
STEP 9:   68°C/10 min
STEP 10: HOLD AT 12°C

5.5 Perform bead-based cleanup of 2nd PCR

In general, perform SPRIselect bead-based purification as stated in the ThunderBolts Cancer Panel Manual (LCN 50-07257), while keeping the previously mentioned constraints in mind regarding the SPRIselect reagent to the PCR ratio. Always prepare EtOH solutions fresh as with time, EtOH absorbs water molecules from air resulting in altered EtOH concentrations.

5.6 Measure NGS-enriched amplicon integrity and quantity

Verify NGS-enriched amplicon integrity and quantify via Agilent Bioanalyzer or Tape Station. Pool templates as directed by Illumina for NGS sequencing. If samples were split at the beginning for 2x 1st PCR reactions, then corresponding 2nd PCR reactions should be pooled as directed in the ThunderBolts Cancer Panel. This round of pooling serves only to bring the two 2nd PCR reactions to like concentration so that sequencing coverage will be similar across panel amplicons. This step should be conducted prior to Illumina directed pooling, and is only necessary if two 1st PCR reactions were setup for same sample templates.

5.7 Pool samples and Setup Illumina Sequencing Run

Please refer to the ThunderBolts Cancer Panel Manual (LCN 50-07257).

6. RainDance ThunderBolts – Instrument and Consumables Check List

6.1 RainDance Materials Needed
- ThunderBolts System/RainDance Source (# 20-04401)
- ThunderBolts Open Source 48-well or 96-well Pack (# 20-07208, # 20-07206)
    - o RainDance Source Chip
    - o RainDance Droplet Destabilizer
    - o RainDance 25X Drop Stabilizer
    - o Carrier Oil

6.2 Required Supplies
- P2, P10, P20, P200, and P1000 pipettes
- 1-20 µl and 20-200 µl 12-channel multi-channel pipettes
- Sterile aerosol resistant filtered pipette tips
- Vortex mixer
- Mini-microcentrifuge for both microtubes and 8-strip 0.2 ml PCR tubes
- 8-strip 0.2 mL PCR tubes and caps (Axygen #PCR-0208-CP-C). Caps must be replaced between thermal cycling steps. ThunderBolts users should use the domed caps that come with the Axygen PCR tubes (Axygen # PCR-0208-C).
- DynaMag-96 magnetic plate (Life Technologies #12331D)
- A thermal cycler with the following specifications, which allows for proper thermal cycling of the emulsion. If you use a thermal cycler that does not meet these specifications, data may be compromised.
    - o Thermal cycler block format should accommodate standard 0.2 ml tubes
    - o Adjustable-height heated lids are recommended
    - o The surface of the heated lid should be hard and free of texture
    - o Adjustable ramp speed
    - o *Thermocycler calibration date should be current*

    NOTE: Centrifugal thermal cyclers are not recommended

6.3 Required Reagents
- TaqMan® Genotyping Master Mix (Life Technologies #4371355)
- Platinum Taq DNA Polymerase High Fidelity (Life Technologies #11304-029)
- 50 mM MgSO4 (included in Life Technologies #11304-029)
- 40 mM combined (10 mM/ea) dNTP Solution Mix (New England Biolabs #N0447L)
- Oligonucleotide PCR primers (Integrated DNA Technologies). All primers should be ordered at 100 uM in TE, pH 8.0. (If ordered thru IDT this is their "Lab Ready" suspension).
- RainDance Custom Sequencing Primers - Read 1, Read 2

- 2nd PCR universal Forward primer
- Multiple 2nd PCR Reverse primers: 2nd PCR reverse primers incorporate a 6-nucleotide barcode for sample indexing. Order sufficient 2nd PCR reverse primers to accommodate the intended level of sample indexing. Consult Illumina website for indexing requirements.
- DNase/RNase-free water (molecular biology grade)
- Trizma Hydrochloride Buffer Solution, pH 8.0 (Sigma #T2694-1L)
- Absolute Ethanol (Sigma E7023-500ML)
- DMSO (Sigma #D8418-100ML)
- Betaine Anhydrous (Sigma #B2629-100G)
- SPRIselect Reagent Kit (Beckman Coulter #B23318)

Disclaimers

The ThunderBolts System/RainDance Source and ThunderBolts Open Source may not perform as intended if the user fails to follow the applicable instructions as provided by RainDance, and RainDance will not provide warranty service or support to a user who did not follow the applicable instructions as provided by RainDance.

The user acknowledges and agrees that consumables are for one-time use, for internal research purposes only, and are not for resale or for other commercial distribution.

In no event shall RainDance be liable for any damages, whether in contract or tort (including, but not limited to, negligence) or under any other legal theory for consequential, special, exemplary, incidental or indirect damages, arising out of or in connection with the use or the inability to use the ThunderBolts System/RainDance Source and ThunderBolts Open Source or the results of using the ThunderBolts System/RainDance Source and ThunderBolts Open Source. The liability of RainDance in connection with the use of the ThunderBolts System and Open Source shall in no event exceed the amount of the invoice for the pertinent consumables.

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