Technical Note

FLDM-00090 Rev 01

Analysis of Advanta Solid Tumor Assay Samples

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An Example Using the SNP/Indel Discovery and CNV Tools in NextGENe Software

To advance our understanding of cancer and make strides toward improved patient care, researchers have a growing need to evaluate somatic mutations, often from limited quantities of available samples. The Advanta[™] Solid Tumor NGS Library Prep Assay with the automated Juno[™] system produces targeted ampliconbased barcoded libraries for subsequent analysis on Illumina[®] NGS platforms, optimizing interrogation of high-value, low-variant-frequency SNPs, indels and CNVs within oncology-relevant genes.

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This technical note provides steps for analyzing samples prepared with the Fluidigm Advanta Solid Tumor NGS Library Prep Assay using NextGENe® Software by SoftGenetics®, LLC. NextGENe Software can detect low-allele-frequency somatic variants along with copy number variations (CNV).









SNP and Indel Discovery

Step A: Perform Reference Setup.

Ensure that the desired genome reference (either Human_v37p.13_105 or Human_GRCh38p.7_108) from the SoftGenetics Reference Database is pre-loaded. Refer to the NextGENe User Manual for details of how to import a reference.

Reference on Local:		References on FTP:	List	
	< >	Horse_v2_1_dna_beta Human_v38.p12+mRN/ Human_GRCh38.p106_c Human_GRCh38_106_c Human_v37.p13+mRN/ Human_v37.p13+mRN/ Human_v37.p10_Major Human_v37.p10_dbsnp Human_v37.3_dbsnp1 Human_v37_3_dbsnp1 Human_RNA.zip	.zip A.zip B.zip ClinVar141.zip JbSNP141.zip A.zip Jp Chr_dbsnp135.zip 135.zip 35_MajorChr.zip 35_dna.zip	Ŷ
Installation Directory:				

Figure 3. Reference import

Step B: Merge paired-end reads using the Overlap Merger.

Use the Overlap Merger to combine the paired-end R1 and R2 FASTQ reads to generate a FASTA file, *_PairMerged.fasta, of merged reads with higher-quality base calls.

- 1 Launch NextGENe and close the Project Wizard.
- 2 Go to the Tools Menu and click Overlap Merger.
- **3** Either load the previously saved settings file by clicking Load or manually change the settings to those shown in Figure 4.
- 4 Click Add and select the R1 and R2 FASTQ files of a sample. Note: Up to 96 samples may be processed together and are merged appropriately if sharing the same base sample name.
- **5** Set the appropriate Output path.
- 6 Click OK when ready to process. A pop-up window appears when the merging is complete.

and printinger				
Input:				
				Add
				Remove
				Remove All
				< >
Jutput:				
				Set
C Merge overlapping contigs				
Merge overlapping paired reads	Ove	erlap min bases	50	
C Merge cell-free paired reads 👘 🔲 Outp	out reverse complemen	it	,	
Ignore low quality ends for non-overlapp	ed pairs	Keep 5' end		
✓ Merged length 80 bp to 300	bp			
Merged length 70 % to 130	% of the longer rea	d length		
Trim Primer			Primer file	
	Save	Load	ок	Cancel

Figure 4. Recommended settings for the Overlap Merger

Step C: Align a sample and generate the Mutation Report.

Samples may be processed individually or as a batch. For batch sample processing, skip to Step E. For a single sample, launch NextGENe and use the Project Wizard to perform the following steps:

- 1 Load the recommended alignment settings file, Advanta_Solid_Tumor_Alignment_Settings.ini.
- 2 Click Load Data. In the Sample files field, select the *_PairMerged.fasta file generated in Step B.
- 3 In the Reference files field, select the desired reference under Preloaded that was imported in Step A.
- 4 Set the desired output path in the Output field.
- 5 Ensure that the path to the Amplicon BED file is set appropriately to the Advanta_Solid_Tumor_assays. bed that provides the genomic locations of the targeted regions without the primers.
- 6 Click Alignment and review the loaded settings. Modify if desired. Recommended: Use very loose parameters for Mutation percentage and SNP allele count for the Mutation filter in this alignment step, outlined in Figure 6, and then use stricter ones in the Mutation Report settings. This still results in the output but prevents the need to re-align if new parameters for curation are desired.

	She	ow Project Lo
	Load data	
Step	Previous run result: Load Previous Run Result To con	ivert to fasta
	Sample files: Form	at Conversior
Application	C: \Users\Administrator \Desktop \Solid_Tumor_Sample 1_PairMerge.fasta	Load
		Remove
Load Data	< >	RemoveA
	,	
ndensation	Human_v37.p13_105	Load
		Preloade
sembly		Remove
		Remove/
ignment	Output:	
	C:\Users\Administrator\Desktop\Solid_Tumor_Sample1_PairMerge_Output	Set
Post	Available disk space: 94 GB free of 214 GB.	
essing	Set Amplicon BED file	
		C+1
Processing	Set Amplicon BED file	

7 Click the Post Processing button to ensure that the Mutation Report is pointed to the correct Settings file location, either Advanta_Solid_Tumor_Mutation_ Report_Settings.ini or another desired Mutation Report settings file saved from a previous run, shown in Figure 7.

Project Wizard - Align	ment	X
	- Alignment	
	Reads: Allowable mismatched bases 1 (0-2)	
Sten	Allowable ambiguous alignments 50	
Step	South 20 have seen the 2 have Inspection it find	
1	Allowable alignments 100	
Application	Allowable alignments 100 (1-1000)	
	Sample trim	
Load Data	from 1 bases to 30 bases	
	✓ Hide unmatched ends	
	Mutation filter Use original Except for homozygous	
Condensation	SNPs Indels HomopolymerIndels	
	Mutation percentage <= 0.5 0.5 0.5	
1	SNP allele count <= 2 2 2	
Assembly	Total coverage count <= 5 5 5	
	Perform in-read phasing	
Alignment	Max gap between two variants 1 (0-3)	
	Phaseable reads percentage >= 50	
Post		
Processing	File type	
	Library size range : from 80 bases to 300 bases	
	454 Sequence:	
	Save matched reads I Highlight anchor sequence Amolguous gain/loss Detect structural variations SV settings>>	
	Default Setting	
		_
Save Settings Lo	ad Settings <<< Back Next >> Cancel Finish	-
		-

Figure 6. Recommended settings for the Alignment and Mutation filter settings

				Show Pr	oject Log:
Step	Post processing Report		Settings		
Application	Mutation	Advanta_Solid_Tumo	or_Mutation_Report_	Setting: Set	Remove
Load Data			J _JISUIDUUUT_SELUI	195.1111 <u>Set</u>	Kemove
Condensation	Save summary rep	ort QC Profile	Add	Remove All	
Assembly	Export	1	Settings		
Alignment					
Post Processing					
	Export BAM		Add	Remove All	
	Output to Geneticis	t Assistant Details	Sample Name		
ave Settings L	Output to Geneticis Run Name	St Assistant Details	Sample Name	Cancel	Finis

Figure 5. Project Wizard single-sample data loading step Figure 3



- 8 Ensure that the Distribution Report is pointed to the correct Settings file location, either Advanta_ Solid_Tumor_Distribution_Settings.ini or another desired Distribution Report settings file saved from a previous run.
- 9 Click Finish to close the Project Wizard.
- **10** In the Projects window, click Run to process the sample.



Figure 8. Final window to launch processing

Step D: View output.

After completion, the NextGENe Viewer automatically opens, displaying the pile-ups as shown in Figure 1 and the Mutation Report (not shown).

When the user clicks the SNP/indel events listed in the report, the viewer automatically changes to the genomic location of the event.

Step E: Perform multiple sample processing.

Many samples can be processed as a batch after merging is completed in Step B.

Refer to the NextGENe User Manual (NextGENe-2.4.2-UG001) for details to set up AutoRun and to create a batch job using the Fluidigm Advanta Solid Tumor template or another settings file that generates the Mutation Report and the Distribution Coverage Report. Use of the Fluidigm Advanta Solid Tumor template automatically applies parameters used to conduct the internal analytical validation.

Generated files include VCF format files, which can be compiled and manipulated as other VCF files, and NextGENe formatted text files, which can be loaded into a spreadsheet program like Excel® as tables for review.

		V
NextGENE Autokun Job Editor - Untitled		~
File Edit Tools		
 SVT_III_Sono_D4_NA24143p05_S15_R1_001_ SVT_III_Sono_D4_NA24143p05_S15_R2_001_ SVT_III_Sono_C5_NA24143p05_S19_R2_001_ SVT_I_Sono_C5_NA24143p05_S19_R1_001_ 	Job name: SVT_XXX_FurSono_F4_NA24143p05_S111_R2_001_fastq_gz ID: 20190618135	5335_937
> SVT Sono C6 NA24143p05 S187 B2 001	Template: Choose Template v	Save
> SVT_IV_Sono_D1_NA24143p05_S91_R1_001 > SVT_IV_Sono_D1_NA24143p05_S91_R2_001	Input sequence files: 1 files loaded	
> SVT_V_Sono_E1_NA24143p05_S110_R1_001 > SVT_V_Sono_E1_NA24143p05_S110_R2_001	C:\Users\Administrator\Desktop\change_validation\sonoma_a\dataset_1\na24143p05\SVTX	Load
> SVT_XIV_Sono_C1a_NA24143p05_S45_R1_0C	R	Remove
> SVT_XIV_Sono_C1a_NA24143p05_S45_R2_00		
SVT_XIV_Sono_C1a_NA24143p05_S48_R1_00 SVT_XIV_Sono_C1a_NA24143p05_S48_R1_00	< > >	
SVT_XVI_S000_C18_NA24143p05_S46_N2_0C > SVT_XVIII_FurSono_C7a_N424143p05_S23_R	Output:	
> SVT_XVIII_FusSono_C7a_NA24143p05_S23_R	C:\Users\Administrator\Desktop\SVT_XXX_FusSono_F4_NA24143p05_S111_R2_001_fastq_	Set
> SVT_XVIII_FusSono_C7a_NA24143p05_S32_R		
> SVT_XVIII_FusSono_C7a_NA24143p05_S32_R	Preprocesses: 0 (Add)	
> SV1_XXV1_S000_L00_NA24143pu5_S24_H1_U S SV1_XXV1_S000_C0b_NA24142p05_S24_B2_0	Reference:	
> SVT XXX FusSono F4 NA24143p05 S111 R	Human_v37.p13_105 S	elect 🔻
SVT_XXX_FusSono_F4_NA24143p05_S111_R2		
✓ Sample File(s)		lemove
None		
 Reference File(s) 	Process & report settings file: Inspect input files to set optimum parameters	
- C:\Users\Public\Documents\SoftGenetic	C:\Users\Administrator\Desktop\settings_files\Advanta_Solid_Tumor_Alignment_Settings.ini	Load
NextGENe Settings File		
Utout Path	Report & Export 2 [Edit details]	
L C:\Users\Administrator\Desktop\SVT X	Output to Geneticist Assistant (E dt details)	
	Run Name SVT_XXX_FusSono_F4_NA2414	43p05_S*
< >>		
	ПК	Cancel
	OK	

Figure 9. The Job Editor is used for batch processing of samples in the AutoRun feature.

CNV Detection

Step A: Generate project files for samples.

Execute the methods listed under the previous section, SNP and Indel Discovery, for all samples that the CNV analysis is to be performed on.

Step B: Generate project files for controls.

Execute the methods listed under the previous section, SNP and Indel Discovery, for a set of control samples that are known to not have CNVs. For this reference, a set of 12 Genome in a Bottle (GIAB) samples that were sequenced using the same methods as the samples to be analyzed for CNV is used as a control set.

Step C: Set CNV Report parameters.

- 1 Launch NextGENe and close the Project Wizard.
- 2 Go to the File menu and click Open NextGENe Viewer.
- **3** After the NextGENe Viewer has opened, go to the Comparisons menu and click CNV Tool.
- 4 In the CNV Tool settings window, click Load Settings and load the Advanta_Solid_Tumor_CNV_Settings. ini file or a different settings file from a previous run.



Figure 10. Open the CNV Tool from the NextGENe Viewer.



Figure 11. CNV Tool settings window

CNV						Х
Method Selection	Data Input	Basic Settings	Advanced Settings	Report Settings		
Input						
	Sample			Control		
Batch Add	Add	<u>R</u> emov	e <u>B</u> atch Add	Add	<u>R</u> emove	
Control Uption			Multiple Cr	natrole		
O Single Cont	rol			Matoh		
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Output Folder:					Set	
Report	🗹 Save CN	V Report	🗹 Save Block	Report		
Format	🗹 TXT		GVF			
Save Settings	Load Setting	s Default	<< >	Run	Cancel	

Figure 12. Data Input for CNV Tool

- 5 Click the Data Input tab and under the Control field, select Batch Add.
- 6 Navigate to the folder that contains the project files generated in Step B and click OK. (The software recursively searches that folder for all project files.)
- 7 Under the Sample field, select Batch Add, navigate to the folder that contains the project files generated in Step A, and click OK. (The software recursively searches for all project files in the selected folder. Up to 48 samples may be processed at one time for CNVs.)
- 8 Set the Output Folder path as desired.
- 9 Click the Basic Settings tab and check that the Input region of interest path is set to the correct location of Advanta_Solid_Tumor_assays.bed, as in Figure 13.
- **10** Modify any additional parameters as desired and click Run.

	Davio Dottingo	Advanced Settings	Report Settings	
gions				
Use segments as defined	in reference file	s		
CDS		Exon		
 Continuous Exon) Continuous CDS		
OROI				
) Set incremental segment	length 10000) bases (>= 1	00)	
Contig	Chromosome			
Input region of interest (*.1	ped)			
C:\Users\Administrator\E)esktop\setting:	s_files\Advanta_Solid	_Tumor_assays.be	Set
				Set
Evolude Chr.X				Set
Exclude Chr X				Set
Exclude Chr X Exclude Chr Y Exclude Chr M				Set
] Exclude Chr X Exclude Chr Y Exclude Chr M				Set
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Exclude Chr X Exclude Chr Y Exclude Chr M				Set
Exclude Chr X Exclude Chr Y Exclude Chr M				

Figure 13. Basic Settings for CNV Tool

Step D: View CNV Report and plots.

After the CNV Tool has finished running, the CNV Report for the first sample is displayed.

Generated files include GVF and NextGENe formatted text files, which may be loaded into a spreadsheet program like Excel as tables for review.

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Sam	SVT_XXV			_									
Cont	SVT_III_S	SVT_IV_S	SVT_I_Sonc	SVT_	SVT_X	SVT_XIV_S	SVT_XVIII_F	SVT_XVIII_FusS	SVT_XX	SVT_≫			
Chr	Chr Start	Chr End	Gene	Exon	Ratio	Total Read	Dispersion	Normalized Like	Deletion	Normal	Duplication	HMM Calls	Normalized Read (Sample;Control)
chr4	1808547	1808676	FGFR3; +	17	0.5529	935.000	0.0790	-0.86;-0.36;-0.37	0.65	2.46	2.43	Normal	517.000;418.000
chr4	1808820	1808927	FGFR3; +	18	0.4547	1203.000	0.0742	-0.53;-0.36;-0.58	1.53	2.50	1.34	Normal	547.000;656.000
chr4	55124883	55125000	PDGFRA; +	2	0.6838	4611.000	0.0602	-1.82;-0.54;-0.16	0.07	1.49	5.14	Duplication	3153.000;1458.000
chr4	55127345	55127472	PDGFRA; +	3	0.7522	7103.000	0.0602	-2.37;-0.70;-0.10	0.02	0.96	6.95	Duplication	5343.000;1760.000
chr4	55129852	55129965	PDGFRA; +	4	0.7405	6818.000	0.0602	-2.27;-0.67;-0.11	0.02	1.04	6.61	Duplication	5049.000;1769.000
chr4	55131106	55131231	PDGFRA; +	5	0.6078	3353.000	0.0602	-1.31;-0.40;-0.25	0.22	2.17	3.54	Duplication	2038.000;1315.000
chr4	55133433	55133549	PDGFRA; +	6	0.8173	5064.000	0.0602	-3.03;-0.92;-0.06	0.00	0.55	9.21	Duplication	4139.000;925.000
chr4	55133703	55133815	PDGFRA; +	7	0.7758	4906.000	0.0602	-2.59;-0.78;-0.08	0.01	0.80	7.70	Duplication	3806.000;1100.000
chr4	55136837	55136955	PDGFRA; +	8	0.6968	41 49.000	0.0602	-1.91;-0.56;-0.15	0.05	1.38	5.45	Duplication	2891.000;1258.00
chr4	55138608	55138705	PDGFRA; +	9	0.7757	3517.000	0.0602	-2.59;-0.78;-0.08	0.01	0.80	7.69	Duplication	2728.000;789.000
chr4	55139756	55139864	PDGFRA; +	10	0.8264	7291.000	0.0602	-3.13;-0.96;-0.05	0.00	0.50	9.59	Duplication	6025.000;1266.00
chr4	55140718	55140821	PDGFRA: +	11	0.8058	5304.000	0.0602	-2.90;-0.88;-0.06	0.01	0.61	8.76	Duplication	4274.000;1030.00
chr4	55140985	55141104	PDGFRA; +	12	0.7208	33602.000	0.0602	-2.10;-0.62;-0.12	0.03	1.19	6.06	Duplication	24221.000;9381.0
chr4	55141003	55141132	PDGFRA; +	12	0.7224	40417.000	0.0602	-2.12;-0.62;-0.12	0.03	1.18	6.11	Duplication	29199.000;11218.
hr4	55141049	55141164	PDGFRA; +	12	0.9242	25356.000	0.0602	-4.79;-1.56;-0.01	0.00	0.12	15.60	Duplication	23433.000;1923.00
chr4	55143577	55143701	PDGFRA; +	13	0.7762	5756.000	0.0602	-2.59;-0.78;-0.08	0.01	0.79	7.71	Duplication	4468.000;1288.00
chr4	55143991	55144088	PDGFRA; +	14	0.8527	3680.000	0.0602	-3.48;-1.08;-0.04	0.00	0.38	10.80	Duplication	3138.000;542.000
chr4	55144046	55144172	PDGFRA; +	14	0.6313	13002.000	0.0602	-1.46;-0.44;-0.22	0.15	1.96	4.00	Duplication	8208.000;4794.000
chr4	55144129	55144225	PDGFRA; +	14	0.9012	7194.000	0.0602	-4.28;-1.37;-0.02	0.00	0.19	13.70	Duplication	6483.000;711.000
chr4	55144534	55144662	PDGFRA; +	15	0.6589	4298.000	0.0602	-1.64;-0.49;-0.19	0.10	1.71	4.57	Duplication	2832.000;1466.00
chr4	55146538	55146664	PDGFRA; +	16	0.7408	7943.000	0.0602	-2.27;-0.67;-0.11	0.02	1.04	6.62	Duplication	5884.000;2059.000
hr4	55151551	55151668	PDGFRA; +	17	0.6999	9618.000	0.0602	-1.94;-0.57;-0.14	0.05	1.36	5.53	Duplication	6732.000;2886.00
chr4	55151948	55152060	PDGFRA; +	18	0.8276	5881.000	0.0602	-3.15;-0.97;-0.05	0.00	0.50	9.64	Duplication	4867.000;1014.000
chr4	55152017	55152145	PDGFRA; +	18	0.6913	13467.000	0.0602	-1.87;-0.55;-0.15	0.06	1.43	5.32	Duplication	9310.000;4157.000
chr4	55153608	55153722	PDGFRA; +	19	0.5683	1061.000	0.0765	-0.93;-0.37;-0.34	0.54	2.42	2.65	Duplication	603.000;458.000

Figure 14: An example of a CNV report. Select a different sample by clicking the drop-down menu on the toolbar. To view the CNV plots, click the CNV Graphs button next to the drop-down menu, including one similar to Figure 2.

For more information

The Advanta Solid Tumor NGS Library Prep Assay: Contact tech.support@fluidigm.com.

Receive a 30-day free trial of the NextGENe software: Contact tech_support@softgenetics.com.

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