SNP Genotyping on the 24.192 Dynamic Array IFC for Gene Expression

Introduction

Single nucleotide polymorphism (SNP) genotyping (GT) has been a widely applicable method for understanding population biology and agricultural biology. Currently Fluidigm offers 48.48, 96.96, and 192.24 Dynamic Array™ IFCs for Genotyping, which are designed to test 48, 96, or 192 samples with 48, 96, or 24 assays, respectively. However, researchers in some cases seek to prioritize the number of assays over the number of samples. In this technical note, we describe a method for genotyping analysis on the 24.192 Dynamic Array IFC for Gene Expression using Fluidigm SNP Type™ chemistry. This method enables analysis of 24 samples and 192 SNP Type assays on one IFC (integrated fluidic circuit). We also characterize performance of this method across various experimental conditions.

Materials and Methods

Materials and methods were as described in the SNP Genotyping User Guide (PN 68000098 O1 or later) unless otherwise specified in the associated protocol in this technical note (Appendix A).

A 96-assay SNP Type panel developed by Fluidigm (PN 101-7773, Advanta™ Sample ID Genotyping Panel) was used for the proof-of-concept study and loaded into the IFC in duplicates but analyzed separately. Twenty-three genomic DNA samples plus one no template control (NTC) were used to encompass a variety of genotypes. The DNA samples, including five Genome-in-a-bottle (GIAB) samples, were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research (Repository ID numbers listed in Table 1). The known calls from high-confidence regions of the five GIAB samples were used to test the genotyping concordance performance.

IFCs were loaded and thermal-cycled on the Fluidigm Juno™ system using a one-step script, or were processed through a two-step workflow using the IFC Controller RX for loading and FC1™ for thermal cycling. IFC images were captured and processed using either a Biomark™ or EP1™ instrument with a custom 24.192 GE IFC definition file. With one IFC we also used the RX loader and Biomark HD in a custom real-time configuration for investigating the effects of PCR cycles on the cluster profiles and genotyping performance.

Table 1. Coriell gDNA samples used in the study. GIAB samples are in bold fonts. NTC = no template control.

	1	2	3
A	**NA12878**	NA05127	NA01531
B	**NA24143**	NA06201	NA22162
C	**NA24149**	NA00130	NA12892
D	**NA24385**	NA00768	NA17316
E	**NA24631**	NA00897	NA17317
F	NA04619	NA00997	NA01707
G	NA20925	NA21080	NA01805
H	NA05124	NA01012	NTC

Data were analyzed using Fluidigm Genotyping Analysis Software v4.5.1. Genotyping calls were first made by automatic calling from the clusters, then improved by manual calling or mapped to an Assay Reference Library (ARL) created from one IFC.

Ground truth variant and wild type calls of the five GIAB samples were obtained from NCBI from sequencing data (ftp://ftp-trace.ncbi.nlm.nih.gov/giab/ftp/release/). NIST v3.3.2 GRCh37 version data were used. In total, 432 genotyping (variant + wild type) calls were found at the 96 loci tested for these samples in the public dataset and used for comparing IFC genotyping concordance performance. Genotyping calls from each IFC run were compared individually to the 432 genotyping calls and the concordance of the full IFC was calculated.

Results and Discussion

We performed a total of eleven 24.192 GE IFC experiments, and compared genotyping call performance across various preamplification and on-IFC PCR conditions (Table 2).

Table 2. Different preamplification and PCR conditions tested. For condition C (real-time imaging), data were analyzed at the different cycles. The cycle numbers in the on-IFC PCR cycle column refer to the modifiable step at 60 °C annealing temperature (see Appendix A for the PCR protocol).

Testing condition	Sample starting concentration (ng/µL)	Off-IFC preamp cycles	Preamp product dilution	On-IFC PCR cycles	Number of IFCs
A	60	14	–	34	4
B	10	14	1:4	34	2
C (real time)	10	14	1:16	40	1
D	5	12	–	25	4

In all testing conditions, distinct sample clusters were formed for all assays and the analysis software produced very accurate automatic calls without any manual correction or ARL (Table 3 on page 3).

Replicated IFC runs with same conditions generated overlapping clusters, demonstrating the robustness of the workflow. An example assay from two IFC runs is shown in Figure 1.

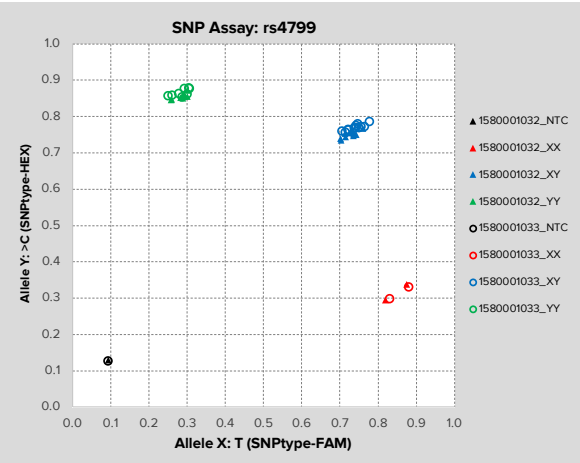


Figure 1. Example of one SNP assay (rs4799) cluster call result for two IFCs (IFC 1: 1580001032 and IFC 2: 1580001033) run under same conditions.

The same conditions on Juno and RX/FC1 also resulted in identical results (data not shown).

There were performance differences under different conditions. Higher concentration of input DNA and/or higher number of on-IFC PCR cycles in general increase the desired signal but may also increase the nonspecific reactions for some assays and cause clusters to merge closely to each other, reducing the genotyping performance.

To further investigate the effect of PCR cycle on genotyping performance, we ran an IFC in a custom real-time configuration and called genotypes at various PCR cycles. Example data in Figure 2 illustrates the progression of the PCR-specific signals over time. In one case, SNP assay rs6107027, an increase in number of cycles caused the cluster to merge closely together whereas in a second assay, rs6965201, the increase in number of cycles improved cluster separation.

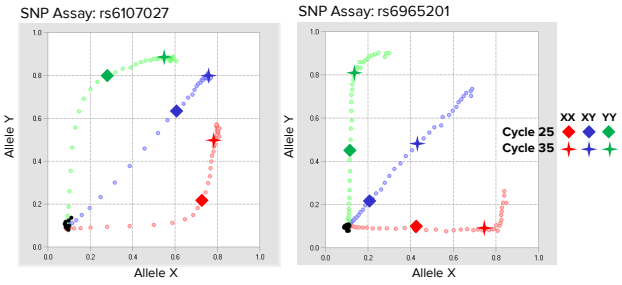


Figure 2. Fluorescent signals at different PCR cycles using condition C.

When comparing the overall IFC genotyping concordances by autocall without ARL at various conditions (Table 3), we found an optimal PCR cycle range that generated the clearest clusters and best overall performance. For the concentrations we tested, the range was 25 ±5 cycles. Therefore, we further tested this number of cycles in condition D (Table 3) with a decrease in number of preamplification cycles.

Table 3. Genotyping concordance performance comparison for different conditions. For testing condition C (real-time), genotyping calls were analyzed at various PCR cycles.

Testing condition	Sample starting concentration (ng/µL)	Off-IFC preamplification cycles	Preamplification product dilution	On-IFC PCR cycles	Genotyping concordance with autocall (no ARL)	Genotyping concordance with ARL
A	60	14	–	34	94.45%	100%
B	10	14	1:4	34	95.14%	100%
C (Real Time)	10	14	1:16	18	95.83%	100%
				20	**98.15%**	100%
				25	**99.07%**	100%
				30	**98.61%**	100%
				35	94.91%	100%
				40	94.44%	100%
D	**5**	**12**	**–**	**25**	**99.13%**	**100%**

It was observed that using condition D, the genotyping concordance with autocall (no ARL) was higher than obtained previously with condition C, but still not 100% (Table 3). The reason was the lack of a sample set that represented all the possible genotypes for one specific assay tested, causing a miscall. This might be an issue when using small sample sets such as the one tested here (n=24) and can be corrected using an ARL from a previous IFC run. When that was applied to the data generated in condition D, the concordance (sensitivity and accuracy) obtained from all IFCs was **100%**.

The samples tested in this study were all high-quality gDNA samples from Coriell. Lower-quality or lower-yield samples may require additional preamplification or on-IFC PCR cycles to achieve optimal genotyping performance. Such custom scripts can be easily configured and saved on a Juno or FC1 instrument.

Conclusion

We have developed a workflow using the 24.192 Dynamic Array IFC for Gene Expression to run 24.192 SNP Genotyping applications and demonstrated the performance of the genotyping concordance with the GIAB samples. Automatic calling can yield highly accurate (>99%) genotyping calls using the optimized off-IFC preamplification and on-IFC PCR conditions from as little as 5 ng/µL gDNA samples. Using Assay Reference Library can further improve the calling accuracy to 100%. We recommend that a new user start at a protocol of 12 cycles of preamplification, load directly onto the IFC, and run the on-IFC PCR at the 60 °C annealing temperature for 25 cycles. Both preamplification and on-IFC PCR cycles may be adjusted if sample concentration or quality is too low.

References

- Fluidigm Genotyping with the 96.96 IFC Using SNP Type Assays or the SNP Trace Panel Quick Reference (PN 100-3912 D1)
- Fluidigm SNP Genotyping User Guide (PN 68000098)

Appendix A: Protocol for SNP Genotyping on the 24.192 GE IFC

Follow the standard SNP genotyping protocol as described in the SNP Genotyping User Guide (PN 68000098 O1 or later) unless otherwise specified. Note that optimal performance requires that reagent mixes are vortexed and centrifuged to ensure proper mixing.

Sample Preamplification (Specific Target Amplification)

For the 24.192 genotyping workflow, samples need to be preamplified before they are loaded onto the IFC.

1 Prepare a specific target amplification (STA) assay pool of 192 assays. This can be stored at –20 °C for 1 year. (Minimize freeze-thaw cycles.)

Component	Volume (µL)	Final concentration
100 µM SNP Type assay STA primer (for each of 192 assays)	2 (x 192 = 384 total)	260.4 nM
100 µM SNP Type assay LSP (for each of 192 assays)	2 (x 192 = 384 total)	260.4 nM
Total	**768**	**—**

2 Prepare a 0.2 µM concentration of the STA assay pool.

Component	Volume for one 24.192 GE IFC (µL)	Final concentration
STA assay pool (from Step 1)	40	0.2 µM
20X SNP Type Sample Loading Reagent (Fluidigm PN 100-3425)	12	4.6x
Total	**52**	**—**

3 Prepare the STA mix.

Component	Volume per well (µL)	Volume for one 24.192 GE IFC with overage (µL)
2x QIAGEN® Multiplex PCR Master Mix (QIAGEN PN 206143)	2.5	75
0.2 µM assay pool (from Step 2)	1.25	37.5
5 to 60 ng/µL DNA (added individually)	1.25	—
Total	**5**	**—**

4 Thermal-cycle the STA reaction (total run time is approximately 1 hr and 20 min).

Temperature	Time	Cycles
95 °C	15 min	1
95 °C	15 sec	12*
60 °C	4 min	

* For lower-quality or lower-yield samples, cycles can be increased.

5 The STA product will be used in the IFC as stock, without dilution*. It can be stored at 4 °C if used within 1–3 days or at –20 °C for later use.

* If the sample concentrations are too high, we generally recommend reducing STA cycles. However, if diluting of STA products is desired, dilute into DNA suspension buffer mixed with 5% 20X SNP Type Sample Loading Reagent.

Assay Mix Assembly

1 Create 50X assays for each set of 192 assays in two 96-well plates. These can be stored for 1 year at –20 °C. (Minimize freeze-thaw cycles.)

Component	Volume per 24.192 GE IFC (µL)	Final concentration
100 µM SNP Type assay ASP1/ASP2 (for each of 192 assays)	3	7.5 µM
100 µM SNP Type assay LSP (for each of 192 assays)	8	20 µM
DNA Suspension Buffer (Teknova T0227)	29 (x 192 = 5,568 µL total needed)	—
Total	**40**	**—**

2 Create 3X assays. This is good for 24 hours when stored at 4 °C.

Component	Volume per 24.192 GE IFC (µL)	Final concentration
50X assays from previous step (for each of 192 assays)	1	3X (450 nM for ASP1/ASP2 and 1.2 µM for LSP)
DNA Suspension Buffer	15.7 (x 192 = 3014.4 µL total needed)	—
Total	**16.7***	**—**

*Volume prepared is enough for six to seven 24.192 GE IFCs.

3 Create assay pre-mix**, which should be** used on the same day. Before combining, bring all reagents to room temperature, vortex well, and centrifuge. Vortex prepped assay pre-mix well before using.

Reagent	Volume per reaction (µL)	Volume for one 24.192 GE IFC with overage (µL)
2X Biotium Fast Probe Master Mix (Biotium PN 31005)	3	690
20X SNP Type Sample Loading Reagent (Fluidigm PN 100-7608)	0.3	69
60X SNP Type Reagent (Fluidigm PN 100-7607)	0.1	23.0
50X ROX™ (Thermo Fisher Scientific PN 12223-012)	0.036	8.3
PCR water	0.564	130
Total	**4**	**920.3**

4 Prepare the assay mix in two 96-well plates labeled A and B.

Reagent	Volume per reaction (µL)
Assay pre-mix (from Step 3 on page 5)	4
3X assay (from Step 2 on page 5)	2
Total	**6**

Choose a Juno/IFC Controller RX Workflow

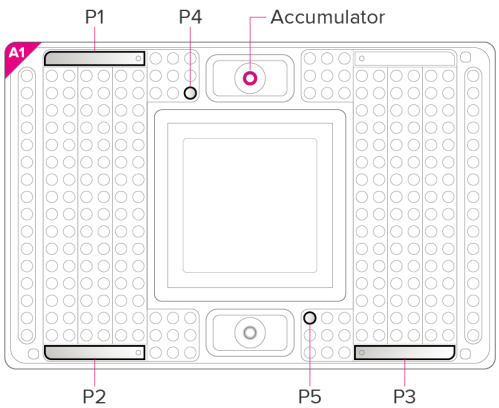
Load and thermal-cycle (PCR)	Image
Juno one-step loading and PCR	Biomark HD/Biomark or EP1

Load	Thermal-cycle (PCR)	Image
RX	FC1	Biomark HD/Biomark or EP1

Prepare 24.192 IFC

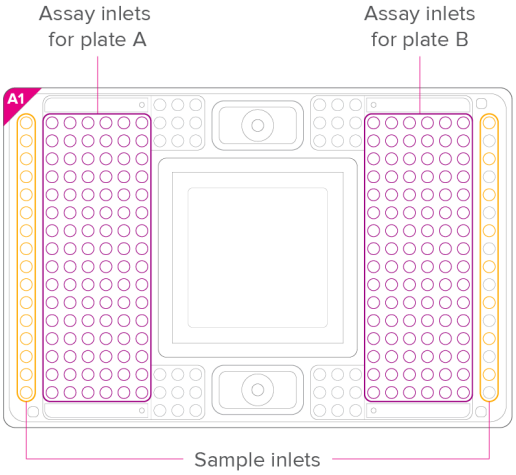
1 Inject Control Line Fluid (Fluidigm PN 100-7602) into the top accumulator.

2 Add 150 µL of Pressure Fluid (Fluidigm PN 100-3463) in positions P1, P2, and P3, and 20 µL of pressure fluid in positions P4 and P5.



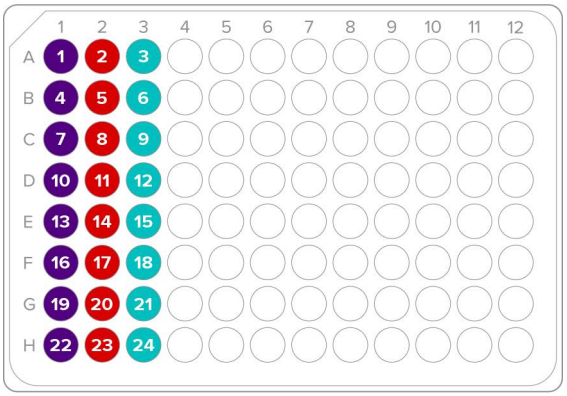
Pressure fluid, 150 µL (P1, P2, and P3)
Pressure fluid, 20 µL (P4 and P5)
Control line fluid, 150 µL (top accumulator only)

3 Transfer 4 µL of each assay prepared to each of the 192 assay inlets.

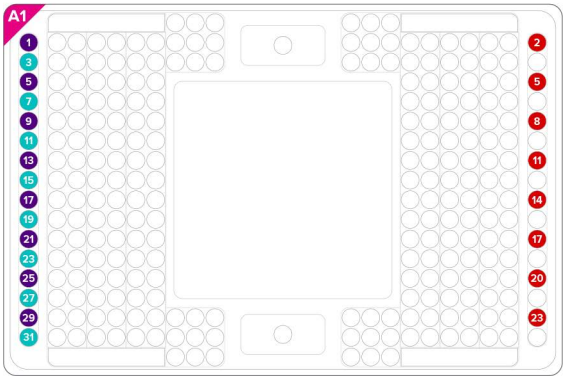


4 Transfer 4 µL of each sample STA product to each of the 24 sample inlets (no STA product dilution is needed prior to IFC transfer).

96-well plate containing sample STA products



Sample inlets in the 24.192 GE IFC



Run the IFC on Juno

Run IFCs on the Juno using the **SNP Type: One Step 24.192** script to load and mix all reagents and samples and complete PCR, then image on Biomark HD, Biomark, or EP1.

Run the IFC on IFC Controller RX and FC1 Cycler

Run the IFCs on the IFC Controller RX using the **Load Mix (158x)** script to load and mix all reagents and samples and on the FC1 cycler using the **SNP Type 24.192 v1** thermal protocol to complete PCR, and then image on Biomark HD, Biomark, or EP1.

PCR Protocol

The PCR protocol is as follows with modifiable final cycle numbers.

Temperature	Time	Cycles
95 °C	5 min	1
95 °C	15 sec	1
64 °C	45 sec	
72 °C	15 sec	
95 °C	15 sec	1
63 °C	45 sec	
72 °C	15 sec	
95 °C	15 sec	1
62 °C	45 sec	
72 °C	15 sec	
95 °C	15 sec	1
61 °C	45 sec	
72 °C	15 sec	
95 °C	15 sec	25* (modifiable)
60 °C	45 sec	
72 °C	15 sec	

* You can increase the PCR cycles if amplification signals are too low or decrease the PCR cycles if clusters are merging. Note that different assays may perform differently.

Analyze the Data

After the run is complete, process data using the SNP Genotyping Analysis software. When annotating the assay information, choose the SBS Plate with SBS96 option in the assay plate setup wizard and then use “192-Assay-SBS96-Left&Right.cdsp” as the dispense map. For information about using the analysis software, see the SNP Genotyping User Guide (PN 68000098).

Appendix B: Instrument Software Requirements and Ordering Information

Fluidigm Instrument and Software Requirements

Operation	Instruments	Software version
IFC loading and PCR	Juno	v3.11.1 with custom script*
	IFC Controller RX/ FC1 cycler	v2.8/v1.6 with custom protocol*
Imaging	Biomark HD, Biomark or EP1	v4.2.2 or later with custom IFC definition*
Data analysis	—	SNP Genotyping Analysis v4.5.1

* Contact techsupport@fluidigm.com for the 24.192 SNP Genotyping Configuration Files (101-8007).

Ordering Information

Vendor	Name	Part number
IFCs		
Fluidigm	Biomark 24.192 GE IFC & Control Line Fluid—2 IFCs	101-7995
Fluidigm	Biomark 24.192 GE IFC & Control Line Fluid—10 IFCs	101-7996
Fluidigm reagents		
Fluidigm	SNP Type 192.24 Genotyping Reagent Kit—10 IFCs **NOTE** This kit is sufficient for processing between six and seven 24.192 GE IFCs when following the protocol documented in this technical note.	100-4136-R
Third-party reagents		
QIAGEN	2x QIAGEN Multiplex PCR Master Mix	206143
Teknova	DNA Suspension Buffer	T0227
Biotium	2X Fast Probe Master Mix	31005
Thermo Fisher Scientific	ROX (50X)	12223-012

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