Single Cell Genetics; from Sorting to Fluidigm

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Comprehensive Flow Cytometry Core Support

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Topics to Discuss

- **Technology of the Fluidigm C1 and BioMark HD**
- Assays/Designs
- How to work with the Core to set up your Fluidigm experiments?

What does the Core has to facilitate your single cell analyses?

Sorting

FACS ARIA II &
Index sorting

Fluidigm C1

Single cell PCR and SNP

IFC controllers
MX (48 x 48)
HX (96 x 96)

BioMark
(qPCR)

Singular
software

All in SHEL Rm 271

What can one do with the Fluidigm C1and the BioMark HD Systems?

The Fluidigm microfluidic technology supports genomics-based applications:

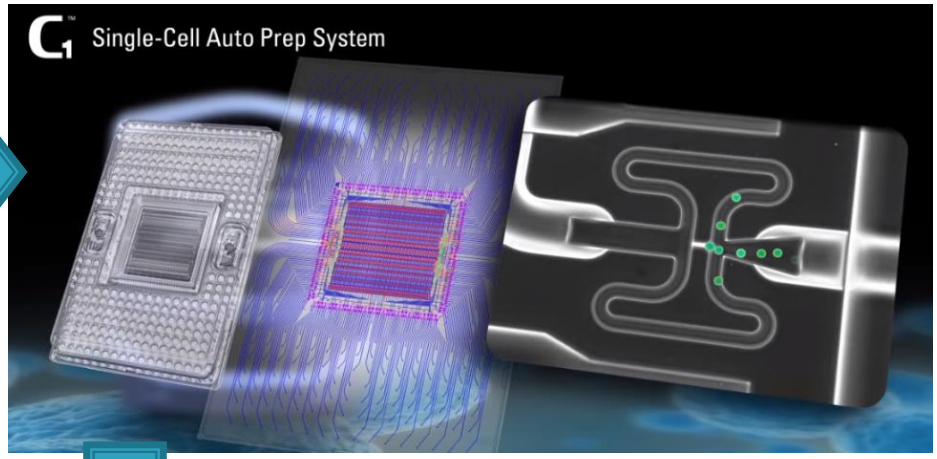
- Single cell gene expression
- High-throughput SNP genotyping
- RNA-seq
- Protein expression analysis
- Digital PCR
- Mutant detection
- More under development

What can one do with the Fluidgim C1 Single Cell Autoprep System?

Watch the C1 video here: Fluidigm C1 general concept

Application	Use in combination with other modules	Protocol
mRNA expression	**BioMark**	**Single-cell Gene Expression brochure**
mRNA sequencing	**Clontech's SMARTer Ultra Low RNA input Kit for cDNA synthesis and Illumina's Nextera XT Sample Preparation Kit for library preparation.**	**mRNA sequencing protocol**
DNA sequencing	**Next-Generation Sequencing**	**C1 DNA Sequencing Brochure**
Chromatin accessibility (ATAC-seq)	**Hudson Alpha**	**view protocol worksheet**

What is done inside a Fluidigm C1 Prep?



This is the first step bulk gene amplification

Applications enabled by the Fluidigm C1 Prep

Specification	
Supported applications	Targeted gene expression Whole transcriptome analysis (mRNA sequencing) miRNA analysis DNA sequencing (see DNA Sequencing Data Sheet, PN 100-7426)
Sample sources	Primary and cultured cells
Sample input	200–1,000 cells
Average cell size	5–25 µm
Cell capture efficiency*	>90% occupancy rate with 1,000 cells input

www.bio-equip.com

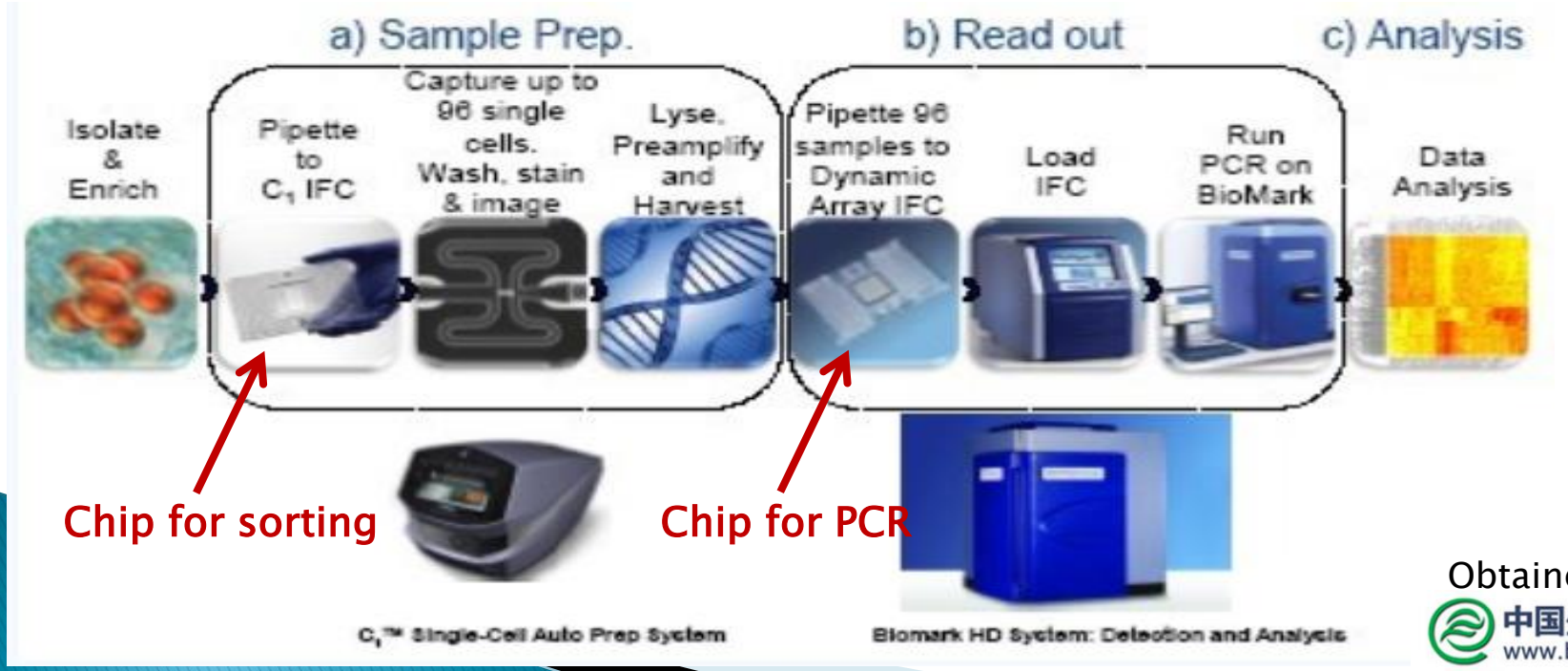
Fluidigm C1 General Workflow

Cell Suspension

- Capture
- RT
- mRNA-seq prep
- Whole genome amp

- qPCR (BioMark)
- Next Gen sequencing (Illumina)

Fluidigm C1 /BioMark Workflow



Obtained from
中国生物器材网
www.bio-equip.com

Fluidigm C1 in Combination with BioMark for Gene Expression

All carry out at the CFCC

Targeted Gene Expression (specific target amplification)	
Workflow	Capture, stain, image, lyse, reverse transcribe, preamplify, harvest, and perform real-time PCR analysis on the Biomark™ HD System
Supported assays	Delta Gene™ Assays TaqMan® Gene Expression Assays (probe-based)
Assay format	Two-step reverse transcription and amplification
Throughput	9,216 datapoints per run
Time to results	Cells to preamplified target: 8 hours (overnight) Real-time PCR: 4 hours Total assay time: 12 hours
Hands-on time	70 minutes
Chip-to-chip R^2 correlation	0.99

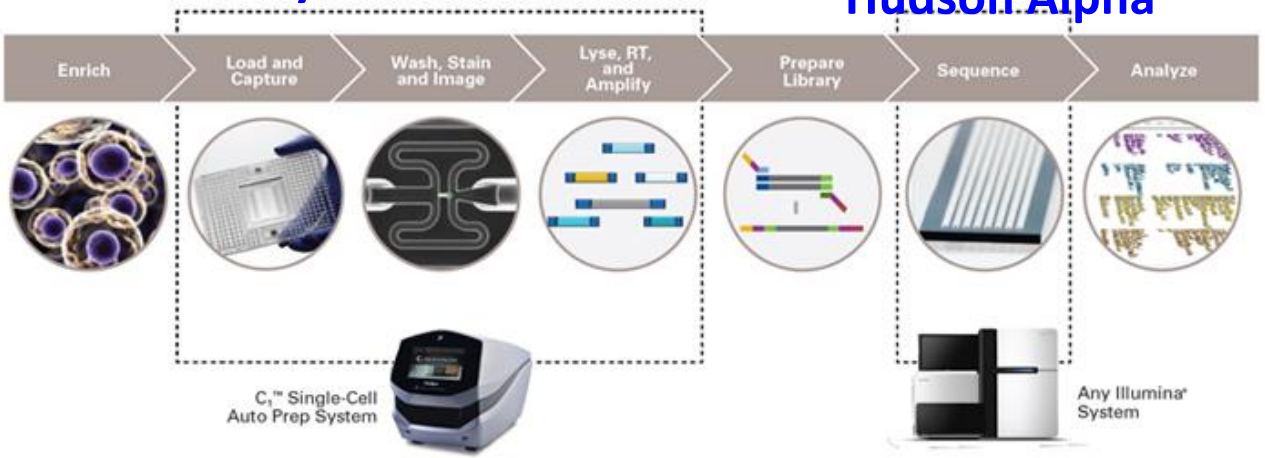
Fluidigm C1 in Combination with Illumina Sequencing for RNA-seq

mRNA Sequencing Application	
Workflow	Capture, stain, image, lyse, reverse transcribe, amplify, harvest, prepare library, and sequence
Supported platforms	Any Illumina® sequencing system
Time to results	Cells to sequence-ready library: 13 hours (overnight)
Hands-on time	3 hours
Typical sequencing performance observed from K562 and HL60 cell lines:	
cDNA concentration per cell	>300 pg/µL
Number of detected RefSeq transcripts with >10 reads mapped	≥4,000 (≥200,000 total single-end reads or ≥400,000 total paired-end reads)
Reads mapped to rRNA/total reads	≤1% rRNA/total reads

Carry out at the CFCC

Heflin Genomics Core
Hudson Alpha

[Oxford Genomics Centre](#)
[*Wellcome Trust Centre for Human Genetics*](#)



The BioMark HD real-time PCR system

IFC controller

BioMark HD

Computer/
Singular data
analysis software

BioMark System is used in combination with the following modules

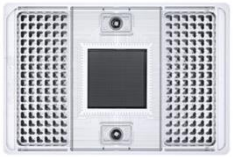
Equipped by the CFCC in SHEL Rm 271

The Biomark HD is compatible with multiple system components to meet a variety of application and sample throughput needs.

IFC CONTROLLER COMPATIBILITY

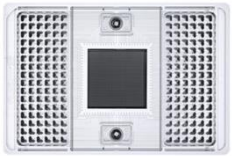
	IFC Controller MX	IFC Controller HX	IFC Controller RX
Gene expression	48.48 Dynamic Array IFC	96.96 Dynamic Array IFC	—
SNP genotyping	48.48 Dynamic Array IFC	96.96 Dynamic Array IFC	192.24 Dynamic Array IFC
Digital PCR	12.765 Digital Array IFC 48.770 Digital Array IFC qdPCR 37K IFC	—	—
Experiment tracking	Barcode		
Gas pressure	Internal compressor		
Interface	USB and ethernet		
IFC Controller MX, HX, RX or WX software	Touchscreen interface for operating and tracking		
Dimensions (approx.)	19 x 9.5 x 13 inches; 48.5 x 24 x 33 cm		

BioMark HD for Gene Expression



IFC Dynamic Array

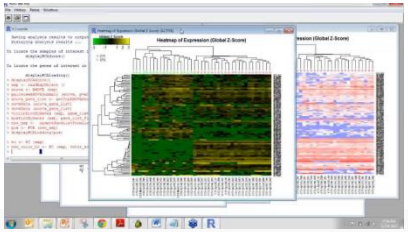
Primers
Samples



WORKFLOW

IFC controller

BioMark HD



Singular data analysis

PRIME | TRANSFER | LOAD | RUN | ANALYZE

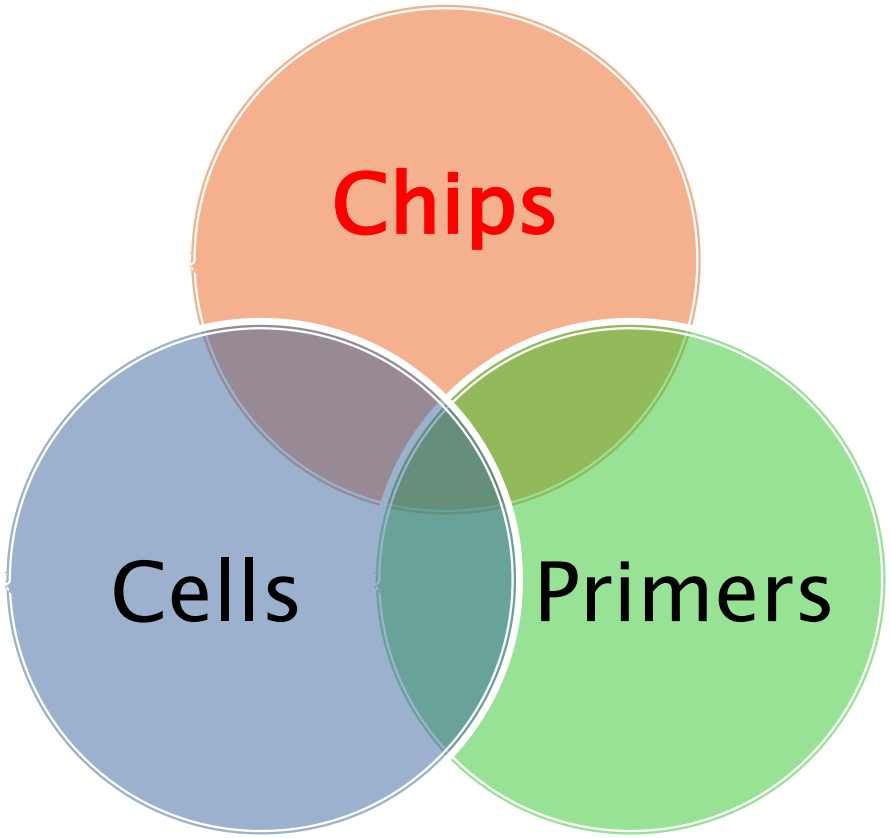
1. **Prime**: prime the Dynamic Array IFC to prepare it for sample transfer
2. **Transfer**: Pipet samples and primer probe sets into the designated inlets of the IFC.
3. **Load**: Load the IFC on the controller, which prepares pressure loading of samples and assays into the reactions
4. **Run**: Run the IFC in BioMark for thermal cycling and end-point real-time fluorescence detection.
5. **Analyze**: View and analyze color-coded expression heat maps, allele maps and cluster plots for single or multiple runs with the Singular software.

BioMark procedure **watch it here**

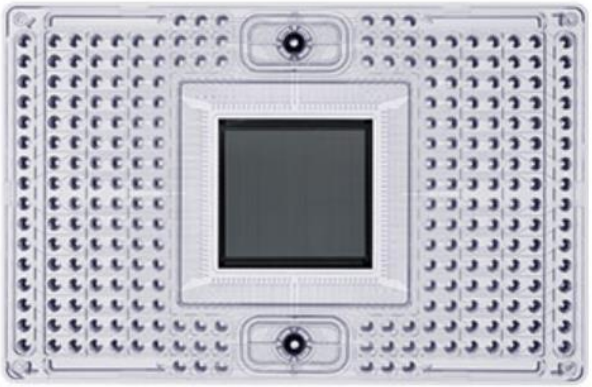
Topics to Discuss

- Technology of the Fluidigm C1 and BioMark HD
- **Assays/Designs**
- How to work with the Core to set up your Fluidigm experiments?

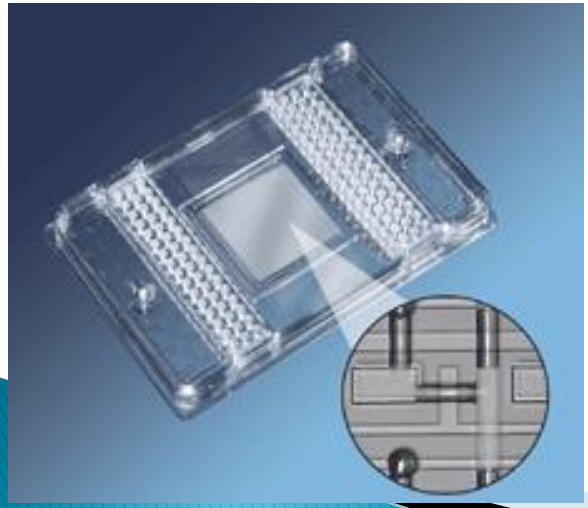
Fluidigm Experiment Design



Two types of chips for gene expression



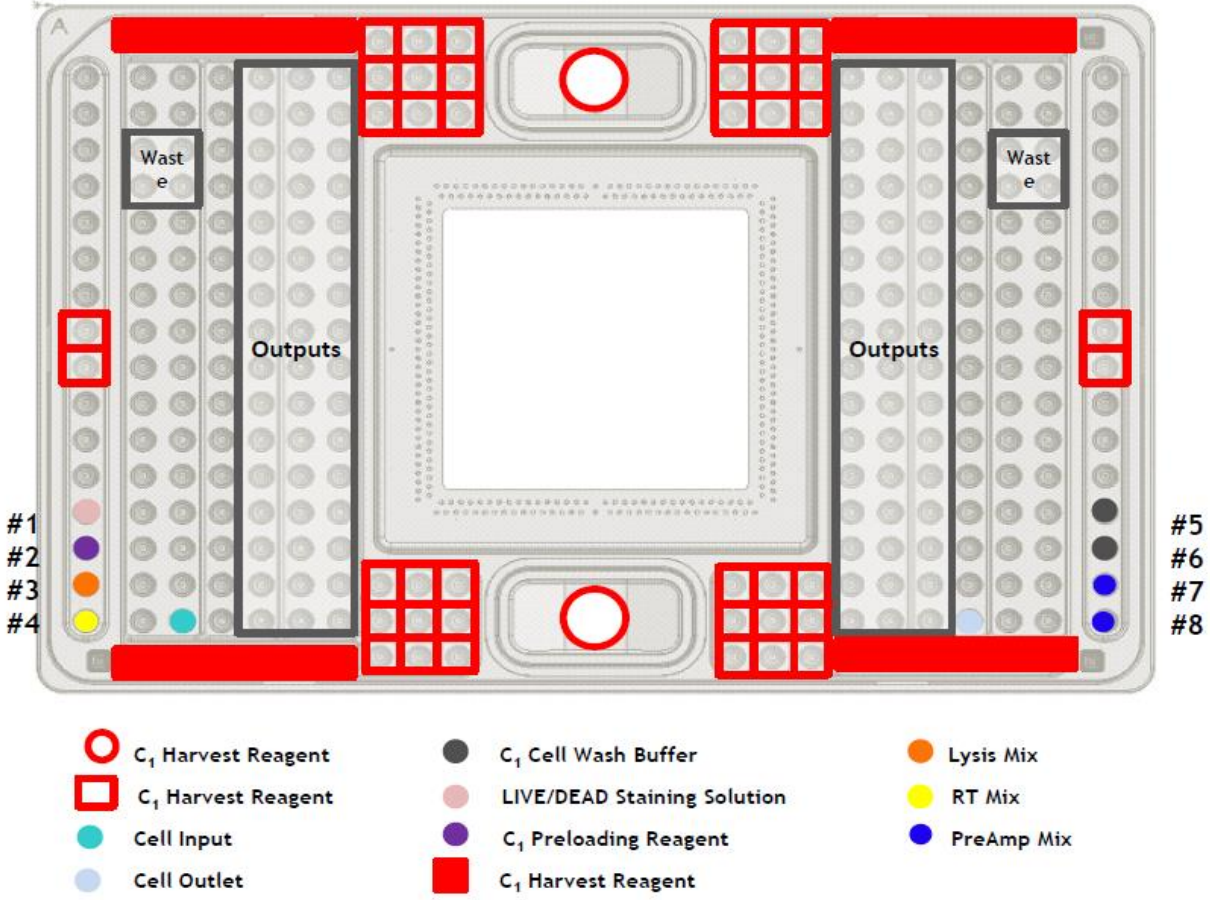
C1 chip used during the Fluidigm C1 step



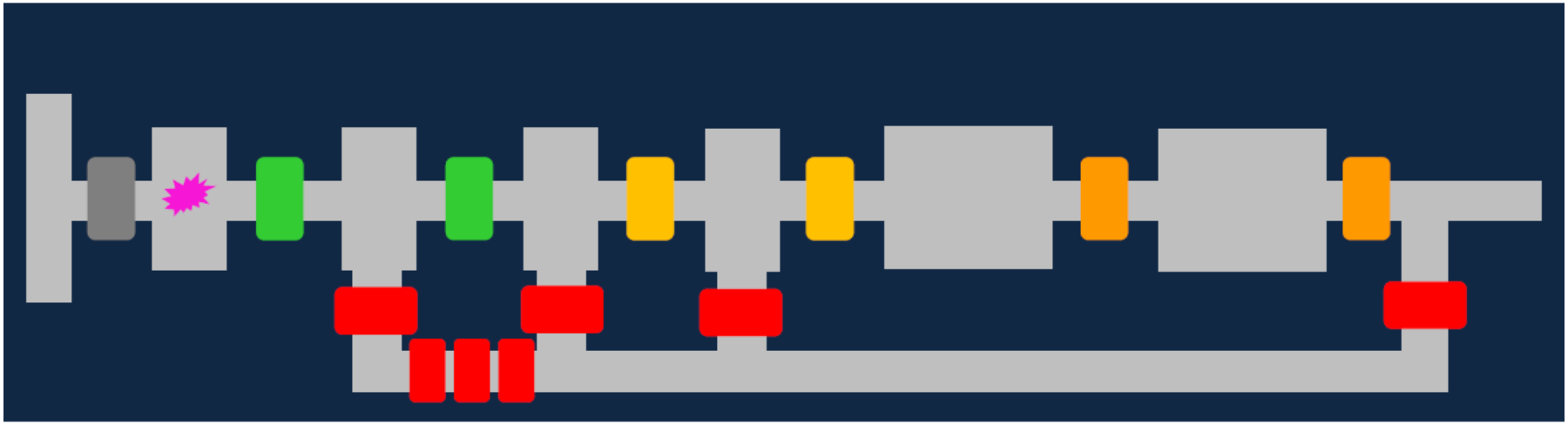
Dynamic IFC chip for PCR

Chip for the Fluidigm C1– single cell sorting, lyse, RT, pre-amp

Pipetting Map for C1 System

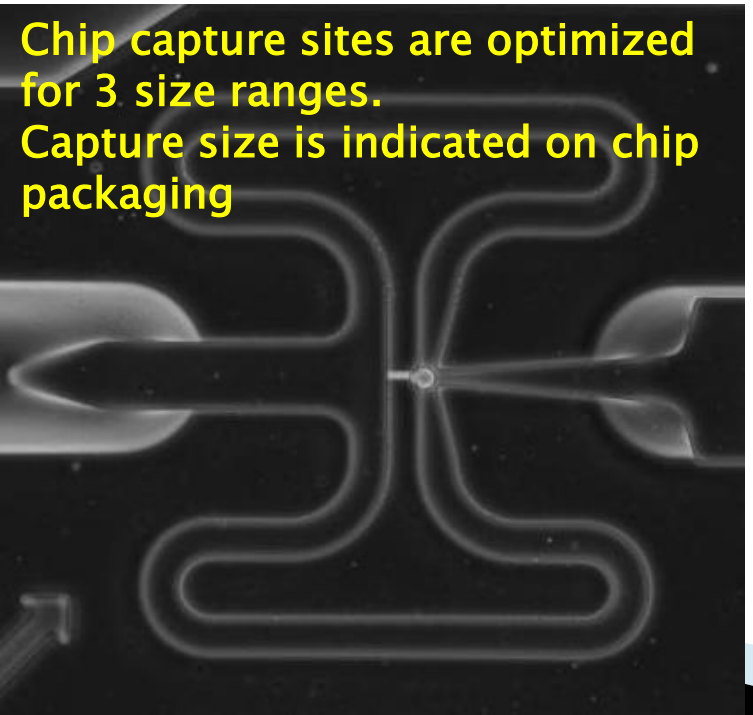


Pre-amplification Implementation in Chip



The C1 chip is optimized to catch your cells

- Capture: Live cells capture more efficiently than dead cells
- Lysis: Efficient for live cells, not optimized for the cross-linking that occurs during fixation
- Transcript quality: maximal for efficiently prepared live cells



Size does matter

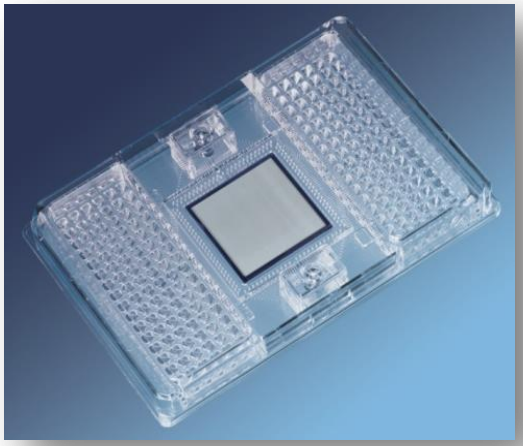
The cell size determines which chip to use for the experiment.
Chips capture three size ranges of cells:
-5-10 µm
-10-17 um
-17-25 um

Cell size must be measured on cells in suspension.
The Core has a Nexelom Cellometer X4 Cell Counter to determine cell count/size.

Chips for IFC controller and BioMark

Dynamic Arrays For GX,GT,CNV

96.96



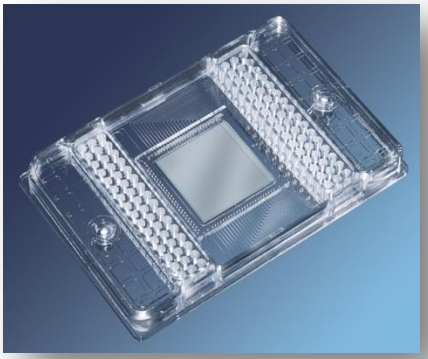
48.48

	96 x 96 array	48 x 48 array
Cost per chip	~ $900 / Chip + reagent	~ $300 / Chip + reagent
Validation capability	Duplication/triplication	Less so
Time	More time for primer design and pipetting	Less time for primer design and pipetting
Information	9,216 data points	2,304 data points

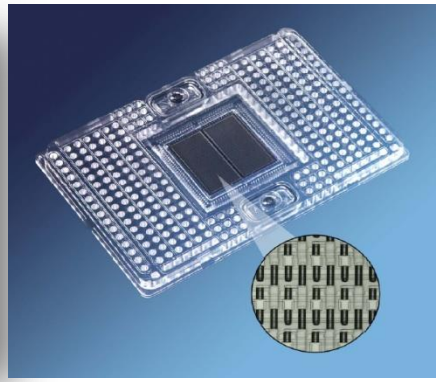
Other Chip Formats for IFC controller and BioMark

Dynamic Arrays
For GX,GT,CNV

48.48

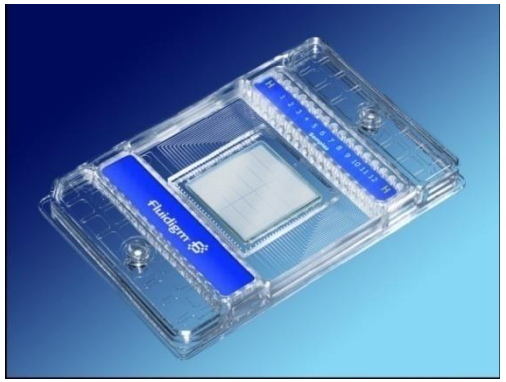


192.24

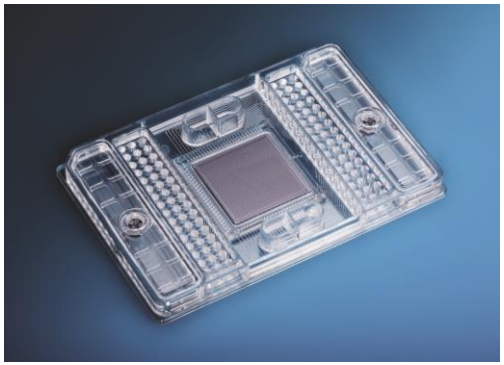


Digital Arrays
For dPCR,CNV

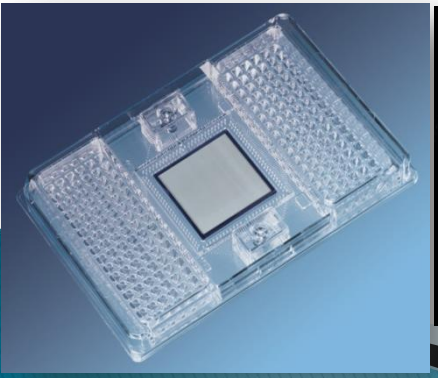
12.765



NGS library prep
Access Array 48.48

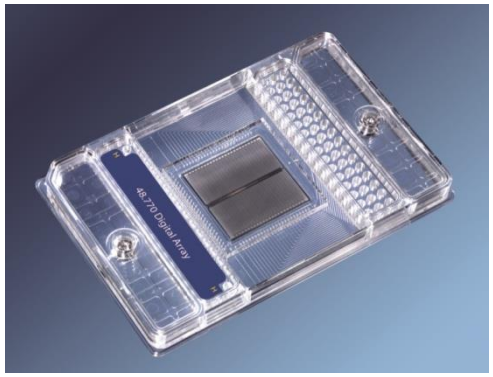


96.96



Flex Six™ IFC

48.770



qdPCR 37K IFC

IF YOU DO NOT WANT HIGH THROUGHPUT

FLEX SIX GENE EXPRESSION CHIP

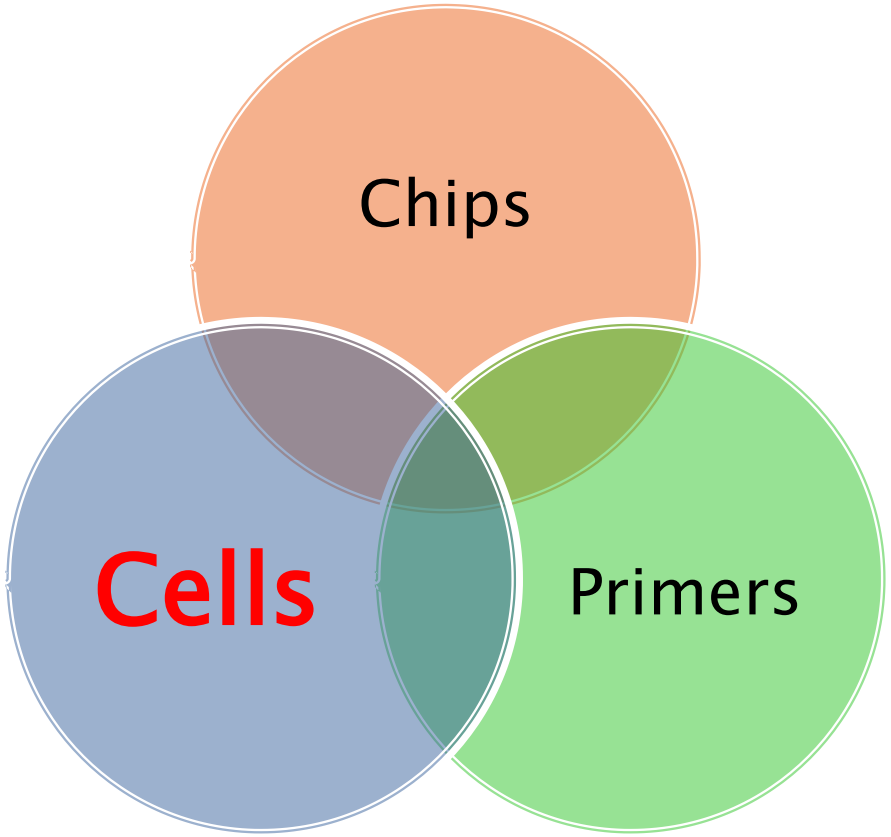
Any 12X12 configurations

Use the chip up to 6 times

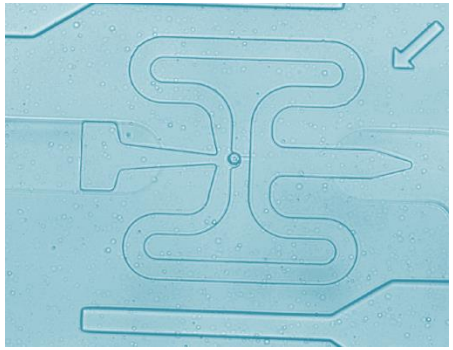
Format Flexibility

- 72 samples by 12 assays
- 12 samples by 72 assays
- Any combination of 12x12
- Or 12x12 multiple times

Fluidigm Experiment Design



Cells



Fluidigm C1

vs

FACS ARIA II

	Fluidigm Sort	FACS Sort
Cost	$400/chip + reagents	$75 assisted sort/hr
Single cell validation	Can be checked fairly quickly under a microscope	In theory, it should be one cell/well, but hard to verify
Easy flexibility to alter analysis template	Yes	Low
Phenotype of cells	Not inherent in the system but can be done with additional steps	Index sorting allows FACS determination of phenotype of each cell

General Guidelines for Cell Prep

How many cells should I load?

- Large chip: 200-300 cells/ µl
- Medium chip: 250-400 cells/µl
- Small chip: 400-800 cells/µl
- In total 1000-5000 cells are loaded into the chip to capture 96

Notes on cell suspension

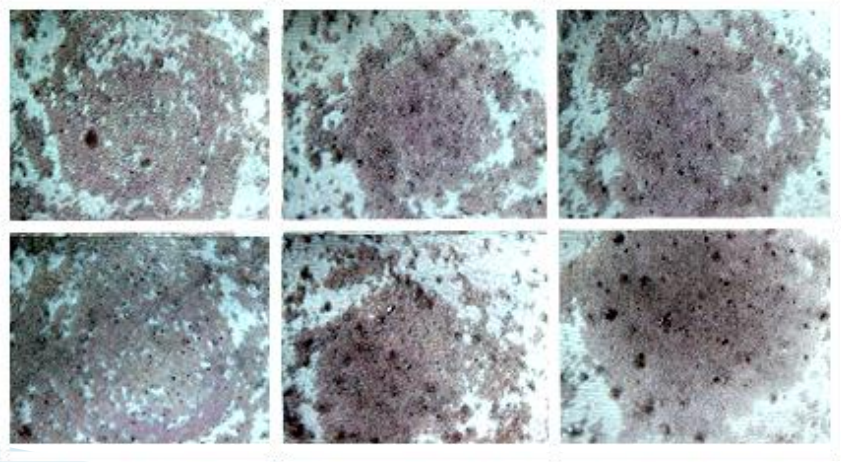
- Debris in cell suspension negatively effects capture
- Non-monodisperse cell suspensions will present multiple cells in capture sites.

What protocols should I use to prepare single cell suspension?

- Cell culture samples: regular PBS/ trypsin harvest
- TrypLE (ThermoFisher) is a more gentle equivalent alternative
- Minimize the time between cell prep and loading on C1
- Do Not vortex the cells
- Minimize cell clumping: triturate shortly before loading
- Filter w/ cell strainers: Pluribead cell strainer (10, 20, 30 µm)
- Add 1-5% BSA for very sticky cells

Dissociation for cultured primary cells

- Freshly derived from living organisms and maintained in culture
- Limited number of divisions before entering senescence
- More sensitive than non-primary cell cultures
- General recommendation : TrypLE cell dissociation
- Alternative recommendation : Acutase “stem PRO acutase” for more gentle dissociation of sensitive cells



Single cells from primary tissues

Enzymes for tissue dissociation: Papain, Collagenase, Elastase

Papain Dissociation system:

- Recommended for epithelial tissues, brain cells
- Worthington Biochem has recommended protocols
- Unlike many other enzymes papain works in the presence of Ca and Mg

Acutase:

- Recommended for embryonic dissociation
- Stem PRO acutase (ThermoFisher) - a marine-origin enzyme with proteolytic and collagenolytic activity for the detachment of primary and stem cell lines and tissues.
- Gentle (compared to trypsin) dissociation for sensitive cells

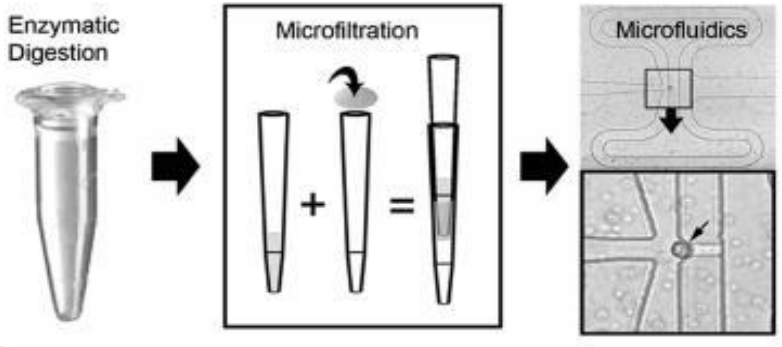
- Equilibration with 95%O2 and 5% CO2
- Important for the survival of tissue during dissociation

Tumor or embryonic tissues- single cell suspension prep

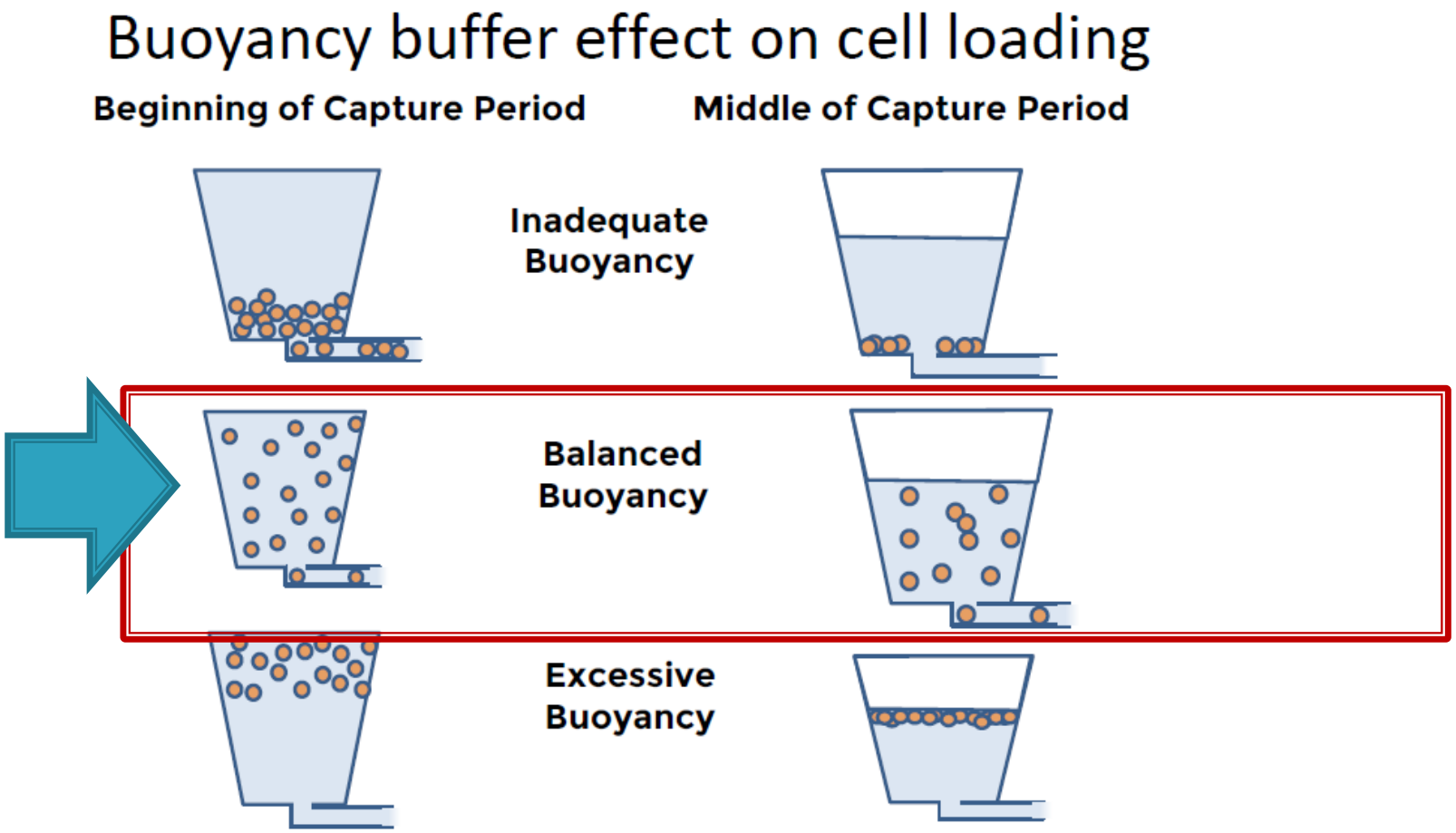
Combined mechanical and enzymatic dissociation :

- Mechanical: Gentle MACS (Mylteni) easy semi automated dissociation of solid tumor tissues into single cell suspensions

- Enzymatic dissociation : collagenase efficiently removes connective tissues

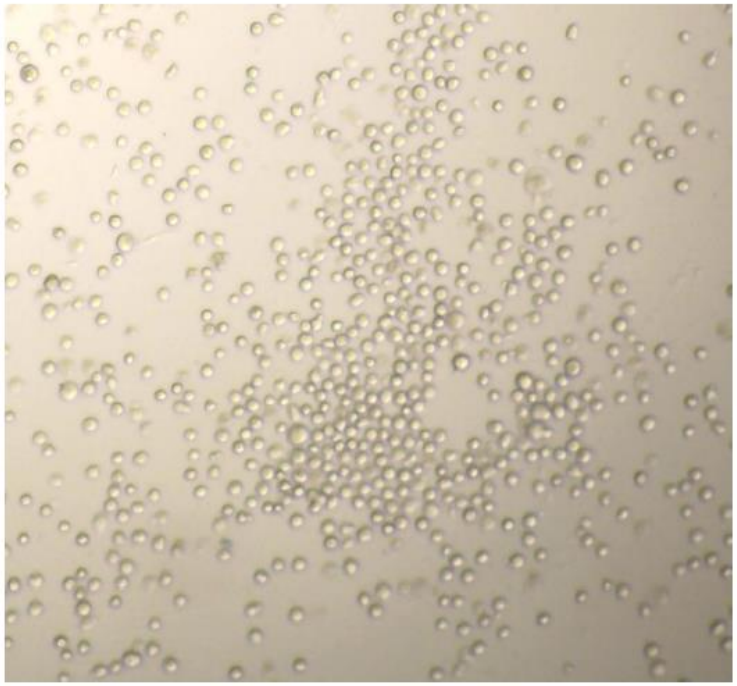


Buoyancy of the single cell suspension is another factor affecting cell capture



Cell Buoyancy Assessment

Inadequate Buoyancy



Cells settled on bottom of well

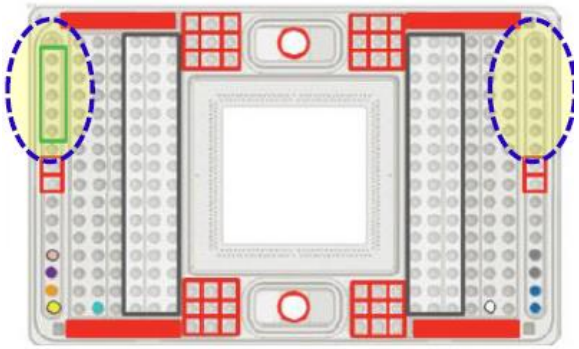
Balanced Buoyancy

Cells suspended through volume of well

Excessive buoyancy resembles inadequate buoyancy, but the cells settle at the top of the fluid volume and no cells are visible at the bottom surface

Cell Buoyancy Testing on C1 chip

- Testing can be done on the upper wells of the C1 chip
- 10 µL per well of sample
- Make titrations from table below
- Let samples sit for 10 min and examine titrations using inverted brightfield microscope.
- The balanced buoyancy solution will have even cell distribution throughout the volume of the well.



Culture cells **Primary immune cells**

	0%	50%	55%	60%	65%	70%	75%	80%	85%	90%
Cells (µl)	10	5	5.5	6	6.5	7	7.5	8	8.5	9
Suspension Reagent (µl)	0	5	4.5	4	3.5	3	2.5	2	1.5	1
Total volume/well	10 µl →									

How do I enrich the samples for C1 experiment during FACS Sorting?

FACS general guidelines:

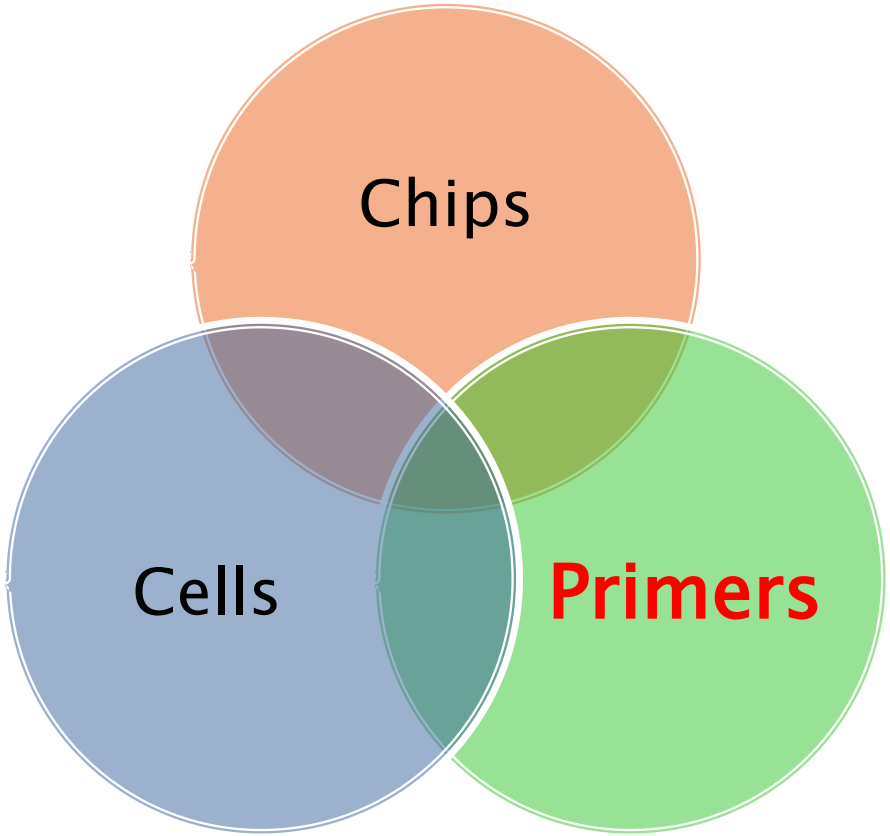
- Fragile cells: larger nozzle and low psi.
- Increased throughput & yield or for small cells: smaller nozzle and high psi

FACS Aria setting up options:

1. High: 70 µm nozzle and 70 psi sheath pressure
2. Medium: 80 µm nozzle and 40 psi sheath pressure
3. Low: 100 µm nozzle and 20 psi sheath

The Core should help you with this but please always remind the Core of your single cell analysis attempt.

Fluidigm Experiment Design



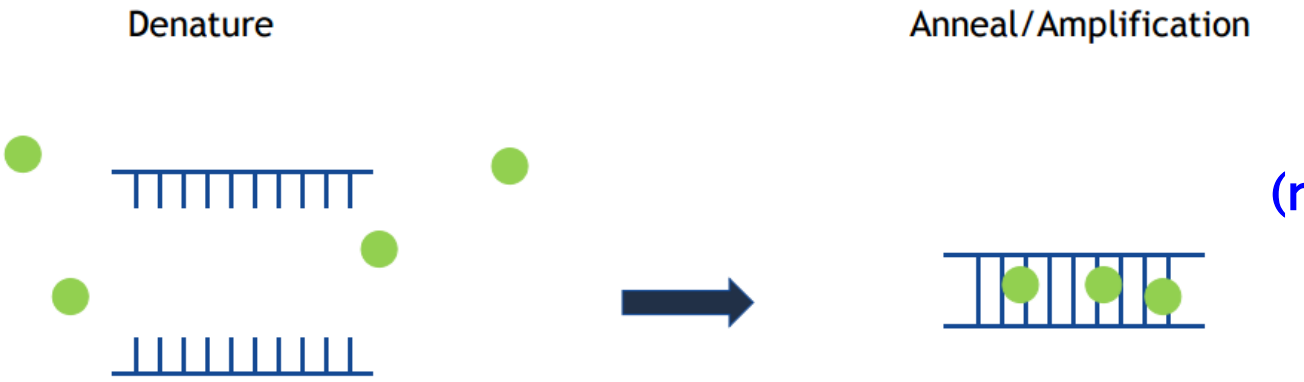
Two types of primers/probes can be used

For Regular qPCR

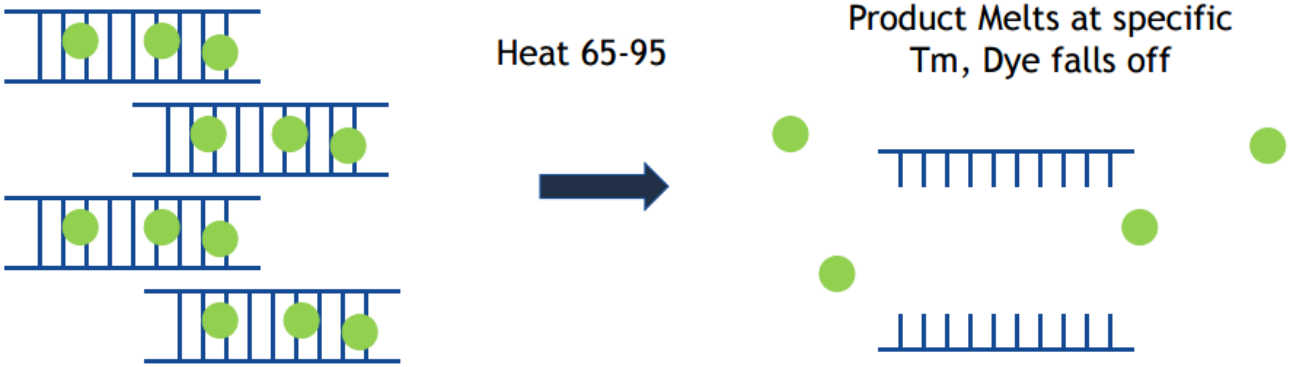
EvaGreen (≈ SYBRGreen)

	SYBR® Green-based detection	TaqMan®-based detection
	Uses SYBR® Green dye (a dsDNA binding dye) to detect PCR product as it accumulates during PCR.	Uses a fluorogenic probe specific to target gene to detect target as it accumulates during PCR.
Specificity	Medium*	High
Sensitivity-low # of copies	Variable*	1-10 copies
Reproducibility	Medium*	High
Gene expression	Low level of quantitation	High level of quantitation
Applications	Gene expression DNA quantitation (pathogen detection) CHiP	Gene expression DNA quantitation CHiP SNP genotyping Copy number variation Pathway analysis microRNA & small RNAs Mutation detection Protein analysis Multiplexing

The EVA Green Approach



Binds to any dsDNA
(not sequence specific)



Melt Curve
Following PCR

EVAGreen Pros and Cons

Pros

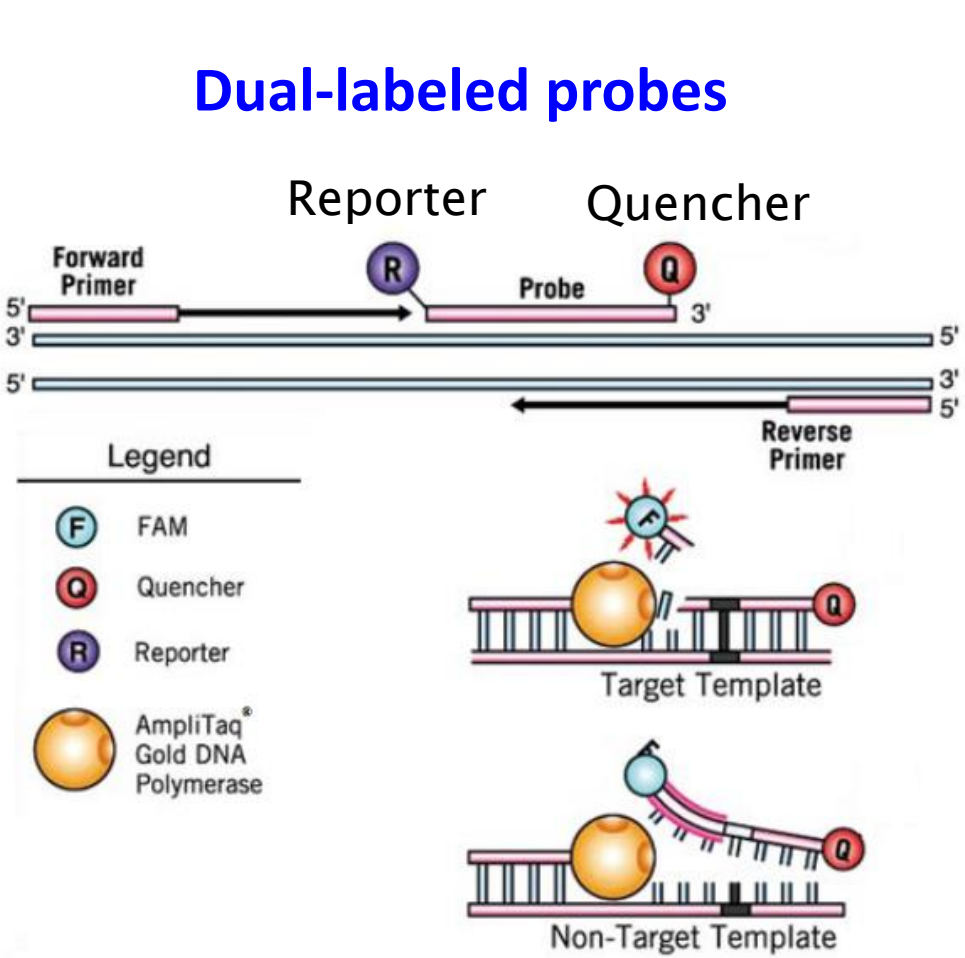
- F/R primers with non-specific DNA-binding dye = A lot more economical than TaqMan

- Flexible, easy to change genes in and out

Cons

- Can be prone to non-specific PCR products (melt curve required)

The TaqMan Dual-labeled Probes Approach



Dual-labeled probes are designed to hybridize to a complementary region of the cDNA.

The probe is flanked by an upstream and downstream primer pair that generates a PCR product.

During PCR, when the polymerase extends the PCR product from the upstream primer, the 5' exonuclease activity of the polymerase cleaves the probe. This separates the fluorescent quencher and reporter dyes

The increase in fluorescence intensity is proportional to the number of probe molecules that are cleaved.

TaqMan Pros and Cons

Pros

- One probe for each target = specificity
- Less likely to report on non-specific PCR products
- Easier to use (all in one tube)

Cons

- A lot more expensive
- Harder to design the primers

EVAGreen Primer Design and Order

1. Conventional qPCR design

2. Fluidigm DELTAgene® Assays (Fluidigm-designed primers for genes/panels)

- Minimize upfront cost of assays
- Uses EvaGreen Chemistry
- Amplicons designed to cross an intron
- Assays predicted to multiplex well

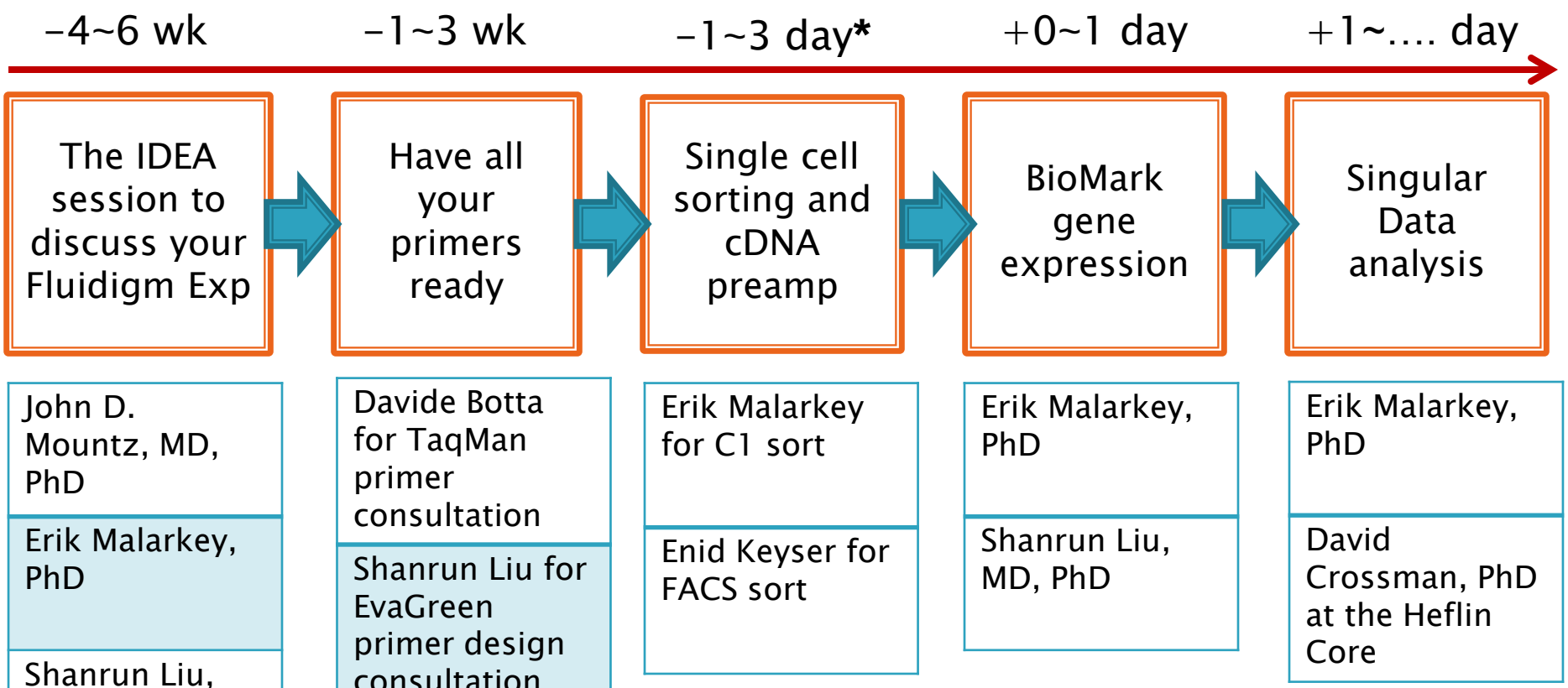
For EVAGreen primers - Order primers through Invitrogen
(http://www.lifetechnologies.com/europe/en/home/products-and-services/product-types/primers-oligos-nucleotides/invitrogen-custom-dna-oligos.html)

Purification: Desalted
Starting Synthesis Scale: 25nmole
Ship Medium: Dry
Normalization: None
Calculate primers to 100 uM using DNA suspension Buffer from Teknova cat # T0221 (CFCC at Shelby has this buffer)

Topics to Discuss

- Technology of the Fluidigm C1 and BioMark HD
- Assays/Designs
- **How to work with the Core to set up your Fluidigm experiments?**

How to work with the Core

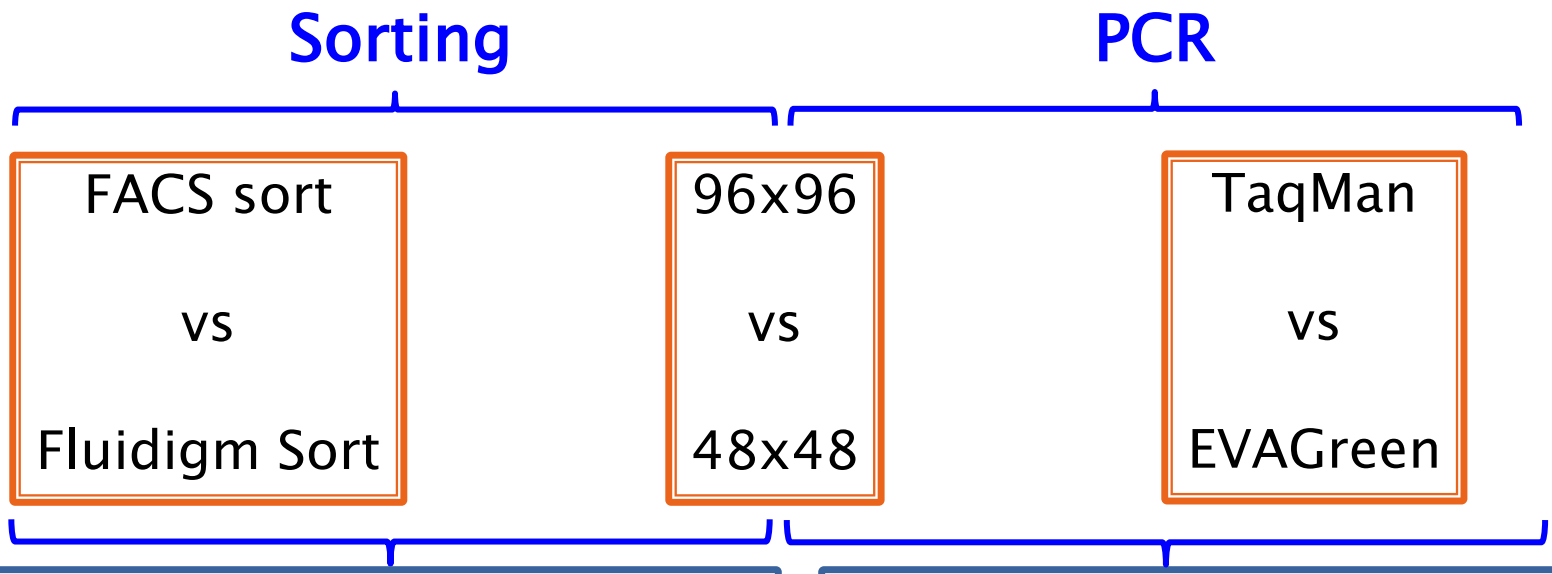


* cDNA can be stored for future use

Who to contact to set up a Fluidigm assay

Name	Specialty	Phone	Email
John D. Mountz, MD, PhD	General discussion	4-8909	jdmountz@uab.edu
Erik Malarkey, PhD	General Fluidigm/BioMark	4-0994	malarkey@uab.edu
Shanrun Liu, MD, PhD	Single cell genomic analysis and EVAGreen primer design consultation	6-2176	shanrun@uab.edu
Enid Keyser	FACS sorting	4-1362	efk@uab.edu
Davide Botta, PhD	TaqMan Fluidigm analysis	5-3324	dbotta@uab.edu
Hui-Chen Hsu, PhD	EvaGreen Fluidigm primer list (~200 pairs)	4-8909	rheu078@uab.edu

Decision points of your Fluidigm assay



You bring the cells to sort via one of the following way

1. FACS sorting into 1xcell/well
2. Fluidigm sorting into 1xcell/well
3. FACS sorting into 1 population, then Fluidigm sorting into 1xcell/well
4. Other methods that suit your purpose.

You bring the primers to the Core: For each Exp, one needs to bring:

1. All primers mixed in 1 tube (100 uM, combined primers, 1 ul each).
2. Individual primer set in individual tube (eg. 96 primer sets in 96 tubes, 1 ul each).

Primers and Reagents provided by the Core?

Primers

1. The Core does not provide any primers

2. The Core offers sequence of EVAGreen primers that has been validated.

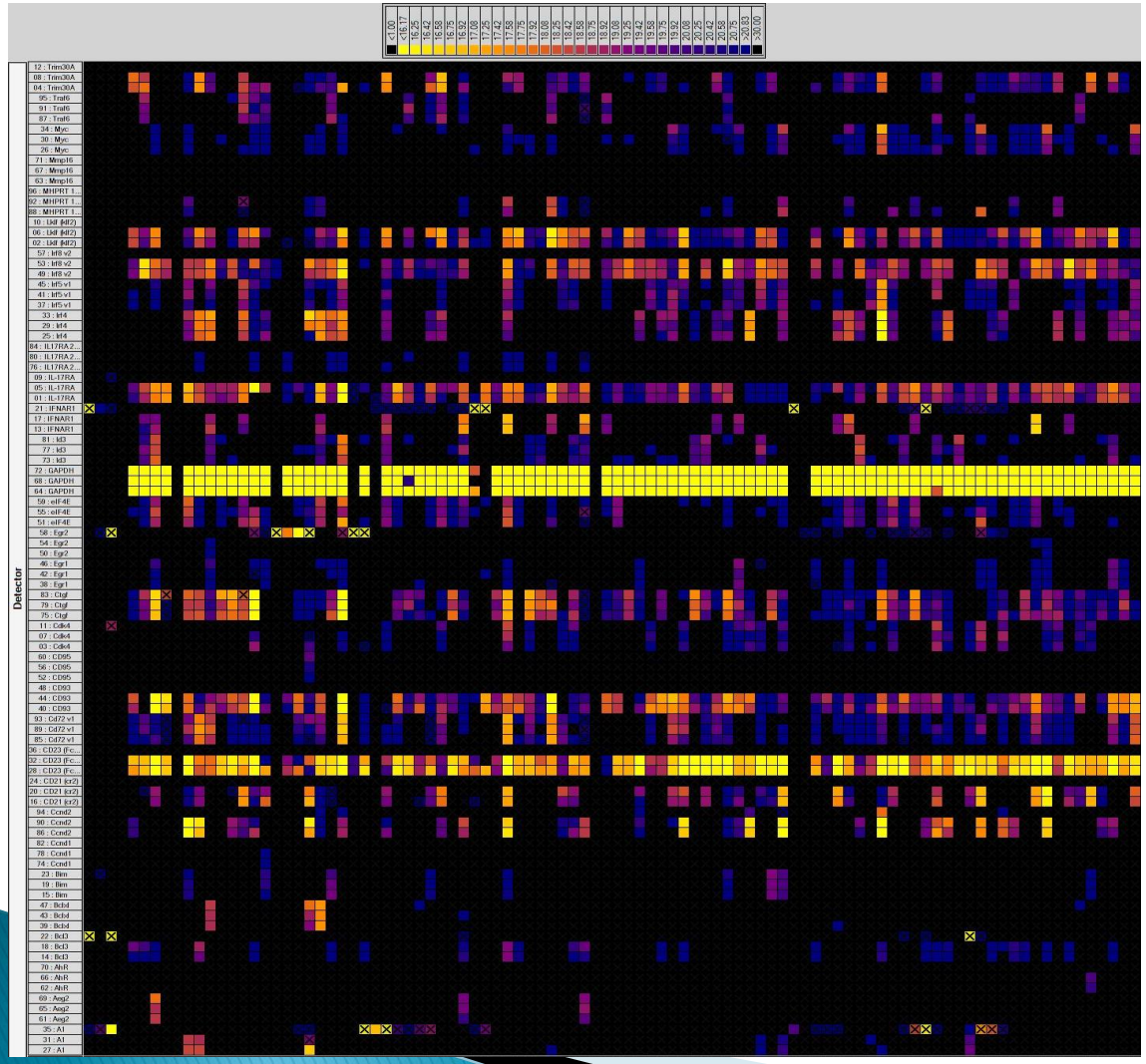
	C1 Chip	C1 AutoPrep kit	96.96 chips + loading kit	48.48 chips + loading kit
Cost/Chip	~$300	~$60	~$900	~$300

C1 sorting ~$400

BioMark gene expression

Total $: For 96x96 gene expression ~$1.300
For 48x48 gene expression ~$700

Our Experience and Tips (96.96 Dynamic Array Assay)



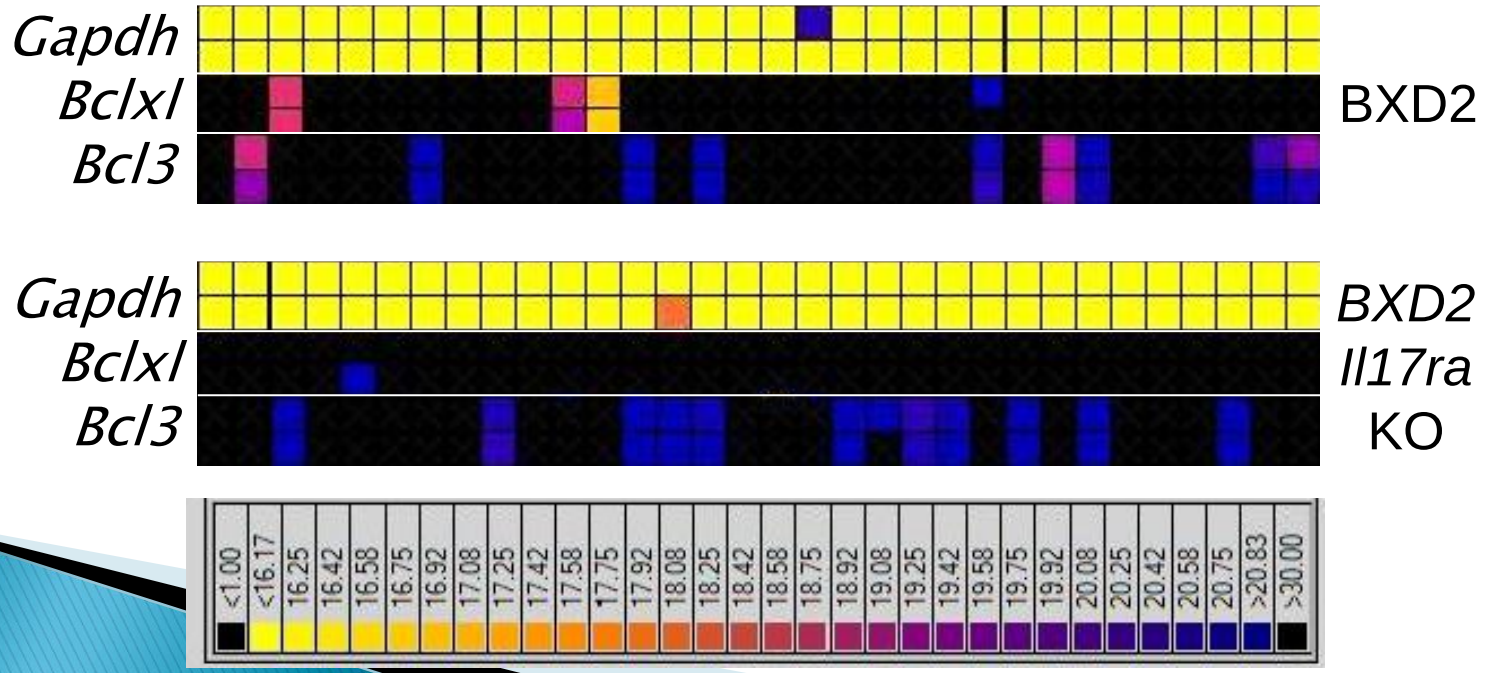
- Importance to do duplicates or triplicates
- Importance to have marker genes of your population of interest.
- Clear heterogeneity exists within the same population
- Primer design is very important
- Cross reference of the results with real-time PCR

Can single cell PCR reveal something not seen by a regular bulk cell PCR analysis?

Bulk real-time PCR results

Bulk Transitional T2 B cells	*Bclxl* (Activation gene)	*Bcl3* (Anergy gene)
BXD2 (B hyperactivity mice)	23.2	37.5
BXD2-Il17ra KO (B anergic mice)	1.5	1.5

Fluidigm/BioMark single cell results



Downstream Application Under Development

BD ARIA Sorter

Amnis ImageStream VA ShEEP grant supported

BD LSRII Analyzer

The HyperCyt autosampler

BioPlex suspension array system

Miltenyi MACS Sorter

Fluidigm C1 Single Cell Prep

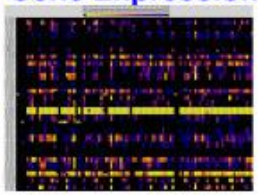
Novel Probes
Tetramers
Dendramers
Nanoparticles
Click-IT
Enzymatic probes

BioMark HD gene expression analyzer

Erik Malarkey, PhD (malarkey@uab.edu)

Single Cell

At CFCC for Gene Expression



In partnership with other UAB Cores & Facilities

Potential applications

Genetic/Epigenetic
Genome
Epigenome
ChIP-seq
RNA-seq
Cloning of genes

Protein
Cytokines
Phospho-protein
Signalome
Kinome
Transcriptome

Cell
Cloning
Transfection
Imaging
Metabolome
Functional assays

Heflin Genomics Core
Hudson Alpha

Thank you for your attention.

Are you ready to take the single cell challenge now?