Precise and Accurate Determination of MicroRNA Precursors by Digital PCR.

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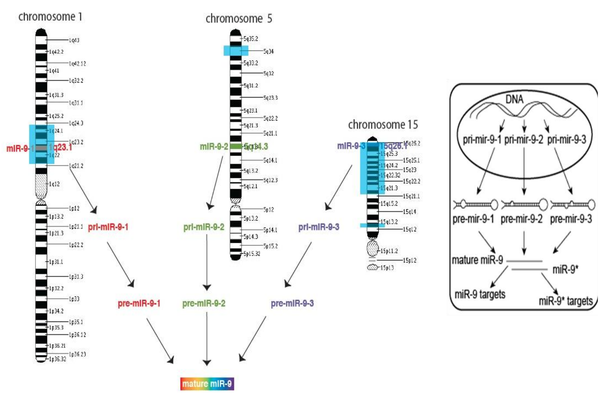
ABSTRACT

There is mounting evidence indicating that microRNAs are fundamental elements of almost every biological process. A majority of methodologies have concentrated on detection of mature microRNAs, which execute microRNA-dependent gene silencing. Much less work has been dedicated to microRNA precursors, despite the fact that they are rudiments of microRNA biogenesis and processing. Challenges of microRNA precursor detection are due to their low abundance, necessity of use of a reference transcript, and sequence similarity. Life Technologies has developed the QuantStudio™ 3D Digital PCR System- a new silicon chip-based digital PCR solution that can be rapidly loaded with little to no dead volume. At the heart of the nanofluidic system is a small chip that enables 20,000 reactions to be run on a single sample. The simplified workflow consists of a three step process, made up of loading the sample, thermal cycling, and detection of the digital results. We measured 3 different precursors of the same microRNA – miR-9 (pri-miR-9-1, -2 and -3) in primary neuronal cell cultures, to show that these challenges can be easily overcome using QuantStudio™ 3D Digital PCR System. Short and long alcohol exposures were used to evoke changes in miR-9 precursors in cultured cells. Our measurements were very reproducible with precision ranging from 1.26-2.33%. Interestingly, one of miR-9 precursors is a embedded in a protein-encoding mRNA transcript, while two others are long non-coding transcripts, thus indicating versatility of digital PCR (dPCR) methodology. In summary, the chip-based QuantStudio™ 3D digital PCR technology offers significant improvement in precision and accuracy of measurement of a mir-9 primary precursor transcripts existing in a much diversified RNA world.

INTRODUCTION

One of the most important microRNAs regulated by alcohol is miR-9 (1). It has been consistently shown that both acute and chronic alcohol exposure affect levels of mature miR-9 in the brain, neuronal cell culture or neurospheres (2). Alcohol exposure upregulates mature miR-9 in adult mammalian brain which triggers post-transcriptional modifications in BK potassium channel splice variants significantly in supraoptic nucleus and the striatum region of the brain (1). Alcohol sensitivities vary with different formation of BK channel isoforms via upregulation of miR-9. Mature miR-9 is a product of 3 different precursors (pre-miR-9-1 ,pre-miR-9-2, and pre-miR-9-3). (Fig. 1)

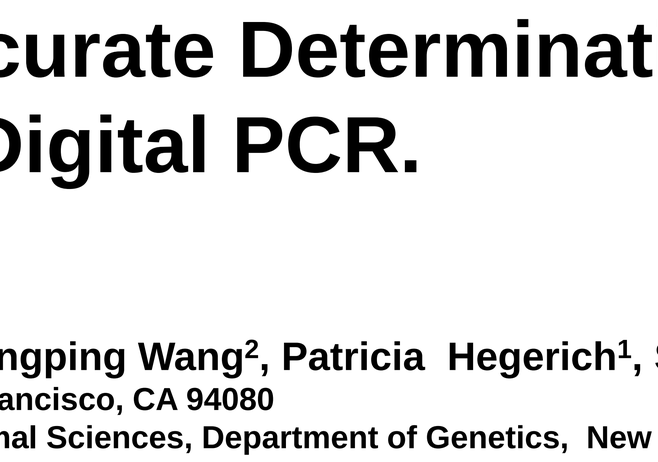
Figure 1. Biogenesis of mature miR-9



The biogenesis of miRNA-9 is a multi-step process in which three primary precursor pri-mir-9-1, pri-mir-9-2, and pri-mir-9-3 are cleaved in the nucleus into three precursor miR-9 (pre-miR-9-1, 2,and 3) by an enzyme complex including Drosha/Pasha. This is followed by processing of pre-miRNAs in the cytoplasm by esportin dicer which cleaves the double stranded pre-miRNA to form mature mirR-9.

Ability to accurately measure levels of these low abundance precursor molecules is essential for understanding molecular mechanisms of alcohol regulation of microRNA.

Figure 2. How Digital PCR Works



QuantStudio™ 3D Digital PCR System is based on a silicon chip that is partitioned into 20000 individual reaction wells, used for nucleic acid detection and absolute quantification. Each sample is distributed into 20,000 individual endpoint PCR reactions per chip where some of these reactions contain target molecule (positive) while others do not (negative). The fraction of negative reaction wells is used to calculate an estimation number of target molecules present.

MATERIALS AND METHODS

Figure 3. Cell Culture and RNA Extraction

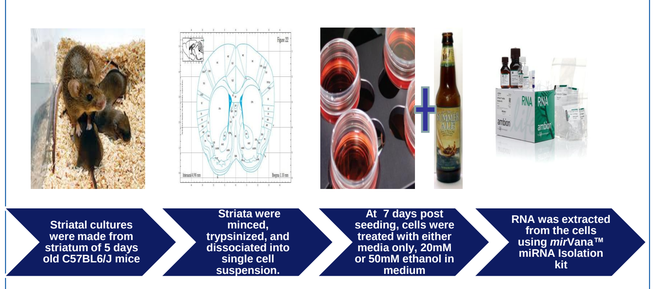


Figure 4. QuantStudio™ 3D Digital PCR Workflow

Table 1. Samples and Assays Information

Sample ID	Sample information	Replicates/Assay : Technical(TR) and Biological(BR)
RT1	T0	TR
RT2	T0	TR
RT4	T0	TR
RT6	15' 20mM exposure	BR
RT7	15' 20mM exposure	BR
RT8	15' 20mM exposure + 1hr withdrawal	BR
RT9	15' 20mM exposure + 1hr withdrawal	BR
RT11	15' 20mM exposure + 6hr withdrawal	BR
RT13	15' 20mM exposure + 6hr withdrawal	BR
RT A	20mM alcohol 6hr exp	TR
RT C	20mM alcohol 6hr exp + 6hr withdrawal	TR
RT E	20mM alcohol 6hr exp + 24 hr withdrawal	TR
	NTC	TR

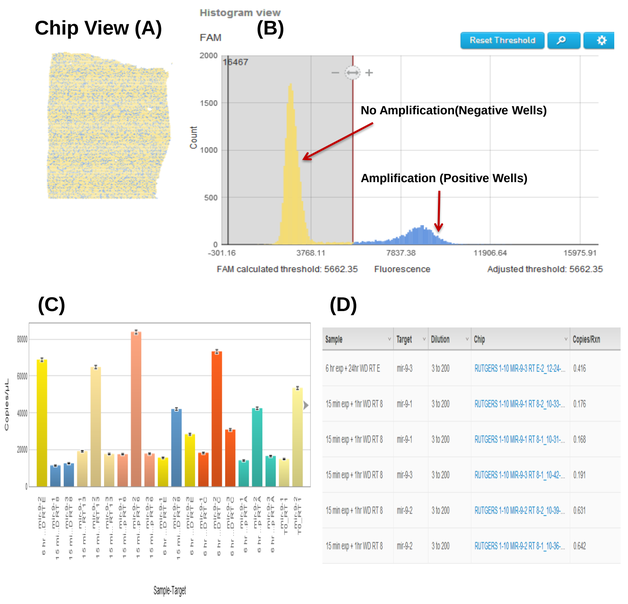
Samples with different alcohol exposure were processed with three mouse TaqMan® Pri-miRNA assays targeting three different loci for mir-9 primary precursor:

Mm04227702_pri : pri-mir-9-1
Mm03306269_pri : pri--mir-9-2
Mm03307250_pri : pri--mir-9-3

Samples were run in duplicates for all three assays either as technical replicates (TR) or with biological replicates (BR) as listed in Table. 1

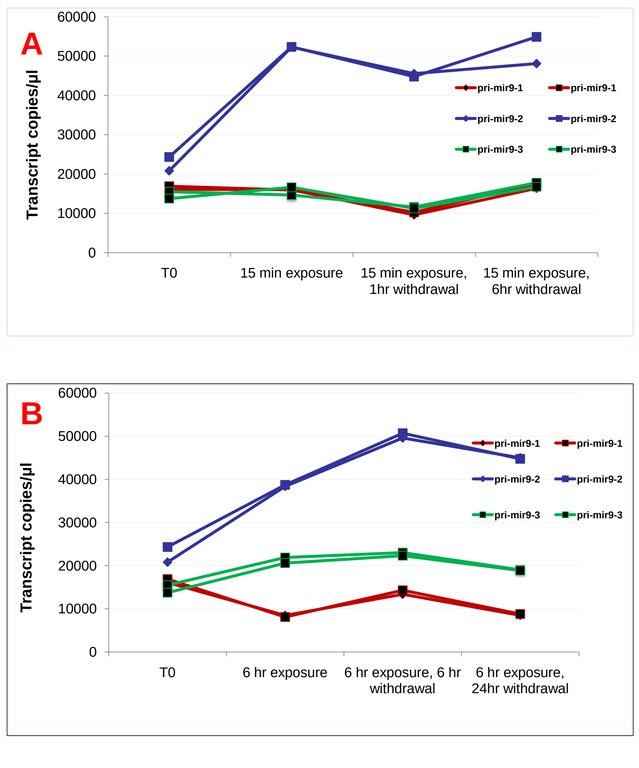
RESULTS

Figure 5. QuantStudio™ 3D AnalysisSuite™ Software



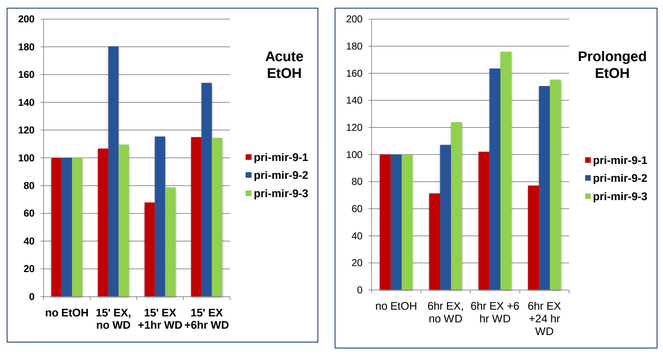
A. Chip view: A Chip QC image representing color by calls. Random distribution of amplified wells FAM™ signal (Blue) and non-amplified wells (Yellow) is ideal.
B. The histogram view: Two distinct peak correspond to population generated with non-amplified (Yellow) wells with lower signal intensity and the amplified wells (Blue) with higher fluorescence.
C. Copies/µl Chart: Software generates Copies/µl for each sample based on copies/rxn generated and dilution factor of the sample.
D. Result Table: Contains information such as dilution factor, chip ID, copies/rxn, copies/ µl, and more for each sample.

Figure 6. Accurate and Reproducible Quantification



- Quantification of three different transcript of primary precursor of mir-9 at :
  - **A:** Acute Ethanol exposure (15 mins). Each data point indicates biological replicates/assay.
  - **B:** Prolonged Ethanol exposure (6 hrs). Each data point indicates technical replicates/assay.
- Replicate measurements were almost identical with precision ranging from 1.26-2.33% .
- pri-mir-9-2 have high expression levels compare to pri-mir-9-1 and 3.

Figure 7. Percentage change for acute and prolonged ethanol exposure



- Pri-mir-9-1 is down-regulated by prolonged alcohol exposure
- Pri-mir-9-2 upregulated by alcohol exposure*
- Pri-mir-9-3 is not affected by acute alcohol exposure but is upregulated by prolonged alcohol exposure

CONCLUSIONS:

The QuantStudio™ 3D Digital PCR System pushes the limits of scientific discovery by allowing for absolute quantification of molecules without compromising quality or precision of measurements. Other key benefits of this systems are:

- Simple workflow with minimal hands-on-time
- Sealed system with minimized contamination
- Minimal Sample Loss
- Enables accurate, reproducible, and highly precise absolute quantification without any reference or standard curve analysis
- Affordable

Here, we have successfully used dPCR to determine alcohol effects on primary precursor of miR-9. Our results encourage more broad use of dPCR to quantify microRNA precursors, mature microRNAs as well as their targets in a variety of biological applications.

REFERENCES

1. Andrzej Z. Pietrzykowski et.al, Posttranscriptional Regulation of BK Channel Splice Variant Stability by miR-9 Underlies Neuroadaptation to Alcohol. Neuron Volume 59, Issue 2, 31 July 2008, Pages 274–287
2. Miranda RC et.al, MicroRNAs: master regulators of ethanol abuse and toxicity? Alcohol Clin Exp Res. 2010 Apr;34(4):575-587

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TRADEMARKS/LICENSING

The QuantStudio™ 3D Digital PCR System is For Research Use Only. Not for use in diagnostic procedures.