

PIXEL SYSTEM™

Whitepaper

INTRODUCTION

Cellular Research is proud to introduce The Pixel™ System, a novel mRNA detection and digital quantitation system capable of detecting individual molecules in single cells or precious samples. Accurate quantification of nucleic acids is necessary in a wide variety of research and clinical applications. Although powerful modern techniques such as PCR, microarray, and next-generation sequencing technologies have been very successful, and have dramatically influenced the pace of research in molecular biology, they generally require large amounts of starting material, generate relative gene amplitude data, and for the most part do not allow for an absolute determination of the actual number of molecules in a specific sample. Direct measurement is just not possible with any modern molecular biology method, including the powerful dPCR approach, which has the additional disadvantage of consuming the sample upon measurement. A sensitive, easy to use, inexpensive and precise method is greatly needed and would be relevant for several research application areas, including gene expression, copy number changes, and absolute quantitation of DNA/RNA molecules in single cells. The high precision Pixel System™ should therefore prove to be extremely useful to molecular biology researchers for the everyday detection and quantitation of nucleic acid performed routinely in their laboratories.

THE PIXEL SYSTEM™

Pixel utilizes Cellular Research's proprietary Molecular Indexing™ Assay, in a fast and simple, single tube, endpoint method. The workflow uses standard thermo-cyclers and well-characterized PCR reagents, making Pixel a powerful, easy, and inexpensive complement to any molecular biology lab interested in direct mRNA quantitation. The Pixel System™ produces accurate and precise quantitative measurements without the need for real time monitoring, intensity based quantitation, or physical partitioning of the sample. Calibration standards are unnecessary because absolute measurements are produced, precisely counting the number of target molecules in an entire sample. Many well known relative quantitation problems, such as PCR bias, variability caused by experimental conditions and sample volume loss are avoided with quantitation using Molecular Indexing™.

WHAT IS MOLECULAR INDEXING™?

The Molecular Indexing™ technology is based on a stochastic labeling process, which occurs before amplification of the sample of interest. Proprietary Poly-T tagged Molecular Index barcodes are used to label every mRNA molecule in the sample individually during the reverse transcription step. Since each synthesized cDNA molecule is encoded with a Molecular Index label, it can be tracked through downstream processing, and PCR biases can be corrected. PCR amplification is used to amplify targets of interest after Molecule Indexing, thereby increasing the concentration of target molecules and removing any concentration based detection limits. This post labeling amplification also serves to virtually immortalize the original sample by creating an abundant sampling pool of the targets of interest. Post amplification, the number of Molecular Index labels contained in the PCR product is detected on the Pixel16 consumable by the Pixel Instrument, revealing the number of copies of the original mRNA target in the starting sample.

WHAT IS THE PIXEL SYSTEM™?

The Pixel System™ is an innovative gene expression analysis tool capable of ultra precise measurements with sensitivity down to and below the level of single cells (Fig.1). The system is particularly useful when sample input is limited, such as for experiments involving single cells or precious samples. For repeated analysis of single cells or small



KEY ADVANTAGES

- Precision - Single-molecule precision provides high accuracy and sensitivity
- Whole Cell or Sample Resolution – the entire sample is tested with no dead volume or sample loss
- Convenience – Ability to count many genes of interest in a single cell or precious sample and re-test for different targets
- Non-destructive - Repeated analysis of sample for different targets
- Ease-of-Use - Simple and streamlined workflow using standard molecular biology equipment and procedures
- Compatibility – the same sample can be analyzed across Pixel, qPCR and NGS technologies

samples, amplification is necessary to avoid sample depletion and to test the contents of the entire sample. In order to enable analysis of the entire sample with no sampling error, the number of molecules in the sample must be increased so that each measurement or aliquot tested is representative of the overall sample. If the number of molecules is not sufficient, statistical noise or sampling error will result in inaccurate measurements, potentially hiding the detection of true biological signals. Unfortunately, amplification reactions introduce bias and often greatly distort the original relative mRNA abundance levels of the sample. Further, precious samples are irreplaceable and all other current techniques (eg. NGS, qPCR or digital PCR) consume the sample, prohibiting multiple measurements or causing data quality degradation over multiple rounds of amplification and detection. In contrast, Molecular Indexing™ permanently labels all mRNA molecules in samples of interest, enabling these samples to be analyzed repeatedly with no quality loss, sampling error or PCR bias.

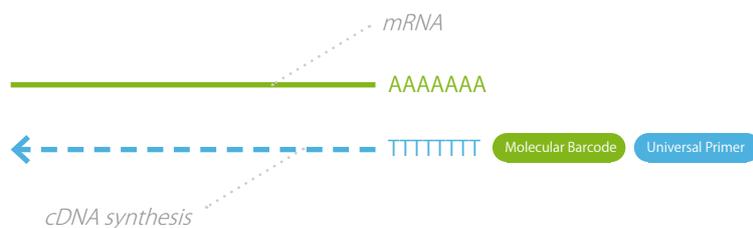
WORKFLOW

Simple Workflow Provides Precise Results in Four Steps

1

MOLECULAR INDEXING™ AND REVERSE TRANSCRIPTION - SECOND STRAND SYNTHESIS

The Molecular Indexing™ workflow begins with a reverse transcription reaction, first synthesizing cDNA using barcoded oligo dT primers and then generating second strand cDNA using a gene-specific primer. The single cell or small sample is combined with the Indexing Master Mix for annealing followed by addition of the RT Master Mix for second strand synthesis. To inactivate excess primers, Uracil DNA Glycosylase (UDG) is added to the mix and incubated. It is during this step where one of the 960 unique Molecular Index labels binds to each mRNA molecule, and incorporates into the final cDNA product making it distinguishable from another mRNA molecule of the same sequence. These labels will be used downstream to determine the true, PCR unbiased, number of target mRNA molecules present in the sample.

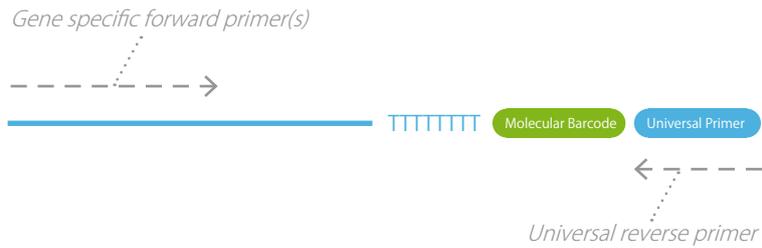


2

MULTIPLEX AMPLIFICATION

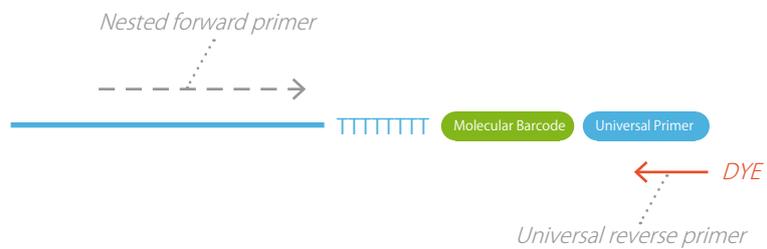
Multiplex amplification occurs when specific transcripts of interest are amplified in a PCR reaction from the cDNA mix in Step 1. This step prepares a large pool of labeled sample, from which target molecules can be analyzed with further amplification using simple PCR. This step also amplifies the unique label attached to each molecule.

3



TARGET AMPLIFICATION

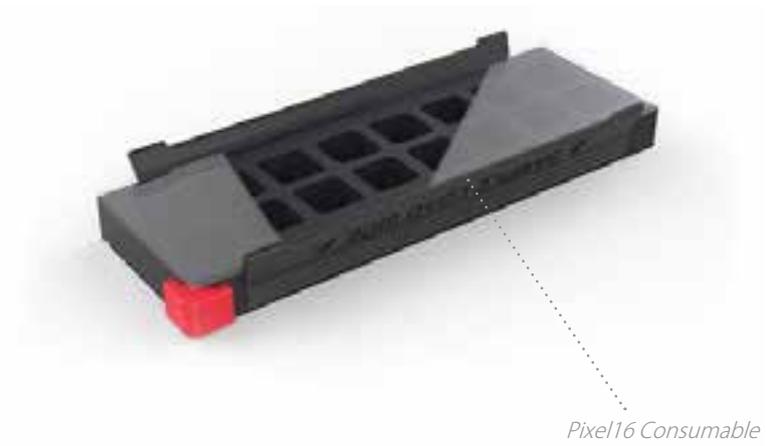
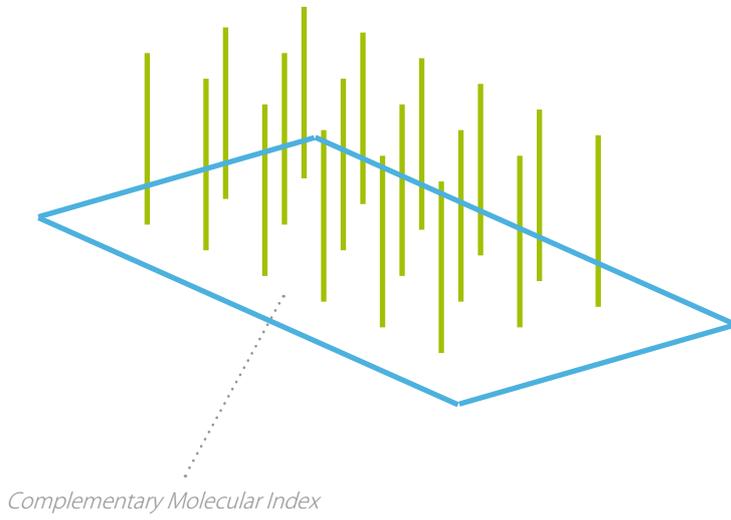
In order to detect the specific gene of interest, target specific PCR primers are used in a nested PCR amplification. This amplification step creates many copies of each individually labeled molecule, which can then be analyzed on the Pixel16 consumable.



4

DETECTION

Detection takes place on the Pixel16 consumable in one of its sixteen wells, with each well being used for an individual sample or experiment. The dye-labeled and amplified molecules from Step 3 is denatured and then transferred into a well on the Pixel16 detector slide to hybridize to the labels. Post hybridization, the sample is vacuum aspirated and the detector is imaged on the PIXEL System™. For each image, a detection threshold is computed to classify detector probes and their corresponding labels as either being present or absent. The number of label probes detected with intensities greater than the threshold is termed k . k is then used to calculate N , the number of molecules in the sample, using probability statistics. N represents the absolute number of molecules of the transcript of interest present in the original sample. All details regarding cell culture, single cell isolation, RNA samples and controls, barcode and primer sequences are available by contacting Cellular Research (Info@Cellular-Research.com).

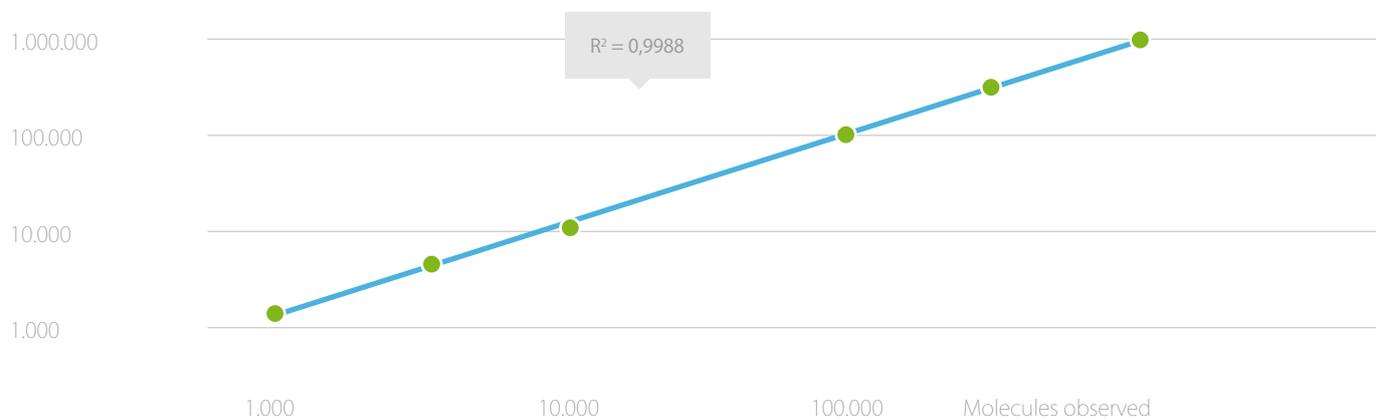


ABSOLUTE QUANTIFICATION OF GENE EXPRESSION

Accurate measurement of gene expression was demonstrated by performing quantitative detection of RPLP0 transcript copy number, using a serial dilution of human liver total RNA (Figure 2A). Samples with a wide abundance range of liver RNA were created by diluting from a high concentration source sample and measured using the Molecular Indexing™ workflow. The true number of copies of RPLP0 mRNA was also determined using digital PCR, and found to be highly concordant with measurements from Molecular Indexing™ (Pearson R-square = 0.9998). Absolute quantities measured by Molecular Indexing™ and digital PCR correspond well, with the latter reporting 14% lower

NUMBER OF RPLP0 MOLECULES EXPECTED VS. MOLECULES OBSERVED IN HUMAN LIVER TOTAL RNA

Molecules expected



PICTOGRAMS OF TOTAL HUMAN LIVER RNA VS. NUMBER OF RPLP0 COPIES



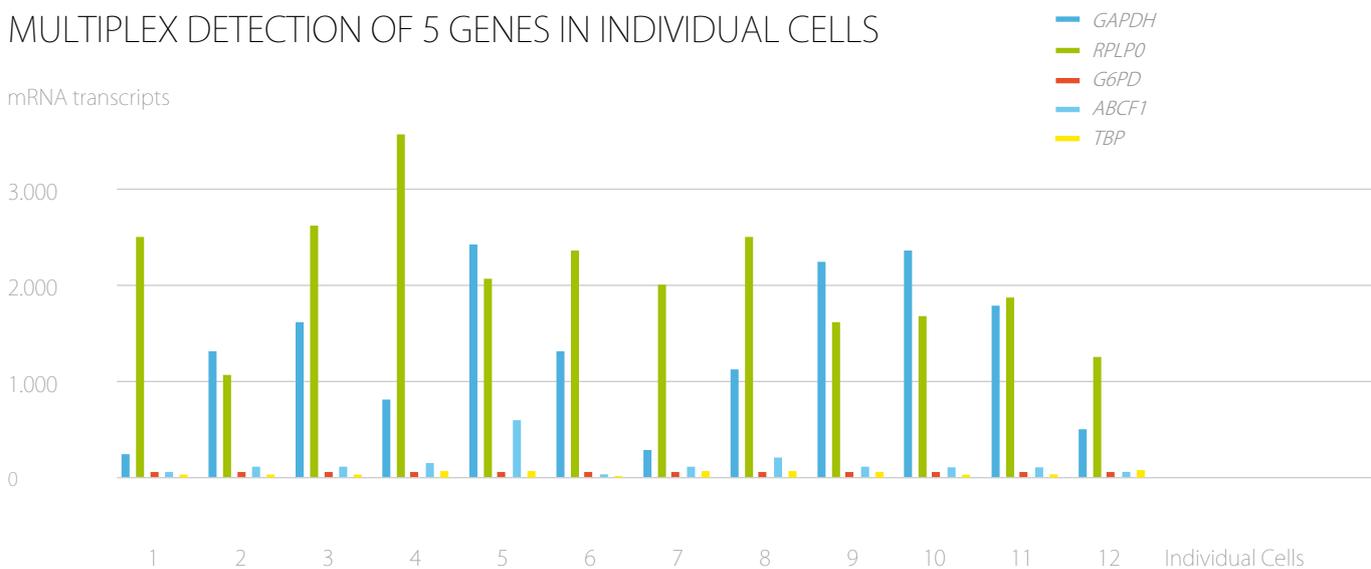
counts on average. For high abundance samples containing RPLP0 transcripts that exceed the measurement range for the set of 960 barcode labels used in this assay, the test sample was first diluted down to the range of approximately 100 to 4,000 molecules prior to reverse transcription and Molecular Indexing™. The total number of molecules present before dilution was then calculated using the dilution factor applied. To determine the sensitivity and accuracy of Molecular Indexing™ under low input conditions, sub-single cell quantities of RNA sample were directly assayed. Testing the gene RPLP0, it is shown that measured values correlate linearly with the 0.5 to 6.3 pg of input liver total RNA tested with both 10-fold (Figure 2A) and 2-fold serial dilutions of starting total RNA (Figure 2B). Both experiments demonstrate very high linearity and a large dynamic range, from total RNA dilutions down to and below single cell amounts, with the 2-fold total RNA dilution showing much greater linearity than traditionally observed in qPCR experiments.

GENE EXPRESSION MEASUREMENTS ON SINGLE CELLS

To demonstrate the value of the Pixel system™ specifically for single cell experiments, absolute quantification measurements were carried out directly from single cells. Cultured K562 cells were individually isolated and lysed in a PCR tube. The standard Pixel protocol was used to amplify five separate genes during the multiplex amplification stage, GAPDH, RPLP0, G6PD, ABCF1 and TBP. Targeted PCR for each gene was performed independently and analyzed across 12 individual cells. TBP expression counts for cells 3-8 were truncated at 600 due to very high expression (in the ~4,000 molecule range) in order to preserve the visual range. The Pixel results identify clear differences in mRNA molecules with low expression abundance as well as a wide overall dynamic range for gene expression.

MULTIPLEX DETECTION OF 5 GENES IN INDIVIDUAL CELLS

mRNA transcripts



CONCLUSION

In recent years single cell gene expression has become one of the fastest growing areas of life science research. One of the principal reasons behind this is that experiments carried out on bulk samples result in an “averaged measurement”, which obscures the detection of differential gene expression patterns across seemingly identical individual cells.

Due to the detection limits of current tools, amplification is always necessary before detection. However, amplification reactions tend to introduce bias into the sample, compromising both the ability to directly measure the true expression level originally present in the cell as well as the ability to accurately compare expression of different genes (since measurements are skewed by differential amplification). Distortions in copy numbers resulting from pre-amplification have been widely demonstrated, and equal efficiencies in the multiplex amplification of so many different genes can not be expected.

In contrast, Molecular Indexing™ is ideally suited to measurements of single cells and precious samples. Fundamentally, Molecular Indexing™ is an absolute measurement method, differentiating it from the NanoString nCounter, qPCR, and massively parallel sequencing methods, which all rely on relative quantification. Absolute measurements do not require calibrations against standard curves, or comparisons with housekeeping genes that may themselves vary in concentration. One of the great strengths of Molecular Indexing™ is that even when genes and samples amplify at different efficiencies the net resulting number of barcoded templates counted at the end of the process is unaffected. Molecular Indexing™ preserves the initial copy numbers, independent of pre-amplification or amplification bias.

Molecular Indexing™ can be conveniently performed in just a single tube, without physical partitioning of the sample. As such, the challenges and volume losses associated with physical separation are avoided. The number of labels in Molecular Indexing™ plays the same role as the number of partitions used for digital PCR, and can be adjusted as necessary. For gene expression measurements in single cells, a set of 960 barcode labels offers a sufficient dynamic range of measurement. Samples of higher concentrations may be diluted to a suitable abundance range prior to measurement.

The Pixel System™ combines Molecular Indexing™ with the simplicity of endpoint PCR to create a highly precise, sensitive, and accurate measurement technique. Molecular Indexing™ delivers an accurate and precise measurement of mRNA gene expression in single cells with single molecule sensitivity and accuracy.

KEY PIXEL SINGLE CELL CAPABILITIES:

- Non-Destructive Assay– ability to re-examine the same sample or cell with no sampling error or loss in data quality.
- Bias-Free Amplification – no bias or sample preparation error regardless of type or number of amplifications
- Whole Cell Resolution – no dead volume, analysis capable of examining the contents of an entire cell with no sample loss
- Absolute Quantitation – ability to absolutely quantify target molecules without external controls or references