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High coverage gene expression profiling on the Applied Biosystems® 3500xL Genetic Analyzer

a sensitive method for detecting gene transcripts

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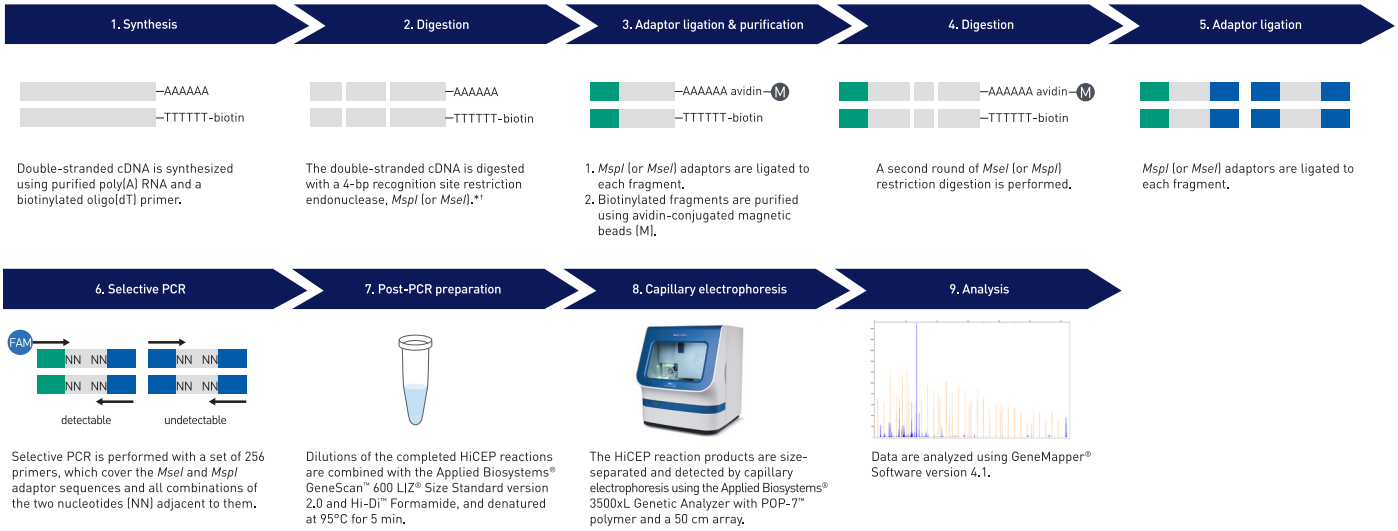
Introduction

Researchers from the Transcriptome Profiling Group at the National Institute of Radiological Sciences, Chiba, Japan (photo) focus on transcriptome analysis applications. The group, led by Masumi Abe, PhD, has developed the high coverage expression profiling (HiCEP) method, a sensitive method for detecting a large proportion of transcripts in both known and unknown genes, with a low false positive rate. This article describes how Dr. Abe's group used the Applied Biosystems® 3500 xL Genetic Analyzer from Life Technologies in a study that demonstrates the accuracy, ease of use, and throughput capabilities of this instrument for performing the HiCEP method.

As an Amplified Fragment Length Polymorphism (AFLP®)-based gene expression profiling method, the HiCEP method requires no prior sequence information and has a reduced rate of false positives with a high degree of detection of both coding and noncoding transcripts. After HiCEP analysis, fragments of interest can be purified and cloned from agarose gels, and sequenced to identify the transcripts. If whole genome sequence information for the organism under study is known, the fragments of interest can be identified by bioinformatic prediction using the sequence information available from public databases and the restriction enzyme recognition sites used in the HiCEP workflow (Figure 1).



* The use of a 4-bp recognition site restriction enzyme generates small fragments, which is important for improving subsequent PCR amplification efficiencies.
† If *MspI* is used for the first digestion, *MseI* is used for the second digestion, and vice versa.

Figure 1. The HiCEP workflow.

Advantages of the HiCEP method for expression profiling

Whole transcriptome expression profiling is often performed using hybridization-based microarray methodologies. These methods have been successful at revealing the patterns of mRNA transcripts within cells and tissues. However, there are a number of limitations to microarray-based approaches such as low sensitivity and specificity, poor dynamic range. Perhaps the biggest limitation, however, is that microarray expression profiling results are restricted to specific sequence annotations and content, and prior sequence information is required. While other techniques such as differential display and arbitrarily-primed PCR reactions do not require *a priori* sequence information, these older techniques are subject to relatively high false positive rates. The HiCEP method was developed to address the above shortcomings in gene expression profiling and provide a sensitive method for detecting a large proportion of transcripts in both known and unknown genes, with a low false positive rate.

HiCEP analysis with the Applied Biosystems® 3500xL Genetic Analyzer

Here, we demonstrate the use of the Applied Biosystems® 3500xL Genetic Analyzer for HiCEP analysis to detect known transcripts unregulated by ionizing radiation (IR). The advanced capabilities of the 3500 family of genetic analyzers, including new thermal control systems, enhanced optical detection, and new consumables designs, provide an easy-to-use platform for the sensitive detection and analysis of HiCEP samples. The optional normalization reagent (Applied Biosystems® GeneScan™ 600 LIZ® Size Standard version 2.0) and compatible run module enable increased precision and accuracy in peak area and height determinations, which was particularly important for the detection of differences in replicate HiCEP reaction preparations.

In this study, the expression of *p21, CyclinG1, and GADD45A* was assessed to demonstrate the accuracy, ease of use, and throughput capabilities of the Applied Biosystems® 3500xL Genetic Analyzer for gene expression analysis. Further, the expression changes detected by capillary electrophoresis were compared to those obtained using TaqMan® Gene Expression Assays for analysis of the *p21* transcript. For each time point and reaction type, a total of 96 replicates were analyzed by capillary electrophoresis (CE). All sample files presented here were analyzed using GeneMapper® Software version 4.1 and JMP® Software version 8.0.2.

mRNA samples were prepared from mouse embryonic fibroblasts (MEFs) at 0, 3, 6 and 24 hours after IR exposure. The peak heights of HiCEP fragment data corresponding to the *p21* transcript from this mRNA are shown in the left hand panels of figure 2 (A and B). Two replicates per HiCEP sample were prepared for each poly(A) RNA sample time point.

Quantitative real-time PCR (qPCR) was performed on cDNA template (10 ng per reaction), synthesized from poly(A) RNA isolated from untreated and IR treated MEFs using an Applied Biosystems® 7500 Real-Time PCR System with TaqMan® Gene Expression Master Mix and TaqMan® Gene Expression Assays directed against mouse *Cdkn1a*

(cyclin-dependent kinase inhibitor 1A (p21), accession no. NM_007669). The reference assay used was a mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) endogenous control (accession no. NM_008084). The relative quantification of gene expression was calculated using the $\Delta\Delta C_t$ comparative method:

$$\Delta\Delta C_t = (C_{t\ sample} - C_{t\ ref})_{ctrl} - (C_{t\ sample} - C_{t\ ref})_{irradiated}$$

This fold change was then $\log_2$ transformed.

Normalization helps differentiate between HiCEP sample replicates

When the same sample is analyzed by CE, a certain amount of variation in signal strength may be observed within a single instrument among different capillaries; or among different injections from the same capillary. For applications that require quantitative analysis, such as HiCEP, minimal signal variation is desired and data analysis can be aided by reducing signal variation. Life Technologies researchers have identified a number of different sources of variation and have elucidated methods to obtain more consistent peak height data on CE instruments from injection-to-injection, and capillary-to-capillary. Advantages of signal height normalization incorporated into the 3500 Series of Genetic Analyzers, including the use of GeneScan™ 600 LIZ® Size Standard version 2.0, are useful for the analysis of HiCEP gene expression data.

An improved understanding of the differences in HiCEP sample replicates is possible following the application of calculated normalization by the Applied Biosystems® 3500xl Genetic Analyzer. This is illustrated by the ability to distinguish replicates within samples (Figure 2A versus 2B). In the un-normalized samples, the dispersed nature of the peak heights—due to injection-to-injection, and capillary-to-capillary variability—makes it difficult to differentiate between replicate 1 (red) and replicate 2 (blue) samples. However, following normalization, the peak height data for each replicate is less dispersed and replicates cluster more tightly together (Figure 2B, left panel). The histograms illustrate the bimodal distribution for certain time points, in particular, this is evident at 3 hour and 6 hour time points (Figure 2B). The comparison circles also indicate that the peak heights are less dispersed for the normalized data than the un-normalized data (Figure 2A, middle panel). The dispersed nature of the peak heights in the un-normalized data is evident from the larger comparative circles observed when compared to the normalized data (Figure 2B, middle panel). The sizes of the comparative circles indicate the 95% confidence interval of the respective group mean of *p21* fragment peak heights.

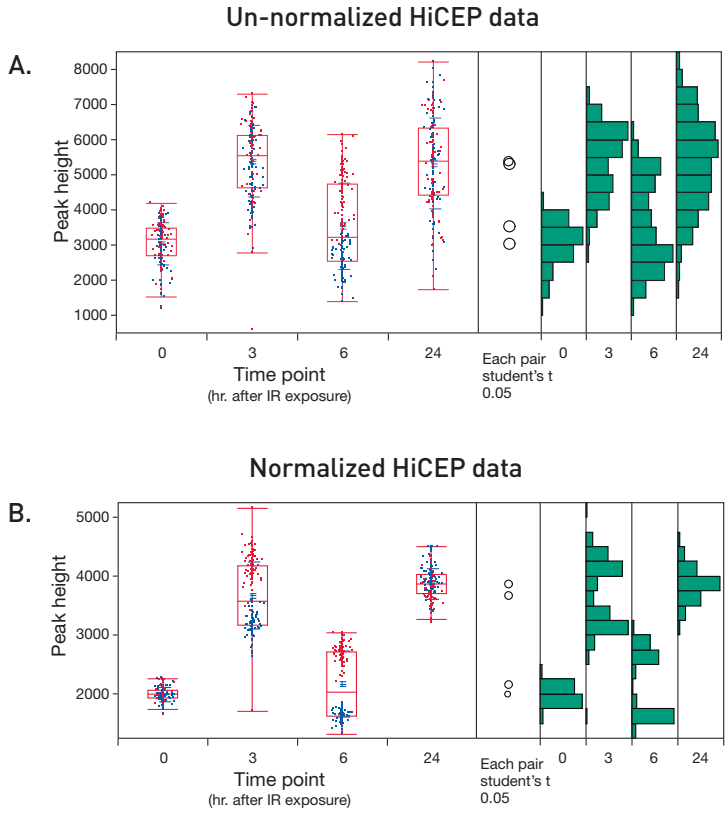


Figure 2. Mouse p21 induction in response to IR, HiCEP data for (A) un-normalized HiCEP data and (B) normalized data. For **(A)** and **(B)**: in the left scatter plot, replicate 1 is indicated in red and replicate 2 is indicated in blue. For both replicates, each time point was analyzed 96 times by CE (represented by points). In the scatter plot are box plots that summarize the distribution of p21 fragment peak heights with the ends of the box indicating the 25th and 75th quantiles. The line across the middle of the box identifies the median sample value and the whiskers extend to the outermost data points. Blue dashed lines identify one standard deviation above and below the group means. For **(A)** and **(B)**: the middle panel shows comparison circles that provide a visualization of the significant differences between each pair of group means of p21 fragment peak heights. Circles for means that are significantly different either do not intersect or intersect only slightly. For **(A)** and **(B)**: the right hand panel shows a histogram distribution of the p21 fragment peak heights at each time point.

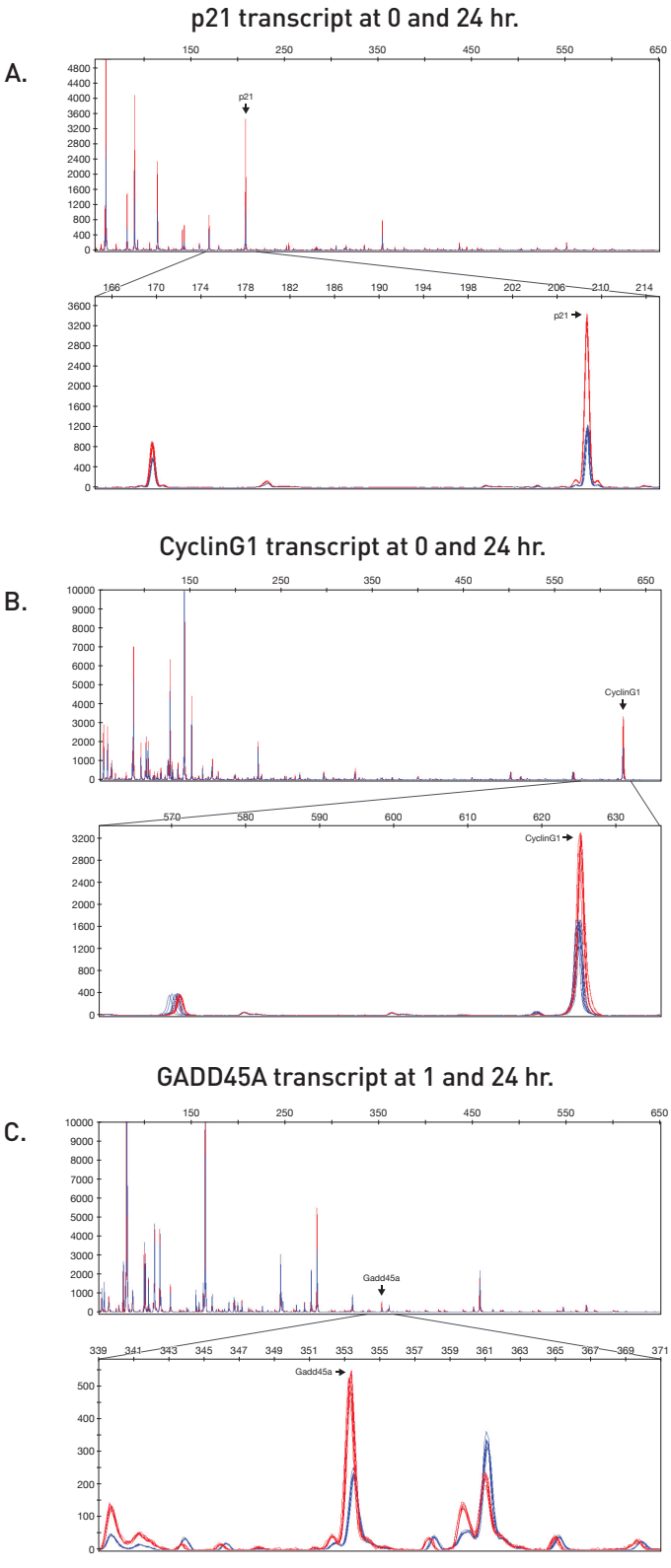


Figure 3. Induction of mouse transcripts in response to IR (5 Gy) exposure. (A) For mouse p21 transcript, the blue and red fragments are from HiCEP reactions prepared from poly(A) RNA prepared at 0 and 24 hours, respectively. **(B)** For mouse CyclinG1 transcript, the blue and red fragments are from HiCEP reactions prepared from poly(A) RNA prepared at 0 and 24 hours, respectively. **(C)** For mouse GADD45A transcript, the blue and red fragments are from HiCEP reactions prepared from poly(A) RNA prepared at 0 and 24 hours, respectively. After HiCEP analysis fragments of interest were purified and cloned from agarose gels and sequenced to identify transcripts.

Normalization of HiCEP data also assists in differentiating between time point group means. The group means of *p21* fragment peak heights were not significantly different between 3 hour and 24 hour time points for the un-normalized data as indicated by overlapping circles that provide a visualization of the pair wise comparisons using Student's t-tests (Figure 2A, middle panel). By applying normalization to the *p21* HiCEP data, all of the time points are significantly different (Figure 2B, middle panel).

HiCEP measures changes in expression levels of genes related to cell stress response

IR can disrupt cellular viability and genomic integrity and trigger cellular stress response pathways that activate proteins for counteracting mutational events that could potentially result in malignant transformation of healthy cells [1]. One of these proteins is p53, which acts to inhibit cell division and apoptosis [2]. Target genes activated by p53 after IR that are involved in growth arrest include two of the genes investigated in this study: *p21 (CDKN1A)* and *GADD45A* (Figure 3A and 3C). A third gene, CyclinG1 (CCNG1), encodes a protein that plays a role in negatively regulating p53 activity. CCNG1 is a transcription target of p53, which becomes transcriptionally unregulated by IR exposure (Figure 3B).

The temporal expression of *p21* observed using the HiCEP method and qPCR (Figure 4) reflects a transcription oscillation of *p21*. This occurs as part of a negative feedback loop that the p53 protein forms with Mdm2, when Mdm2 targets p53 for degradation [3]. An oscillation period of 6 to 7 hours was observed for p53 in response to ionizing irradiation with the Mdm2 protein oscillating out of phase [4,5]. The initial spike in *p21* transcript at the 3 hour time point is likely the transcriptional response to the initial induction of p53

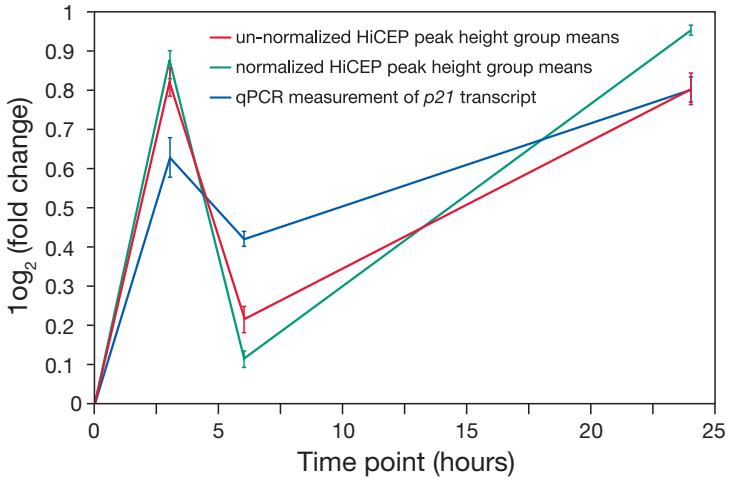


Figure 4. Mouse p21 fold-change in response to IR. The red line indicates the fold change from the 0 hr time point for un-normalized HiCEP peak height group means. The green line indicates the fold change of normalized HiCEP peak height group means, and the blue line indicates the fold change for qPCR measurement of p21 transcript from mRNA prepared 0, 3, 6, and 24 hours after IR exposure.

after irradiation. The decrease in *p21* transcript levels at the 6 hour time point likely reflects an out-of-phase expression with the second peak in transcription of *p21* at 24 hours after IR exposure, correlating with an in-phase pulse of p53 activity.

HiCEP and qPCR were used to measure fold change in expression of *p21* for sample mRNA prepared 0, 3, 6, and 24 hours after IR exposure (Figure 4). The red line indicates the fold change from the 0 hour time point for un-normalized HiCEP peak height group means. The green line indicates fold change of normalized HiCEP peak height group means, and the blue line indicates the fold change for qPCR. Interestingly, between the normalized and un-normalized data for p*21* HiCEP data, there was no significant difference in fold change when it was calculated based on group means. Moreover, the kinetics of the *p21* mRNA response to IR was found to be similar using both the HiCEP method and qPCR.

HiCEP provides a sensitive method for global gene expression profiling

Taken together, these analyses indicate that the normalization features incorporated into the 3500 Series Genetic Analyzers improve the consistency of HiCEP data and facilitate the comparison of replicate samples between injections and between capillaries within the same injection. For CE instruments in general, the HiCEP method provides a sensitive method for global gene expression profiling that detects a large proportion of transcripts in both known and unknown genes, with a low false positive rate. Further, unlike microarray analysis, this AFLP® technology-based method requires no prior sequence information making the HiCEP technique suitable to researchers working on organisms with little or no sequence annotation available in the public databases.

Acknowledgement

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Ordering Information

Product Name	Part Number
Applied Biosystems® 3500xL Genetic Analyzer for Resequencing & Fragment Analysis	4440463
POP-7™ Polymer for 3500/3500xL Genetic Analyzers	4393714
3500 Genetic Analyzer Capillary Array, 50 cm	4404685
GeneScan™ 600 LIZ® Size Standard v2.0	4408399
Hi-Di™ Formamide	4311320
Mse I	15494-016
T4 DNA Ligase	15224-041

Go to **www.appliedbiosystems.com/3500** to learn more about the Applied Biosystems® 3500 Series Genetic Analyzers.

Go to **hicepweb.nirs.go.jp/english/what/index.html** to learn more about the Transcriptome Profiling Group at the National Institute of Radiological Sciences, Chiba, Japan.

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