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Clinical Application of Automatic Gene Chip Analyzer (WEnCA-Chipball) for Mutant KRAS Detection in Peripheral Circulating Tumor Cells of Cancer Patients

Suz-Kai Hsiung^{1,2}, Shiu-Ru Lin^{1,2}, Hui-Jen Chang^{1,2},
Yi-Fang Chen³, and Ming-Yii Huang^{4,5}

¹Department of Medical Research, Fooyin University Hospital, Pingtung,

²School of Medical and Health Science, Fooyin University, Kaohsiung,

³Gene Target Technology Co.Ltd, Kaohsiung,

⁴Department of Radiation Oncology, Kaohsiung Medical University Hospital, Kaohsiung,

⁵Department of Radiation Oncology, Faculty of Medicine, College of Medicine,
Kaohsiung Medical University, Kaohsiung
Taiwan, ROC

1. Introduction

KRAS is an important oncogene that participates in the mitogen-activated protein kinase (MAPK) pathway. The MAPK pathway is involved in various cellular functions, including cell proliferation, differentiation and migration. Mutations in KRAS are found in many types of malignancies including lung cancer (Fong et al., 1998; Slebos & Rodenhuis, 1989; Chen et al., 2003; Siegfried et al., 1997), colorectal cancer (Calistri et al., 2006; Weijenberg et al., 2008; Wang et al., 2007), and pancreatic cancer (Smit et al., 1988; Gocke et al., 1997). As early as 1989, Slebos *et al.* have identified that the KRAS mutation status can be used for lung cancer detection or prognosis prediction (Slebos & Rodenhuis, 1989). In 1995, Yakubovskaya *et al.* detected 12 different KRAS mutations in nearly 60% of tissue specimens of non-small cell lung cancer (NSCLC) patients (Yakubovskaya et al., 1995). As for pancreatic, stomach and breast cancers, there have been a number of studies reporting KRAS mutations (Smit et al., 1988; Gocke et al., 1997; Deramaudt & Rustgi, 2005; Carstens et al., 1988; Lee et al., 2003; Shen et al., 2008). The predictive value of KRAS mutation in metastatic colorectal cancer patients treated with cetuximab plus chemotherapy has recently been shown in that patients with tumor KRAS mutation were resistant to cetuximab and had shorter progression survival and overall survival times compared with patients without mutation (Lievre et al., 2006; Lievre et al., 2008). Additionally, NCCN Clinical Practice Guidelines in Oncology Version 3, 2008, strongly recommends KRAS genotyping of tumor tissue (either primary tumor or metastasis) in all patients with metastatic colorectal cancer before treatment with epidermal growth factor receptor (EGFR) inhibitors. KRAS mutational analysis has advantages over attempts to predict responsiveness to anti-EGFR antibodies.

To date, detection of KRAS mutations are limited to traditional techniques. The traditional techniques such as direct sequencing, polymerase chain reaction and restriction fragment length polymorphism are complicated and can easily be used only in tissue samples, which limits KRAS mutation detection in clinical applications. In order to improve the mutant KRAS detection efficiency, we successfully developed an Activating KRAS Detection Chip and colorimetric membrane array (CLMA) technique capable of detecting KRAS mutation status by screening circulating carcinoma cells in the surrounding bloodstream (Chen et al., 2005; Wang et al., 2006; Chong et al., 2007; Yang et al., 2009; Yen et al., 2009; Yang et al., 2010). However, the sensitivity still needs further improvement. In addition, the digoxigenin enzyme used on the colorimetric gene chip platform is too costly for routine laboratory diagnosis, and the high criteria of the operation techniques have prevented its widespread availability for clinical applications. Therefore, we have developed the next generation gene chip operation platform named the weighted enzymatic chip array (WEnCA), as shown in figure 1. The technical difference between the WEnCA and CLMA system includes the different weighted value for each gene target on the gene chip of the WEnCA system, dependent on the importance of each gene during the cancer development process. Furthermore, the conventional digoxigenin system was replaced by the biotin-avidin enzyme system to lower the cost. The manual operation process of the WEnCA system has been successfully established and published (Tsao et al., 2010; Yen et al., 2010). The proposed platform may benefit post-operative patients or facilitate patient follow-ups, and also bring breakthrough improvements in the prediction and evaluation of the therapeutic effects of anti-EGFR drugs. However, as the technical threshold of chip array remained relatively high, human errors during clinical examinations were commonly seen, and the propagation of associating operations somehow became restricted.

The analysis of gene overexpression has led to fundamental progress and clinical advances in the diagnosis of disease (Chen et al., 2005; Wang et al., 2006). The techniques that are commonly used to study gene overexpression include Northern blot, reverse transcriptase *polymerase chain reaction* (RT-PCR), and real-time PCR (Chong et al., 2007; Yen et al., 2009; Yanget al., 2009). Since Northern blot involves complex steps and a large numbers of samples, its application is limited to research instead of clinical diagnosis. On the other hand, since RT-PCR and real-time PCR are performed through a series of simple steps, they are applied extensively for the detection of a single gene, as with the hepatitis virus and infectious pathogens (Yang et al., 2010; Tsao et al., 2010). Although the invention of PCR ranks as one of the greatest discoveries of all time, most PCR techniques have a few common problems: (1) contamination, i.e., false positive results from oversensitive detection of, say, aerosolized DNA or previous sample carry-over; (2) RT-PCR is regarded as only semi-quantitative, since it is difficult to control the efficiency of sequence amplification when comparing different samples; and (3) interference is caused by annealing between the primers. RT-PCR or real-time PCR is used extensively in the detection of a single-gene target (Yen et al., 2010; Harder et al., 2009; Sheu et al., 2006). For the detection of multiple targets or gene clusters, PCR-related techniques tend to have the disadvantages of being time-consuming, cumbersome and costly.

The rapid development of biotechnology in recent years has made gene chips an important tool in clinical diagnosis or drug efficacy evaluation (Popovtzer et al., 2008). Our previous study has developed and evaluated a membrane array-based method for simultaneously detecting the expression levels of multiple mRNA markers from circulating cancer cells in the peripheral blood for cancer diagnosis (Chen et al., 2006). In those studies, the expression

levels of molecular markers were simultaneously evaluated by RT-PCR and membrane array. Data obtained from RT-PCR and membrane array were subjected to linear regression analysis, revealing a high degree of correlation between the results of these two methods ($r=0.979$, $P<0.0001$) (Chen et al., 2006). However, even though the array-based chip technology has proven to be a powerful platform for gene overexpression analysis, some drawbacks still exist and may hinder its practical applications. Two of the critical issues are its tedious sample pretreatment and time-consuming hybridization process. Sample pretreatment process including cell lysis, DNA/RNA extraction and several tedious washing process requiring well-trained personnel and specific instruments, which indicate that the array methods can be only operated in a central lab or medical center, and also limited its applicability for clinical diagnosis. Besides, the manual operation may cause the fragile RNA samples to be degraded by the surrounding RNases (Chirgwin et al., 1979; Chomczynski, 1993). Recently, magnetic bead-based extraction has been widely employed for high-quality RNA extraction. When compared with the conventional methods, the high-quality RNA samples can be stably extracted by simply applying an external magnetic field. Regarding to the hybridization process, it is another time-consuming process due to slow diffusion between target and immobilized probes for conventional array technology. It has been reported that proper mixing is important to achieve an efficient hybridization (Southern et al., 1999). The rotation of the array was reported to be effective in reduction of hybridization time (Chee et al., 1996). Regarding to the above-mentioned issues, there is a great need to develop a rapid and automatic sample pretreatment platform to isolate specific RNA samples from cells and efficient hybridization for array-based methods.

With the rapid advancements in the field of fluid manipulation technology, and especially biomedicine development in recent years, automated and rapid biomedical analysis is now considered to offer the greatest potential and market value (Chen et al., 2003; Siegfried et al., 1997). In terms of biomedical applications, the automatic biomedical analysis system that integrated of several fluid manipulation device including transportation, mixing and heating, which based on the "Lab-on-a-chip" concept, has the advantages of high detection sensitivity, portability, low sample/test sample consumption, low power consumption, compact size, and low cost. Compared to the conventional analysis techniques, it represents a significant breakthrough. With a variety of innovative techniques, a wide range of precision fluid manipulation devices have been integrated to control biological fluids such as whole blood, reagents and buffers, to reduce the size of the biochemical analytical instruments, and integrate the processes into a one-step automated system that facilitates the rapid conducting of biomedical analysis from samples to results (Calistri et al., 2006). In this research, the integrated fluid manipulation technology is adopted to operate the WEnCA platform (figure 1), significantly reduce detection time and errors arising from human operation. Thus, the bottleneck that was preventing the commercialization of the chip detection technique has been overcome. In the current study, we developed an automatic gene chip analyzer which named Chipball (as shown in Fig. 3b), and we have introduced an automatic WEnCA operating platform to improve the manual operations. The system is designated the 'WEnCA-Chipball system', as shown in figure 2. In order to understand the difference between test results obtained by operating the WEnCA-Chipball and WEnCA-manual systems, and to assess the clinical applications of the WEnCA-Chipball system a number of screenings were evaluated. The WEnCA-Chipball platform can be automatically operated to effectively reduce the manual errors and limitations due to current technical criteria.

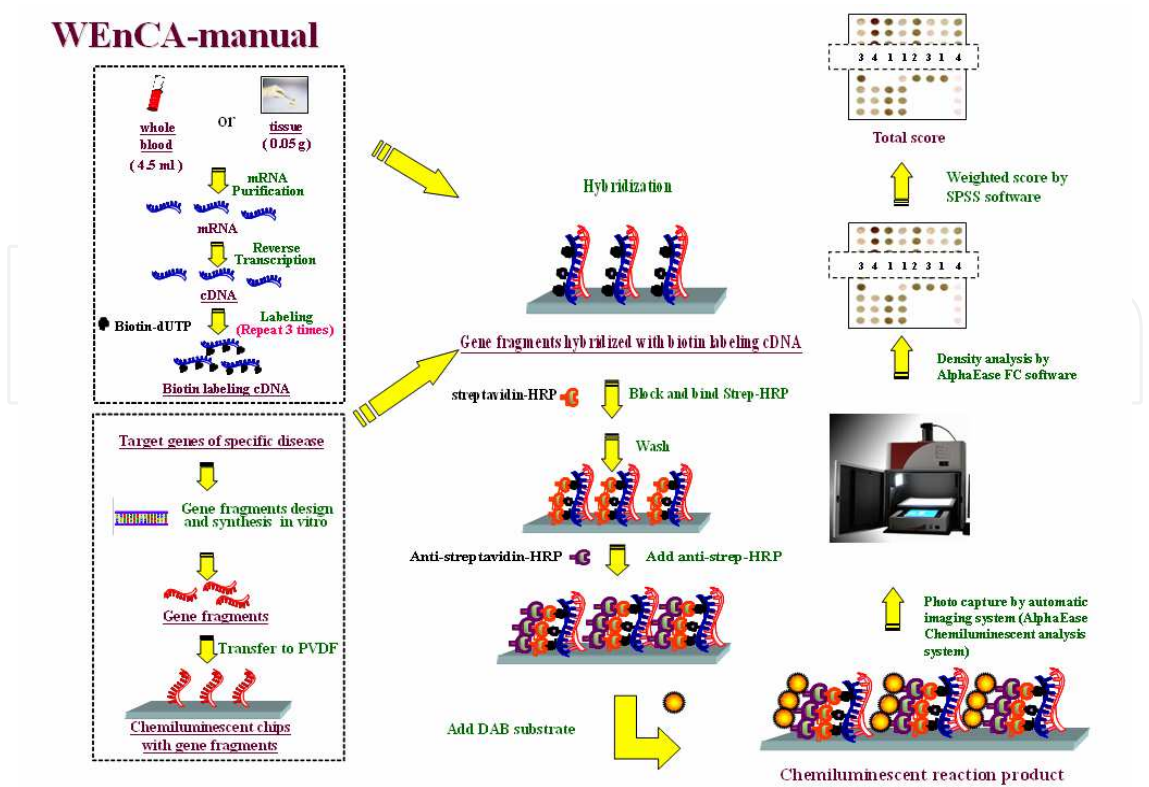


Fig. 1. The manual operation platform of Weighted Enzymatic Chip array (WEnCA) (Hsiung, et al., 2009).

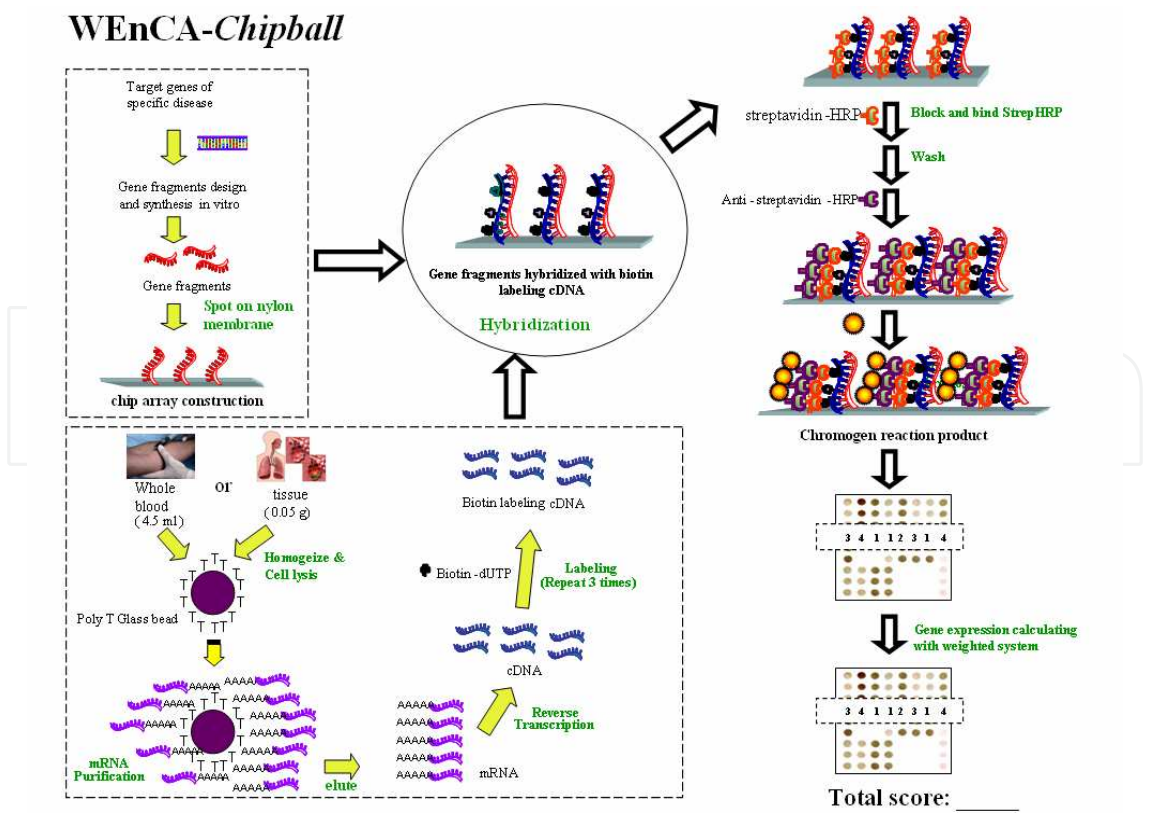


Fig. 2. The automatic WEnCA-Chipball operation platform (Hsiung, et al., 2009).

In addition, the activated KRAS expression in blood samples of 209 lung cancer patients was determined according to the experimental procedure shown in Figure 3 and then analyzed by both WEnCA-manual and WEnCA-Chipball; the results were compared and the clinical applicability of WEnCA-Chipball was defined. Further comparisons were performed on the sensitivity, the specificity and the accuracy of the WEnCA-manual and WEnCA-Chipball; the application, the operation time, and the cost of the two platforms were investigated to evaluate the clinical applicability potential of WEnCA-Chipball.

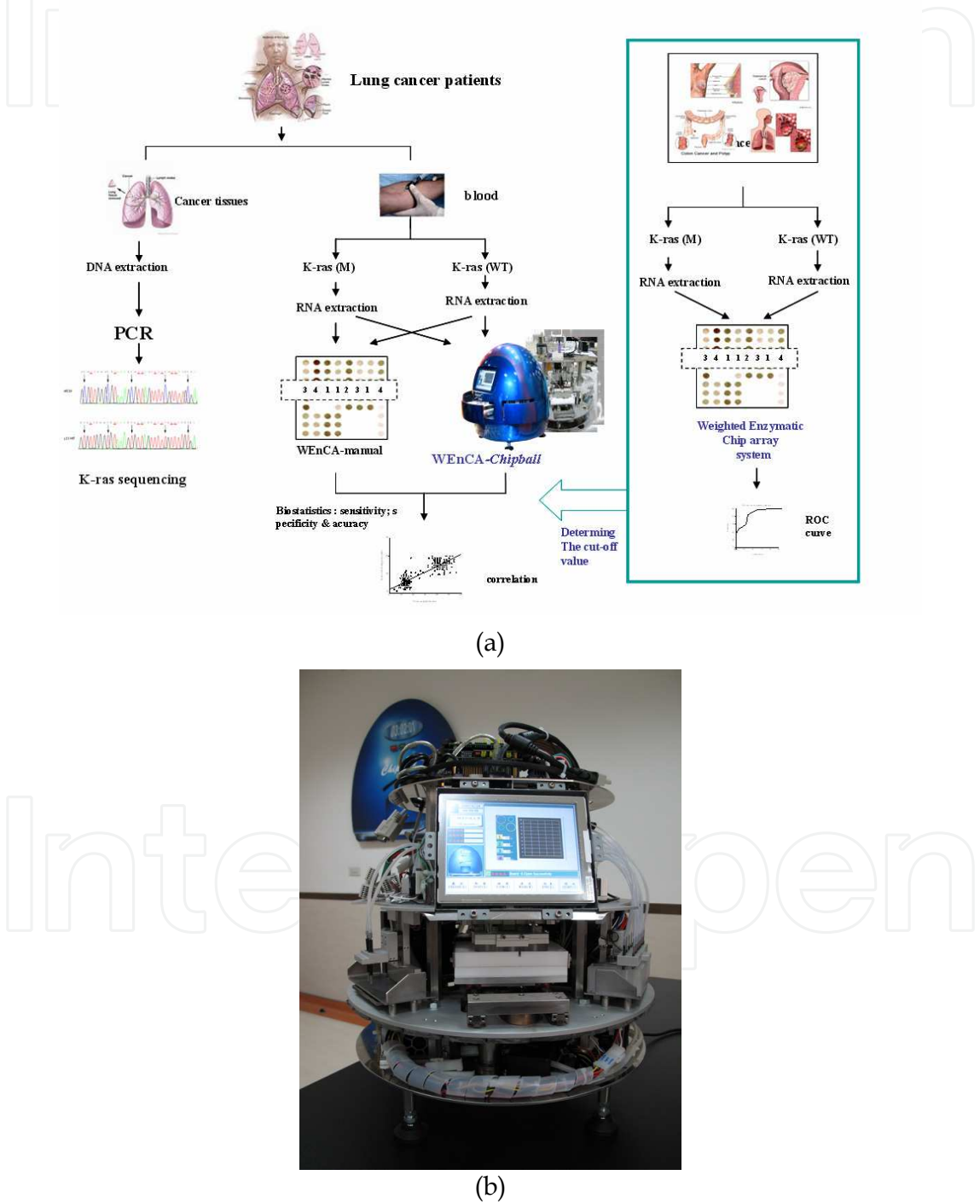


Fig. 3. (a) The research flow chart of current study (Hsiung, et al., 2009). (b) Photograph of the proposed automatic gene chip analyzer.

2. Materials and methods

2.1 Specimens collection

Initially, cancer tissues from two hundreds selected cancer patients including 85 patients with breast cancer, 64 patients with colorectal cancer (CRC), and 51 patients with non-small cell lung cancer (NSCLC) cancer who had undergone surgical resection or biopsy between January 2007 and December 2008 were enrolled into this study. The data from the 200 cancerous patients were used for the analysis of sensitivity, specificity and diagnostic accuracy of WEnCA-Chipball. Tissue samples from various cancer patients were divided into two groups, one group of 100 cancer tissues with *KRAS* mutation including 32 CRCs, 51 breast cancers and 17 NSCLCs and the other group of 100 cancer tissues without *KRAS* mutation including 32 CRCs, 34 breast cancers and 34 NSCLCs were used to determine the cut-off-value of weighted enzymatic chip array method for further circulating tumor cells (CTCs) analysis of 209 lung cancer patients. In order to clinically evaluate and compare both two systems, CLMA and WEnCA-Chipball; blood specimens were collected within test tubes containing anticoagulant sodium citrate from 209 lung cancer patients. To avoid contamination of skin cells, the blood sample was taken via an intravenous catheter, plus the first few milliliters of blood were discarded. Total RNA was immediately extracted from the peripheral whole blood, and then served as a template for cDNA synthesis. Sample acquisition and subsequent usage were approved by the Institutional Review Boards of three hospitals. Written informed consent was obtained from all participants.

2.2 Total RNA isolation and cDNA synthesis

Total RNA was isolated from the collected cancer tissue specimens using the acid - guanidium-phenol-chloroform (AGPC) method according to the standard protocol. The RNA concentration was determined spectrophotometrically based on the absorbance at 260 nm. First-strand cDNA was synthesized from total RNA using the Advantage RT-PCR kit (Promega, Madison, WI) and then reverse transcription was performed in a reaction mixture consisting of Transcription Optimized Buffer, 25 mg/mL Oligo (dT)15, Primer, 100mM=L PCR Nucleotide Mix, 200 mM=L MLV Reverse Transcriptase, and 25 mL Recombinant RNasin Ribonuclease Inhibitor. The reaction mixtures were incubated at 42°C for 2 h, heated to 95°C for 5 min, and then stored at 48°C until the analysis.

2.3 Establishment of membrane array-based method

The rapid development of biotechnology in recent years has made gene chips an important tool in clinical diagnosis or drug efficacy assessment (Popovtzer et al., 2008). Visual OMP3 (Oligonucleotide Modeling Platform, DNA Software, Ann Arbor, MN) was used to design probes for each target gene and β -actin, the latter of which was used as an internal control. The probe selection criteria included strong mismatch discrimination, minimal or no secondary structure, signal strength at the assay temperature, and lack of cross-hybridization. The oligonucleotide probes were then synthesized according to the designed sequences, purified, and controlled before being grafted onto the substrates. The newly synthesized oligonucleotide fragments were dissolved in distilled water to a concentration of 20 mM, applied to a BioJet Plus 3000 nL dispensing system (BioDot, Irvine, CA), which blotted the selected target oligonucleotides and TB (*Mycobacterium tuberculosis*) and the β -actin control sequentially (0.05 μ L per spot and 1.5 mm between spots) on SuPerCharge nylon membrane (Schleicher and Schuell, Dassel, Germany) in triplicate. Dimethyl sulfoxide

(DMSO) was also dispensed onto the membrane as a blank control. In addition, the housekeeping gene was β -actin while the bacterial gene was derived from *Mycobacterium tuberculosis*. Both served as positive and negative controls, respectively, and blotted on the membrane. After rapid drying and cross-linking procedures, the preparation of membrane array for target genes expression was accomplished. Our previous study developed and evaluated a membrane array-based method simultaneously detecting the expression levels of multiple mRNA markers from circulating cancer cells in peripheral blood for cancer diagnosis (Wang et al., 2006; Yen et al., 2009; Tsao et al., 2010). We have carried out membrane array analysis using normal human adrenal cortical cells with KRAS mutation, and obtained 22 upregulated genes most closely related to the KRAS oncogene through bioinformatic analysis. The Activating KRAS Detection Chip for detecting the activated KRAS from peripheral blood was successfully constructed. Although this method is a convenient way of directly using peripheral blood for detecting KRAS activation, and has achieved major breakthroughs in clinical applications, the sensitivity of this technique is only about 84% (Chen et al., 2005).

The colorimetric membrane array (CLMA) was reported in clinical applications for diagnosis of cancer (Harder et al., 2009; Sheu et al., 2006). By the CLMA method, the interpretation importance of each gene is equally included in the diagnosis and each gene is calculated by the same value; this does not evaluate or differentiate the importance of each gene for specific disease diagnosis. That is a major limitation of this technique in clinical application (Tsao et al., 2010). In addition, the cost of the digoxigenin enzyme used on the CLMA platform was too high for routine laboratory diagnosis, and the high criteria of the operation techniques prevented its widespread availability for clinical applications.

Therefore, as mentioned above, our team developed a new generation gene chip operation platform designated as WEnCA. The technical difference between the WEnCA system and the conventional membrane array includes the different weighted value for each gene target on the gene chip, dependent on the importance of each gene during the carcinogenesis of cancer. Furthermore, the conventional digoxigenin system was replaced by the biotin-avidin enzyme system to lower costs.

2.4 Configuration of integrated automatic gene chip analyzer

In order to realize the concept of automatic performing the gene chip operation procedure from samples to images, an integration system composed of several modules including fluid manipulation, temperature controlling, magnetic controlling, actuation, image acquiring and operation platform was investigated, which can perform the critical procedure of array-based gene chip operation such as sample pretreatment, DNA/mRNA purification, reverse transcription, probe labeling and hybridization process, and the image of the gene chip can be acquired automatically after the hybridization as well. The framework of the proposed automatic gene chip analyzer was shown in Fig. 4. Regarding to the Lab-on-chip concept, we have designed an operation platform to provide the interaction fields of the fluid such as samples and reagents, and gene chip operation. The operation platform also was considered as an interface between the sample/reagents and instrument, so that the fluid can be manipulated by utilizing the external devices. In addition, a vessel device contains corresponding reagents to specific process was included in the system. Briefly, the major functions of the proposed system were samples/reagents manipulation, cell lysis, mRNA collection/purification, reverse transcription, probe labeling, and gene chip hybridization.

The images of gene expression can be acquired accordingly. As mentioned above, several modules were designed to achieve these functions. For sample/reagents transporting, samples and reagents can be manipulated and transported through the micro piezoelectric pump device, the volume can be controlled precisely and the operation process can be performed in sequence. By utilizing the fluid manipulation device, the reagents can be sucked and transported from the vessel to the operation platform in specific area, and the reactants can be manipulated between the reaction chambers, the wasted fluid also can be excluded from the operation platform accordingly. Since the temperature control is the critical issue for the gene chip operation, the temperature of each operation process such as cell lysis and hybridization can also be controlled by embedded heaters and thermal sensors, the temperatures, heating/cooling rates and thermal distribution can be well controlled. Compare to the time-consuming and instrument-intensive conventional method of mRNA purification, the commercial magnetic beads were utilized to realize the automatic mRNA purification in this system, and a magnetic controlling device was designed for the magnetic beads manipulation, so that the mRNA can be collected accordingly. Furthermore, for the purpose of interaction enhancing, an active mixing device for shaking mechanism was added into the system. By utilizing the simplified design, the operation platform can be rotated to generate the mixing effect of the samples and reagents inside the operation platform. Finally, the images of the gene chip representing the gene expression can be obtained after all the operation process, and the images can be recorded by the image acquiring device, which including the CCD (Charged-couple device) and image analysis software. The image data can be stored and transmitted to the central laboratory via internet.

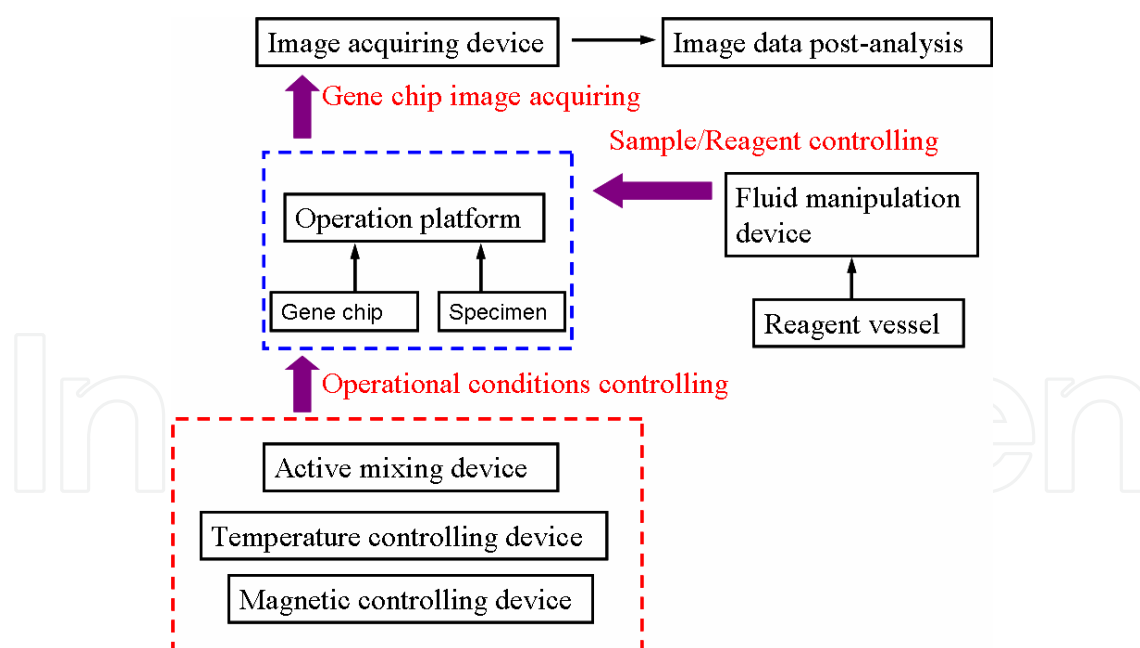


Fig. 4. The framework of the proposed automatic gene chip analyzer.

2.5 Design of the operation platform

In this study, for the purpose to provide the interface between sample/reagents and modules which can control the critical parameters of each process, an operation platform has been designed to perform the sample manipulation and gene analysis. For easy

fabrication and low cost, the material utilized for the operation platform was Polymethylmethacrylate (PMMA), the width and length of the substrate was 10 cm each, and the thickness was 1 cm. As shown in Fig. 5, we have divided the platform into four chambers for specific operation process, including sample pretreatment area, sample purification area, transcription and probe labeling area, and hybridization area. The four areas were fabricated by a micro-milling machine, the diameter and depth of each chamber has been calculated precisely to ensure the volume was sufficient for each process. Initially, a membrane array device with specific gene probes was first integrated into the hybridization area, and then the operation platform was placed onto a telescopic loading tray structure, which was designed in this system for the orientation and operation of the platform with external controlling device. Each area on the platform was corresponding to an external module for its specific operation process. For instance, a temperature controlling device embedded onto the tray structure including a set of heater and thermal sensor was placed underneath the sample pretreatment area for cell lysis application. We have set up three temperature controlling device corresponding to area I, III and IV for the adjustment of operation temperature, and a simple design of magnet lift-up mechanism to control the magnetic force and collect the magnetic beads in area II for mRNA purifying application. In order to transport the reagents into the operation chamber and manipulate the sample/reagents between the chambers, several commercial piezoelectric pumps were utilized. Sets of sucking needles were inserted into the reagent vessels and operation chamber before the piezoelectric pumps were activated, and corresponding samples/reagents can be transported to the specific chamber by activating specific pump. After the samples/reagents transportation in each chamber, a mixing mechanism was required for the sample interactions. The tray structure and operation platform can be clockwise rotated simultaneously by utilizing a cam and electric motor device. The rotation speed can be adjusted within a dynamic range from 50 to 200 rpm. As shown in fig. 5, the

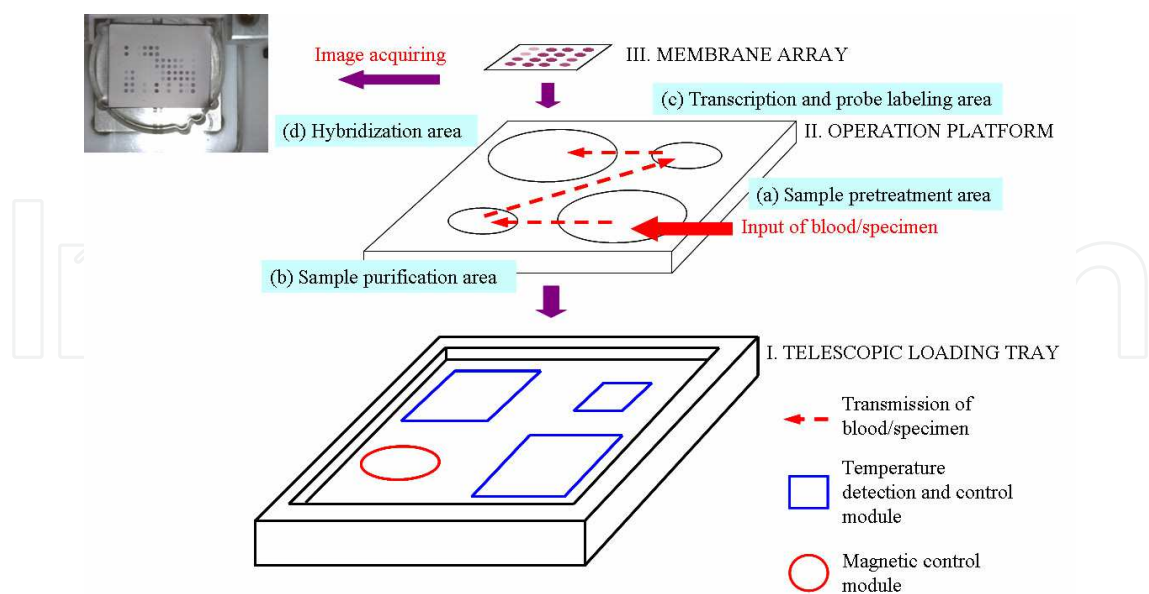


Fig. 5. Illustration of the fluidic operation platform, which divided into four areas, the blood/specimen can be operated sequentially through the four operation process. The membrane array device was firstly integrated into the hybridization area, and then the operation platform was placed onto the telescopic loading tray for further external control.

image of the gene expression on the gene chip can be obtained after finished all operation process. The darkness of each probe can reveal the interaction between pretreated DNA/RNA sample and probe with specific sequence on gene chip. The image can be recorded by a CCD, and then the recorded image can be sent to the commercial image analysis software for further analysis. The darkness of each probe can reveal the expression of specific sequence for the gene information analysis.

2.6 Operating procedure of automatic gene chip analyzer

Firstly, a sample pretreatment process from whole blood to mRNA was required, as shown in Fig. 6. In order to breakdown the sample cells and isolate mRNA from the specimen, the fluid manipulation device delivers the whole blood and lysis buffer to the first reaction chamber (sample pretreatment area), as shown in Fig. 6(a). The fluid manipulation device also delivered the magnetic beads, binding buffer, and washing buffer from reagent vessel to the first reaction chamber (Fig. 6b). The samples were then mixed by the active mixing device to ensure that the samples react effectively and to enhance the mRNA conjugation with the magnetic beads. As shown in Fig. 6(c), biotin poly dT and streptavidin magnetic beads were used to isolate the mRNA. The reacted samples and the beads that have conjugated mRNA onto the surface can then be delivered to the second reaction chamber (sample purification area) by the fluid manipulation device. In this area, magnetic controlling device was utilized to manipulate magnetic beads and to separate the target mRNA samples from the surroundings (Fig. 6d). The mRNA-conjugated magnetic beads can be collected by the external magnet and then washing buffer can be transported into the area by the fluid manipulation device for further washing process (as Fig. 6e). The remaining waste fluid excluding the mRNA-conjugated beads can be transported by the fluid manipulation device to the waste collection area. The elution buffer was then delivered through the fluid manipulation device to the reaction chamber for the further mixing reaction. The mRNA-conjugated magnetic beads were demagnetized and suspended in the elution buffer after the external magnet descended. As shown in Fig. 6(f), after the mixing and elution process, the magnet activated again to separate the beads and target mRNA samples. Hence the buffer contained the purified mRNA samples that have been extracted and released were then delivered through the fluid manipulation device to the third reaction chamber (transcription and probe labeling area). The required temperature for the transcription can be regulated by the temperature controlling device allowing the mRNA to be converted into stable cDNA for chromogen labeling for the bio-molecular test target. The reacted samples and buffer solution were then delivered by the fluid manipulation device to the hybridization area for the hybridization process. Meanwhile, prior to deliver the samples to the hybridization area, the gene chip was placed in the hybridization area for the pre-hybridization procedure. The labeled cDNA samples then entered the reaction chamber contained the Express Hyb hybridization solution where the required temperature for the hybridization reaction was regulated by the temperature controlling device. Subsequently, samples and reagents including biotin-labeling mixture, washing buffer, blocking buffer, streptavidin conjugation, detection buffer, DAB, and ddH₂O were delivered into the chamber through the fluid manipulation device. Finally, after all processes of the hybridization reaction were completed, the image of gene chip can be obtained and acquired by the image acquiring device and image/information processing system for the further gene expression information analysis. A detailed operation process can be seen in Table 1. As the result, the overall operation time can be decreased less than 8 hours, which was shorten by 70%

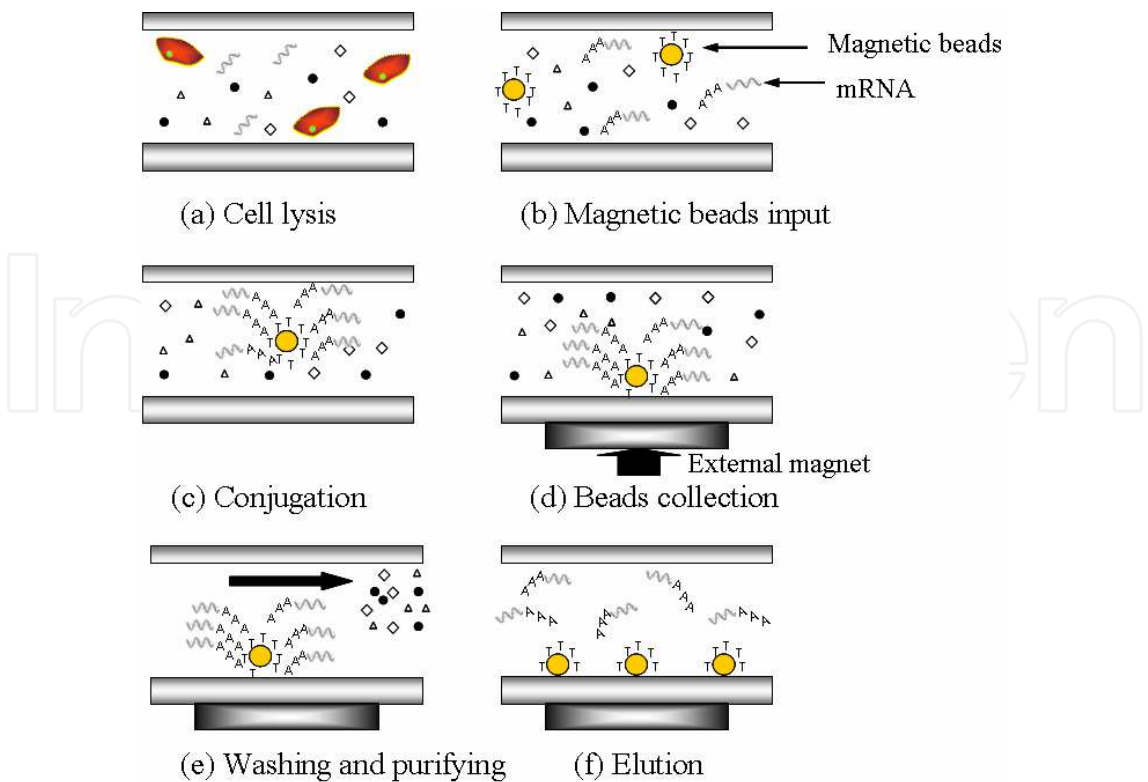


Fig. 6. Illustration of the purifying and separation process from whole blood to mRNA samples.

Areas	Reagents	Volume (ml)	Time (min)	Temperature (°C)
Sample Pretreatment Area	Lysis Solution	1.02	15	60
	Whole Blood	4		
	Magnetic Beads	0.25	1	Room Temperature
	Binding Solution	0.25	4	
Sample Purification Area	Washing Buffer I	0.25	3	
	Washing Buffer II	0.25	3	
	Elution Solution	0.25	5	
Transcription and Probe Labeling Area	RT Reagents	0.25	40/5	42/75
	DIG-Labeling solution	0.25	60	37
Hybridization Area	Hybridization solution	3	30	42
	Washing Buffer I	2	10	
	Washing Buffer II	2	10	
	Washing Buffer III	2	10	Room Temperature
	Blocking Buffer	2	10	
	Anti-DIG AP Buffer	5	10	
	Detection Buffer	2	10	
	NBT/BCIP	1	3	

Table 1. Detailed operation process of the automatic gene chip analyzer.

when compared to the conventional manual method, and also represented the great potentials and advantages of the proposed automatic gene chip analyzer for gene diagnosis applications.

3. Results

3.1 Comparison between colorimetric membrane array and weighted enzymatic chip array method

In order to verify the sensitivity, specificity and accuracy of the activating KRAS detection chip, we enrolled 209 NSCLC patients (pathologically proved) to detect the activating KRAS from their peripheral blood specimens. All specimens were tested by both the CLMA and WEnCA methods. We also analyzed tissue samples of 209 cases of patients with KRAS mutations by a traditional PCR-combing direct sequencing method to be a standard reference. Experimental results indicated that there were 71 cases with KRAS mutations by sequencing analysis, and a total of 59 patients tested positive by the CLMA, while the WEnCA tested positive in a total of 66 cases. Moreover, in 138 NSCLC cases without KRAS mutation, CLMA detected 133 cases as negative, and WEnCA detected 130 cases as negative. After statistical analysis, the CLMA sensitivity was 83%, specificity 96%; and WEnCA sensitivity could be raised to be 93%, while the specificity still is maintained at around 94%. The examinational comparison results also compared the ability of peripheral blood detection results of two technology platforms where 3 cancer cells /cc blood were detected by the WEnCA, and 5 cancer cells /cc blood by the CLMA. These findings suggest that the WEnCA platform has a higher detection rate for activated KRAS oncogene, and great potential for further investigation and clinical application.

To determine the cutoff value of the Activating KRAS Detection Chip by the WEnCA method, we analyzed 200 cancer tissues of which 100 had the KRAS mutation and the others had wild-type KRAS. The 200 tissues collected underwent mRNA extraction and first cDNA labeling before reacting to the Activating KRAS Detection Chip by the WEnCA-manual method. After signal development, each gene spot density was normalized using the density of β -actin on the same chip. Next, the result obtained from the cancer tissue with KRAS mutation was divided by the normalized value obtained from the sample spot of the tissue without mutant KRAS to obtain the ratio. A ratio higher than 2 was defined as being positive for gene overexpression. In terms of analysis using WEnCA, to determine the weighted value of each gene spot, we divided the percentage of each gene overexpression in the 100 cancer tissues with the activating KRAS mutation to provide four classes. The gene spot that showed overexpression in over 80 cancer tissues had a weighted value of 4 (3 in 70–80 cancer tissues, 2 in 60–70 cancer tissues, and 1 in 50–60 cancer tissues). After the reaction through WEnCA, the positive gene spots were multiplied by their respective weighted values to obtain the total score of the chip. Then underwent analysis using the receiver operating characteristic curve can be obtained with a positive reaction cutoff value of 20. Results showed that the sensitivity reached 96% and the specificity reached 97%.

3.2 Detection limitation of the WEnCA-manual and WEnCA-Chipball assay

Evaluating the detection limitation of WEnCA-manual and WEnCA-Chipball system, with the addition of 100, 25 and 12 cancer cells that possessed the activated mutant KRAS into 5cc of blood, which obtained total scores higher than the cutoff value 20 in both systems. In addition, when only 6 cells were added, in which case the total score equaled 8 in WEnCA-

manual and 5 in WEnCA-Chipball system, which are both lower than 20. Therefore, no significant difference was found between the detection limitations of the two systems.

3.3 Clinical assessment of the accuracy of WEnCA-manual and WEnCA-chipball system

To further understand the practical clinical detection of the WEnCA-Chipball system, we obtained blood samples of 209 pathology-proven lung cancer patients and analyzed the KRAS pathway-related genes overexpression in those blood specimens by previously constructed Activated KRAS Detection Chip using both the WEnCA-manual and WEnCA-Chipball systems. The paired cancer tissue with KRAS mutational status then served as the reference standard. As shown in Table 2, the results are as follows: 74 cases of the 209 clinical samples were identified with activated KRAS by the WEnCA-manual method, and the WEnCA-Chipball system test results showed in a total of 71 cases. Among them, 66 were positive through WEnCA-manual and 63 through WEnCA-Chipball. Moreover, among the 138 paired cancer tissues with wild type KRAS, 130 were negative through both WEnCA-manual and WEnCA-Chipball system. According to the results, we can obtain the sensitivity, specificity and accuracy of WEnCA-manual were 93%, 94% and 94%; the sensitivity, specificity and accuracy of WEnCA-Chipball were 89%, 94%, and 92%, respectively. As the results in Table 3, using WEnCA-Chipball, the average total score of the positive sample was 6.1 lower and the average total score of the negative sample was 3.9 lower while the overall average total score was 4.7 lower than the WEnCA-manual. Regarding to the operation time, the WEnCA-Chipball system takes 7.5 h to complete all tasks, while the operation time of the WEnCA-manual system is around 72 h, which was approximately 9 folds than the time required for the automatic system. The operating cost of the WEnCA-manual system was approximately 5 times more expensive than that incurred for the WEnCA-Chipball system. There was no difference in the detection limitation between the two systems. We believe that the WEnCA-Chipball operating system has considerable potential in clinical medicine applications.

		WEnCA-Chipball (WEnCA-manual)		
		Negative	Positive	Total
KRAS	Wild Type	130 (130)	8(8)	138
	Mutation	8 (5)	63(66)	71
Total		138 (135)	71(74)	209

Table 2. The sensitivity, specificity and accuracy of WEnCA-Chipball and WEnCA-manual system

Method \ Mean score	WEnCA-manual	WEnCA-Chipball	Difference (Chipball- Manual)
Positive specimens	46.1	40	-6.1
Negative specimens	13.8	9.9	-3.9
Total specimens	25.2	20.6	-4.7

Table 3. Comparing the total score of Activating KRAS Detection Chip analyzed by WEnCA-manual and WEnCA-Chipball system

4. Discussion

In recent years, target therapy has rapidly developed. Research and development for the targeted therapy drugs, such as Iressa and Cetuximab, have been proven efficient in advanced NSCLC (Thatcher, 2007; Chang, 2008). Many studies report that KRAS mutations are highly-specific independent predictors of response to single-agent EGFR tyrosine kinase inhibitors (Iressa) in advanced NSCLC; and, similarity to anti-EGFR monoclonal antibodies (Cetuximab) alone (Rossi et al., 2009; Tiseo et al., 2010). However, at the present time, therapeutic targets such as HER2/neu, EGFR, KRAS, Raf, etc., are analyzed using RT-PCR combining direct sequencing, fluorescence in situ hybridization (FISH), real-time PCR, and other methods (Hilbe et al., 2003; Cappuzzo et al., 2007; Akkiprik et al., 2008). The above methods have disadvantages such as inadequate sensitivity, and the need to collect patients' cancer tissues as specimens, which make medicinal effect evaluations prior to clinical treatment cumbersome. RT-PCR and real-time PCR are applied for the detection of single genes, and most PCR techniques have a few common problems: (1) contamination, such as false-positive results from oversensitive detection of aerosolized DNA or previous sample carry-over; (2) RT-PCR is regarded as only semi-quantitative, since it is difficult to control the efficiency of sequence amplification when comparing different samples; and, (3) interference is caused by annealing between the primers. RT-PCR or real-time PCR is used extensively in the detection of a single-gene target. For the detection of multiple targets or gene clusters, PCR-related techniques tend to be time-consuming, labor-intensive, and costly. Therefore, the current study successfully developed the WEnCA-Chipball to effectively address and solve those problems.

In the WEnCA-Chipball system, the total operation time from input of samples to completion of the image analysis was about 7.5 h, which is a substantial decrease in time when compared to the three days required for the original manually operated membrane array, and significantly minimizes the occurrence of human errors. The WEnCA-Chipball system not only provides an innovative automatic system for clinical target therapy efficacy evaluation, but also improves the clinical usability and accuracy compared to the manual method. Thus, it has been proven to be a practical means to assess the drug efficacy of clinical target treatment.

The WEnCA-Chipball system developed by this research team not only retains the advantages of the Lab-on-a-chip, but also overcomes the problem of the microfluidic chip's unsuitability for continuous operation and linkage to an interpretation system. As the world's first automatic chip analyzer, it will be useful in the future for the molecular diagnosis of infectious diseases, the detection of CTC through chip replacements, or the assessment of drug efficacy.

5. Future trends

Medical automation technology is the future trend that can reduce labor, operation errors, and time-consumption. WEnCA-Chipball is suited for clinical application to detect mutant KRAS in CTCs before target therapy. The specialized automatic gene chip detecting system would be designed for the fast and accurate detection of KRAS in CTCs in each human cancer specimen. This is the challenge to meet for the years ahead.

The WEnCA-Chipball system, through a built-in computer system, will not only instantly produce the results of the chip analysis but also connect to a global network. The detection

results can be transmitted locally in any operation area and stations around the world through common software used in data transmission and interpretation. The station networks around the world can be completed through the prevalent WEnCA-Chipball system. The WEnCA-Chipball system is believed to be capable for extensive applications in clinical medicine, and holds great potential for future development.

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Phone: +86-21-62489820
Fax: +86-21-62489821

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