C/Tech SARS-CoV-2 RT-PCR Technology

(Catalogue No. C02-01-1180-100)

CE mark Approved

October 2020

Table of Contents

Kit contents

The C/Tech SARS-CoV-2 RT-PCR Technology detection kit was designed for use in research laboratories. The kit can also be deployed at clinics or hospitals, centralized laboratories or contracted testing providers. It is based on the Real-time PCR (RT-PCR) technology developed by Hai Kang Life Corporation (HKLife), targeting the ORF1ab and the Nucleocapsid (N) genes in the virus genome and utilizing fluorescence probes for detection.

C/Tech SARS-CoV-2 RT-PCR Technology

(Cat. No. C02-01-1180-100)

2 x 730 µl SARS-CoV-2 RT-PCR Mastermix

1 x 40 µl RT-PCR Enzyme Mix

1 x 250 µl Positive Control*

1 x 1 ml Nuclease-free Water

***Important**: *This component contains high copy number RNA template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.*

Equipment and consumables to be supplied by user

Real-time PCR machine

SARS-CoV-2 detection kits were validated using the BIO-RAD CFX96™ fluorescent quantitative PCR system and the Applied Biosystems 7500 Fast Real-time PCR system.

Vortex and microcentrifuge

Test samples (collected and extracted by user)

Sample collection

SARS-CoV-2 detection kits are recommended for use with HKLife Virus Col Sample Collection kit. However, they are designed to work well with all collection processes that yield high quality clinical samples. Throat swab is the recommended sample type. Other compatible sample types include nasal swab, saliva, sputum and BALF.

Extraction

SARS-CoV-2 detection kits are recommended for use with HKLife Virus Ex RNA Extraction kit. However, they are designed to work well with all processes that yield high quality RNA and DNA with minimal PCR inhibitors.

Consumables

Pipettes and filter-head fitted pipette tips
0.2 ml PCR tubes
Quantitative PCR tubes (8-well PCR strips or 96-well PCR plate)
1.5 ml and 2.0 ml centrifuge tubes
96-well cold blocks
Disposable powder-free gloves
10% bleach

Kit storage and stability

Store Mastermix and Enzyme Mix at -20℃. Store Positive Control at -80℃. Fluorescent probes are sensitive to light so additional covering of the reagent tube with aluminum foil is recommended to avoid light. Thaw frozen reagents just before use. Each kit can be stored at -20℃ for up to 1 year. Use of the kit after the expiry date stated on the packaging box is not recommended.

Note: Repeated freeze-thaw of reagents may reduce reagent sensitivity. Depending on the frequency of testing, you can store the reagents in appropriate volumes to avoid repeated freezing and thawing.

Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and RNA/DNA integrity. Always run at least one negative control with the sample. To prepare a negative control, replace the template RNA sample with the nuclease-free water provided with the kit.

Disclaimers and warranties

These products are developed, designed and sold for research purposes. Under the Emergency Use Authorization (EUA), they are cleared for *in vitro* diagnostics use. As an auxiliary diagnosis of COVID-19, they should be used in combination with other diagnostic methods and with comprehensive evaluation of clinical symptoms.

Hai Kang Life Corporation Limited warrants the products manufactured by it are free of defects in materials and workmanship when used in accordance with the applicable instructions for a period equal or shorter than one year from the date of shipment of the product(s) or the expiration date marked on the product packaging under the storage conditions recommended in the instructions and/or on the package. Application protocols published by Hai Kang Life Corporation Limited are intended to be only guidelines for the buyers of the products. Buyers are expected to validate the kit to their individual application. Hai Kang Life Corporation Limited makes no other warranty, expressed or implied. There is no warranty of merchantability or fitness for a particular purpose.

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Principles of the test

Real-time PCR (RT-PCR)

SARS-CoV-2 specific primers and probes are included in the Mastermix. The ORF1ab signal can be detected through the FAM channel, the N-gene signal through the HEX channel and the RNase P signal through the Cy5 channel.

The real-time PCR procedure in both kits utilizes the TaqMan® method. During PCR amplification, forward and reverse primers hybridize to the SARS-CoV-2 cDNA. A fluorescent probe is included in the same reaction mixture which consists of a probe labeled with a 5'-dye and a 3' quencher. This probe hybridizes to the template at the region to be amplified and during PCR amplification, the probe is cleaved separating the reporter dye and quencher. The resulting increase in fluorescence can be detected on a range of real-time PCR platforms (see non-exclusive list in 'Equipment and consumables to be supplied by user'). The number of cycles required before the fluorescence increases above a preset threshold value is termed the Ct value and is related to the quantity of initial template in the sample. There is a direct relationship wherein the smaller the Ct value, the more initial template in the sample so this kit can be used for semi-quantitative analysis.

Test controls

Positive controls

In vitro transcribed (IVT) RNA products, ORF1ab (12800 bp-14000 bp), N gene (28274 bp-29533 bp) and RNase P gene fragments, are provided as positive control templates for both kits. The positive control should be used in every experiment run wherein at least one positive control reaction must be included in the run. A positive result indicates that the test reaction worked properly in that particular test run. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false-positive results. This can be achieved by handling this component in a separate laboratory environment away from the other reagents. Care should also be taken to avoid sample contamination when adding the positive control to the run. This can be avoided by first adding negative controls then test samples, and sealing these wells before adding the positive control into the positive control well.

Negative control

To validate any positive findings at least one negative control reaction should be included for each experiment run. For this reaction the nuclease-free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run.

Internal control

To ensure that the sample's negative results do not come from sampling failures, all clinical samples need to undergo internal control (RNase P gene) testing to confirm sample quality. A positive result for the sample internal control indicates collection and extraction of the sample has passed QC. It is possible that some samples may fail to exhibit RNase P growth curves due to low cell numbers in the original clinical sample. A negative RNase P signal does not preclude the presence of SARS-CoV-2 virus RNA in a clinical specimen.

Spiking Positive Control

Negative test results have several possibilities: 1. The sample does not contain the target fragment; 2. The quantity of target fragment in the sample is lower than the detectable level; 3. The sample contains PCR inhibitors. The purpose of adding positive control template to the test sample (Spiking control) in an experiment is to test whether PCR inhibition took place. If the amplification result of the Spiking control is close to 40 (or near the Ct value of the negative control), this indicates that PCR inhibitors are present in the sample, the negative test results cannot be taken as correct. Sample RNA extraction and PCR amplification has to be repeated.

Spiking control is an optional control and can be performed if you question the negative test result for your sample.

Special precautions for nucleic acid testing

Before the experiment:

1. The operator should first make sure of personal protection in accordance with Biosafety Level 2 Laboratory's personal protection requirements (medical protective masks or N95 masks, latex gloves, isolation clothing over work clothes, medical protective caps, hand hygiene, protective glasses as appropriate). The experiment should be carried out in a Biosafety Level 2 Laboratory;
2. There should be designated areas for different operations: reagent preparation, nucleic acid extraction, sample addition, amplification and post-amplification areas. A one-way system should be enforced whereby a person is not allowed to go back to the reagent preparation area after being in the amplification area. There is a risk of contamination in the amplification and post-amplification areas so there should be strict physical separation from the other areas. Regular environment monitoring is recommended to ensure the work area is contamination-free;
3. Work clothes worn in different operating areas should not be mixed. In particular, clothes from the amplification areas are recommended to be used only in the amplification areas;
4. The thermal cyclers and real-time PCR instruments should be cleaned with 10% bleach prior to experiment. They should be wiped dry and allowed a further 10-15 min to dry completely before use;
5. To minimize risk of cross-contamination, the biosafety cabinet should be cleaned with 10% bleach and irradiated with UV lamp for 30 minutes prior to the experiment. The required tip boxes, centrifuge tubes, PCR reaction tubes and other materials need to be sprayed with 10% bleach before being placed into the cabinet;
6. Each operating area should have its own set of pipettes, tip boxes, centrifuge tubes and PCR reaction tubes and their mixed use should not be allowed.

During the experiment:

7. Once thawed, keep all reagents on ice during the whole test procedure. Prepare the reaction mixture in the reagent preparation area, aliquot after thorough mixing. Centrifuge and gently cap before transfer to the sample addition area by another person or through a transfer window;
8. In the sample addition biosafety cabinet, add samples one by one into the corresponding reaction tubes and tightly cap the tubes after each addition. Each batch of tests must also include at least one positive control and one negative control. The order of addition should be negative control, RNA samples then positive control; positive control is added last to avoid contamination;
9. Preparation of reaction mixture are done by up-down pipetting slowly to avoid risk of aerosol production and contamination;
10. Avoid passing over the top of uncapped tubes while pipetting;
11. Change tips every time after a component is added and use tips with fitted filter. Avoid repeated use that would result in volume error and cross-contamination. Prepare a 'Sample addition table' prior to sample addition to make sure the sample addition process is efficient and correct;

12. To reduce risk of cross-contamination arising from gloves, change gloves whenever there is accidental contact with reagents or samples, after sample addition and before and after post-amplification handling;
13. Add 20µl nuclease-free water to all empty tubes and cap them to avoid tube deformation during reaction that may affect the experiment.

After the experiment:

14. After completion of the experiment, disinfect the laboratory workbench and floor with 10% bleach; the waste consumables should be immediately cleared from the experimental area which is subsequently UV irradiated for not less than 30 minutes;
15. Work clothes should be washed every day and those from different operating areas washed separately;
16. All coronavirus-related medical waste has to be autoclaved before being collected as general medical waste by professional institutions. Garbage cans with cover should be used and garbage bags should be labeled "New coronavirus medical waste" with related information;
17. Garbage should be cleared on the same day; no overnight garbage in the laboratory;
18. Perform environmental monitoring – during high volume testing periods, routine environmental monitoring with swab sampling should be performed regularly.

Test procedure

1. Prepare reaction mixtures per sample according to Table 1. Aliquot 15µl to each PCR tube.

Table 1. RT-PCR reaction

Component	Volume (µL)
RT-qPCR Mastermix	14.6
RT-qPCR Enzyme Mix	0.4
Total volume	15

2. Add 5µl RNA sample, Nuclease-free water or Positive Control to each tube. Flick the tube wall to mix the solution and then centrifuge to pull-down all reagents to the bottom of the tube.
3. When all PCR reactions are ready, load all PCR tubes into the real-time PCR instrument.
4. Select the FAM channel (Reporter: FAM, Quencher: None), HEX channel (Reporter: HEX, Quencher: None) and Cy5 channel (Reporter: Cy5, Quencher: None) to monitor the ORF1ab, N-gene and internal control, respectively. Set passive reference dye channel to 'None'. (VIC or JOE channel can be used in place of HEX if necessary)
5. Amplify according to the amplification profile below (Table 2).

Table 2. RT-PCR amplification profile

Temperature	Time	Signal	Cycle
50℃	15 min	-	1 cycle
95℃	5 min	-	
95℃	15 s	-	40 cycles
60℃	34 s	FAM, HEX and Cy5 signal collection	

6. Perform data analysis as presented in 'Interpretation of results'

Note: Mix reagents thoroughly before use. Do not vortex Mastermix after addition of enzyme as this could cause loss of enzyme activity. It is advisable to run samples in duplicate to ensure reliability of results.

Interpretation of results

General baseline threshold setting: either automatic setting of instrument or select the region with stable fluorescence signal after Ct = 5 and prior to exponential amplification, and set the threshold to just exceed the highest point of the fluorescence curve of the negative control.

Data analysis:

- The acceptance criteria for positive and negative controls are given in Table 3.

Table 3. Ct values for controls for valid results

Control	Ct value (Optical channel)		
	ORF1ab (FAM)	N (HEX/VIC/JOE)	RNase P (Cy5)
Negative	≥ 40	≥ 40	≥ 40
Positive	≤ 35.0	≤ 35.0	≤ 35.0

- If the results for all controls do not meet the given criteria, the results from the entire run are considered INVALID and repeat testing must be performed. In cases where positive controls do not meet criteria, there is a possibility of positive control degradation from long-term storage or repeated freeze-thaw, and new controls are recommended.

- Sample Ct values detected in both FAM channel (ORF1ab) and HEX/VIC/JOE channel (N) are ≤ 39.0 (positive for ORF1ab and N) and typical amplification curves are observed; the results are valid and the sample can be directly reported as **POSITIVE**.

- Sample Ct value for the RNase P gene Cy5 channel is < 40.0 (positive for RNase P), both FAM channel (ORF1ab) and HEX/VIC/JOE channel (N) have no Ct or Ct values of 40.0; the results are valid and the sample can be directly reported as **NEGATIVE**.

- Sample Ct values for the FAM channel (ORF1ab), HEX/VIC/JOE channel (N) AND Cy5 channel (Cy5) have no Ct or Ct values of 40.0; the results are **INVALID** and the sample should be resampled for repeat experiment.

- Detection of a Ct value of 39.0 < Ct < 40.0 in the FAM channel (ORF1ab) and HEX/VIC/JOE channel (N); a redo is recommended. If the Ct values are still between the given values and the amplification curves have observable exponential growth, the sample can be reported as weak positive.

Interpretation of results

- Interpretation of results according to Table 4 where positive results for individual genes in terms of Ct values are as defined above.

Table 4. Result interpretation

ORF1ab gene	N gene	RNase P gene	Interpretation	Suggestion
+	+	±	Positive	/
Only one is positive		±	Inconclusive	Redo
-	-	+	Negative	/
-	-	-	Invalid	Redo

Note:

- *For interpretation of test results, a comprehensive analysis is needed. The test results should be considered in combination with patient symptoms, medical history, other laboratory examinations and treatment responses.*
- *Incorrect sampling, processing and transport, as well as sample types and nucleic acid extraction methods can have an impact on the accuracy of the results; particularly in leading to false negative results, which may require confirmation using different tests.*

Specificity

C/Tech SARS-CoV-2 RT-PCR Technology detection kit is an *in vitro* diagnostic kits for detection of the coronavirus SARS-CoV-2. The primers and probes are designed to have 100% homology with the 66 genome sequences available on the GISAID database, the 32 genome sequences available on the NCBI database and the 86 genome sequences available on the Chinese National Genomics Data Center database at the time of design. As of 8 Sepember 2020, 100% identity with all available SARS-CoV-2 sequences was again confirmed. Other than SARS-CoV-2, there is high sequence homology only with bat coronavirus for three primers and one probe, and with pangolin coronavirus for one primer as indicated in Table 5. Sequence homology for all primers and probes with all other known coronaviruses, including other human coronaviruses, were less than 80%.

Table 5. Sequence homology of primer/probe set with known coronavirus*

Primer / probe	Coronavirus	Percentage homology
ORF1ab_Reverse1	Bat coronavirus RaTG13	89.5
	Pangolin coronavirus	89.5
ORF1ab _Forward2	Bat coronavirus RaTG13	95.2
ORF1ab_Reverse2	Bat coronavirus RaTG13	100
N-gene_Probe2	Bat coronavirus RaTG13	100

** Excluding SARS-CoV-2*

For specificity validation, a total of 58 pathogens were used (Table 6). All target templates tested negative using either ORF1ab or N-gene primer sets.

Table 6. List of pathogens tested in specificity validation

Virus				
229E	H1N1	FMDV	RSV (A,B)	CMV (DNA)
OC43	H3N2	Norovirus	NDV	Adv (1,2; DNA)
HKU1	N1	RHV	HPIV (1-4)	N9 (DNA)
NL63	H9	MPV (A,B)	HBV (DNA)	H5 (DNA)
SARS	H7	EV (A-D)	HSV1 (DNA)	
MERS	FluB			
Bacteria				
H. influenzae	S. epidermis	M. tuberculosis	B. cepacia	K. oxytoca
P. aeruginosa	S. salivarius	B. pertussis	E. aerogenes	M. catarrhails
S. pneumoniae	C. pneumoniae	Acinetobacter	E. cloacae	S. aureus
S. pyogenes	M. pneumoniae	S. marcescens	E. coli	S. maltophilia
L. pneumophila				
Fungi				
Candida albicans	P. jirovecii (PJP)			

229E, Human coronavirus 229E; OC43, Human coronavirus OC43; HKU1, Human coronavirus HKU1; NL63, Human coronavirus NL63; SARS, Severe acute respiratory syndrome-associated virus; MERS, Middle-East respiratory syndrome-associated virus; H1N1, Influenza A virus subtype H1N1; H3N2, Influenza A virus subtype H3N2; N1, Influenza A virus subtype N1; H9, Influenza A virus subtype H9; H7, Influenza A virus subtype H7; FluB, Influenza B virus; FMDV, Foot and mouth disease virus; RHV, Rhinovirus; MPV, Metapneumovirus; EV, Enterovirus; RSV, Respiratory syncytial virus; NDV, Newcastle disease virus; HPIV, Human parainfluenza virus; HBV, Hepatitis B virus; HSV1, Herpes simplex virus 1; CMV, Cytomegalovirus; N9, Influenza A virus subtype N9; Adv, Adenovirus; H5, Influenza A virus subtype H5

All data are available upon request

Performance evaluation

Analytical sensitivity

The contrived sample condition was nucleic acids extracted from pooled oropharyngeal (OP) swab samples and spiked with inactivated SARS-CoV-2 virus. The pooled OP swab matrix was first confirmed negative using C/Tech SARS-CoV-2 RT-PCR and then a total of four dilutions of inactivated virus were prepared in the negative matrix. Samples were extracted using HKLife Virus Ex Extraction Kit and tested using the ABI 7500 Fast Real-time PCR system. Four replicates per concentration were tested.

The four dilutions tested were 1000, 500, 200, 100 copies/ml, and a provisional LoD of 200-500 copies/ml was obtained. A final LoD of 250 copies/ml was confirmed by testing concentrations 250 and 500 copies/ml in replicates of 20 (Table 7).

Table 7. Sensitivity of C/Tech SARS-CoV-2 RT-PCR

Target level (Copies/ml)	Valid tested replicates	ORF1ab			N			RNase P (internal control)		
		n	Mean Ct	Detection rate (%)	n	Mean Ct	Detection rate (%)	n	Mean Ct	Detection rate (%)
500	20	20	31.6	100	20	31.8	100	20	29.3	100
250	20	20	33.1	100	19	33.0	95	20	29.4	100

Clinical evaluation

a) Individual clinical samples

The performance of C/Tech SARS-CoV-2 RT-PCR was evaluated using clinical samples. A total of 39 nasopharyngeal/oropharyngeal swabs were collected and tested to be positive for SARS-CoV-2 using EUA-approved kits. Another 50 samples were determined to be negative specimens. The set of 39 positive samples were then randomized with the 50 negative samples and subjected to a blind trial (Table 8).

Table 8. Clinical evaluation results for C/Tech SARS-CoV-2 RT-PCR

Clinical sample	Number tested	Number detected	% detection
Positive	39	39	100
Negative	50	0	0

Positive Percent Agreement: 100% (95% CI: 91.0% - 100%)
Negative Percent Agreement: 100% (95% CI: 92.9% - 100%)

b. Sample pooling

The clinical performance of C/Tech SARS-CoV-2 RT-PCR was further evaluated in pools consisting of 4 or 10 specimens. Testing included both positive and negative pools wherein the positive pools consisted of one positive specimen with the remaining specimens being negative and the negative pools consisted of only negative specimens. The positive specimens were taken from those validated in the above validation test and equal volumes of all specimens in a pool were combined prior to extraction. All specimen pools were evaluated following the C/Tech SARS-CoV-2 RT-PCR standard protocol in a randomized blind trial and the results are shown in Tables 9 and 10. The Positive Percent Agreement and Negative

Percent Agreement were calculated in relation to the individual specimen result for each evaluated pool.

Table 9. Pooled specimens test (Pool size of 4)

Pooled Test Result	Individual Specimen Result	
	Positive	Negative
Positive	30	0
Negative	0	30

Positive Percent Agreement: 100% (95% CI: 88.6% - 100%)
Negative Percent Agreement: 100% (95% CI: 88.6% - 100%)

Table 10. Pooled specimens test (Pool size of 10)

Pooled Test Result	Individual Specimen Result	
	Positive	Negative
Positive	30	0
Negative	0	30

Positive Percent Agreement: 100% (95% CI: 88.6% - 100%)
Negative Percent Agreement: 100% (95% CI: 88.6% - 100%)

Scatter plots for the Ct values of individual specimens versus the respective pooled specimens are shown in the following figures. A linear relationship is seen between most of the Ct values for both pool sizes.

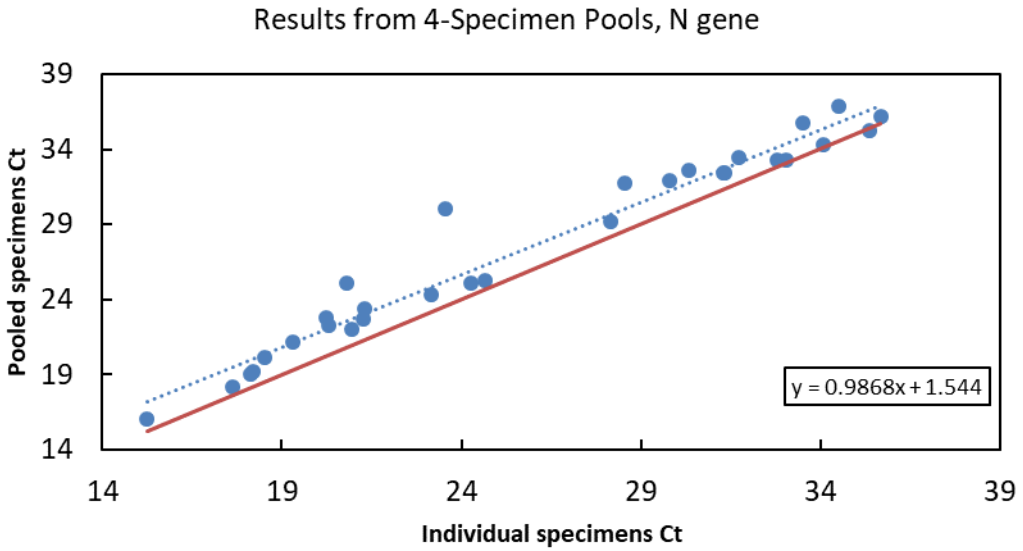

Figure 1. Ct values for individual specimens and pooled specimens for pool size of 4. The 95% confidence intervals from Passing-Bablok regression analysis for intercept and slope were (0.020, 3.050) and (0.928, 1.051), respectively.

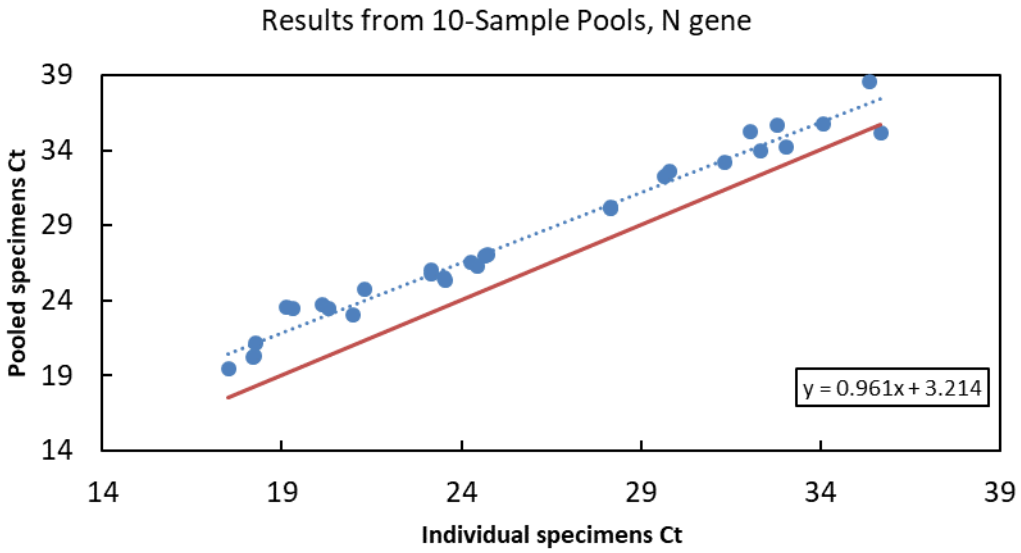

Figure 2. Ct values for individual specimens and pooled specimens for pool size of 10. The 95% confidence intervals from regression analysis for intercept and slope were (2.001, 4.917) and (0.899, 1.011), respectively.

About Hai Kang Life

Founded in 1999, Hai Kang Life Corporation Limited was established as a biotechnology company and set out to commercialize research undertaken on the molecular diagnostics of infectious diseases. It is now a recognized *in vitro* diagnostics (IVD) solutions provider. Its first innovative biotechnology product, a H5 Avian Influenza Virus Detection Kit, went on to receive international recognition for utilizing advanced molecular biological techniques to provide immediate and sensitive detection of the genetic virus that was and continues to present a threat worldwide.

Our mission is to revolutionize the practice of clinical diagnostics, providing effective platforms for point-of-care applications focused on personalized medicine and pre-emptive surveillance of emerging pathogens and diseases.

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EC REP

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