ALLSHENG

tNGS

Pathogen Targeted High-Throughput Sequencing

Infectious diseases have always been a major disease threatening human health and life in the world today. At present, the global incidence rate of infectious diseases is rising, and the pathogens are diversified and complex. Early, rapid and accurate identification of pathogenic microorganisms is the prerequisite for effective prevention and treatment of infectious diseases. Pathogen targeted high-throughput sequencing (tNGS), combined with high-multiplex PCR amplification and high-throughput sequencing technology, can simultaneously detect dozens to hundreds of known pathogenic microorganisms in samples, as well as detect the virulence and resistance genes of pathogenic microorganisms. For the detection of low concentrations of pathogenic microorganisms, especially their virulence and drug resistance genes, tNGS has the advantage of higher sensitivity and lower cost of detection, compared with pathogenic metagenomic next generation sequencing (mNGS). It also takes into account the DNA and RNA processes, and has advantages in excluding host nucleic acid interference and detecting low concentrations of pathogenic microorganisms.

Introduction

Allsheng Auto-NGS 200 is an automated NGS library preparation workstation that can achieve temperature control and precise pipetting. This workstation consists of functional blocks such as a fluorometer, robot arms, temperature control, PCR, and a computer. It can be controlled to run by programming and simulate manual operation processes for library preparation. This workstation can support up to 96 samples simultaneously. Except for the reagent preparation, which needs to be done manually, all other steps can be automatically completed in Auto-NGS 200.

Material and Method

2.1 Experimental Material

Automated NGS library preparation workstation Auto-NGS 200; tNGS reagents from company A; Qubit 1X dsDNA HS Assay Kit, main consumables include 96-well PCR plates, PCR caps, 96 square well round bottom 1.0 mL plates, 200 μL automated tips, 96-channel storage tanks, 8-strip PCR tubes (0.2 mL), 100 mL storage tanks, 96-well storage tanks, etc.

2.2 Experimental Method

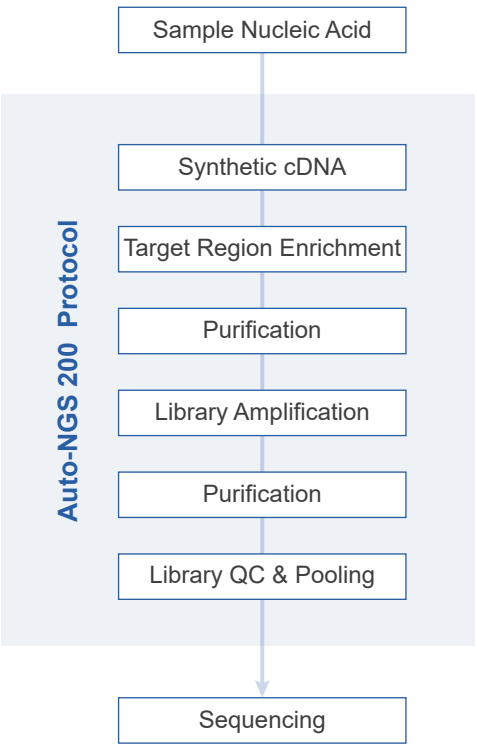


Figure 1 tNGS Experimental Process

❶ Experimental Solution Design

a. Linearity test: Dilute 10 times (high concentration) of nucleic acid extracted from a single target (H1N1 culture); dilute 100 times (medium concentration); dilute 1000 times (low concentration) test for pathogen detection. Manual and automated synchronous testing, comparing the detection differences between the two methods.

b. Detection limit test: Using the detection limit of the kit as a reference, sequence after library preparation on low concentration samples.

c. Contamination test: Arrange the samples and NTC in a plum blossom pile pattern in the consumables, and determine the experimental contamination situation based on the sequencing results.

❷ Tabletop Layout for Automated NGS Library Preparation Workstation

As shown in Figure 2, the tabletop layout of the experimental steps established according to the tNGS kit experimental process. The tabletop layout mainly consists of three parts, from left to right: the first part (A) is the tip area; the second part (B) is the reaction area, which is equipped with a cooling block, PCR, mixing block, magnet, etc. for library amplification and purification steps; the third part (C) is the fluorescence detection area, which is used for concentration determination and normalization of library preparation products.

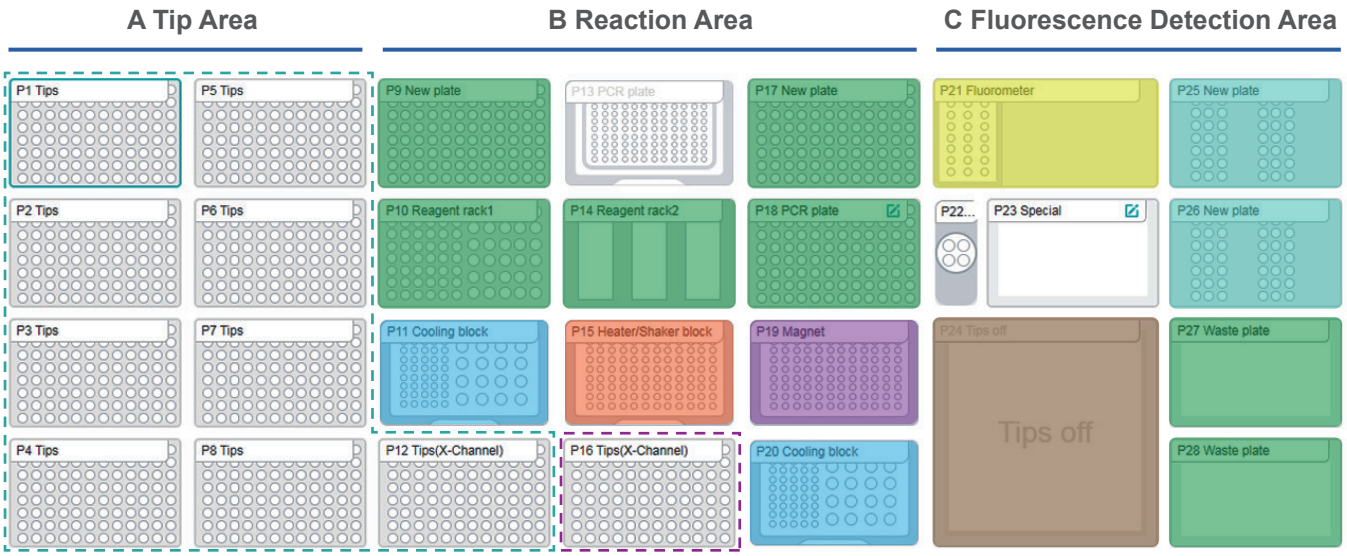


Figure 2 Auto-NGS 200 96 Samples tNGS Tabletop Layout

- P1~P8, P12 200 μL TIP holders
- P16 50 μL TIP holder
- P9, P10, P14, P17, P18, P27, P28 SBS standard tray
- P11, P20 cooling block
- P15 heater / shaker block
- P19 magnet
- P21 fluorometer
- P24 TIP off box
- P25, P26 fluorometer consumables

Result

3.1 Linearity Test

Dilute 10 times (high concentration) of nucleic acid extracted from the H1N1 culture; dilute 100 times (medium concentration); dilute 1000 times (low concentration). Transfer nucleic acids of different concentrations to PCR plates according to the form shown in Table 1. The same applies to manual library preparation, comparing the differences in detection.

Nucleic acid layout-automation	1	2	3	4	5	6	7	8	9	10	11	12
A	Low	Low	Low	Low	Low	Low	Medium	Medium	Medium	High	High	High
B	Low	Low	Low	Low	Low	Low	Medium	Medium	Medium	High	High	High
C	Low	Low	Low	Low	Low	Low	Medium	Medium	Medium	High	High	High
D	Low	Low	Low	Low	Low	Low	Medium	Medium	Medium	High	High	High
E	Low	Low	Low	Low	Low	Low	Medium	Medium	Medium	High	High	High
F	Low	Low	Low	Low	Low	Low	Medium	Medium	Medium	High	High	High
G	Low	Low	Low	Low	Low	Low	Medium	Medium	Medium	High	High	High
H	Low	Low	Low	Low	Low	Low	Medium	Medium	NTC1	Medium	Medium	NTC2

Table 1 Layout of Nucleic Acid Samples

1 Library Concentration and Fragment Analysis

The workstation running program is established according to the kit instructions. The library concentration is detected in Auto-NGS 200 built-in fluorometer after the library output, as shown in Figure 3, the library concentration of Auto-NGS 200 is slightly higher than that of the manual library. There is no significant difference between the CV (coefficient of variation) of the automated library and that of the manual library (P<0.05).

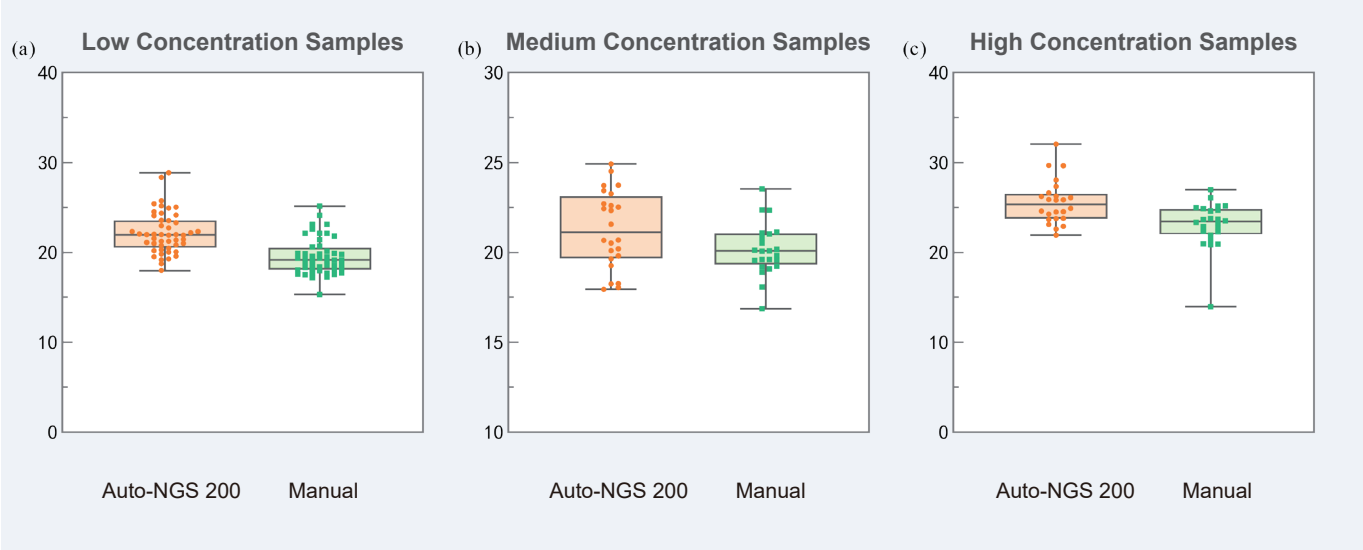


Figure 3 Library Concentration

Randomly select 4 samples of library products for library fragment analysis in the Agilent 4150 TapeStation system, and compare them with manually library preparation products. The fragment results are shown in Figure 4, and the fragment length and average fragment size are similar to the results of manual library preparation.

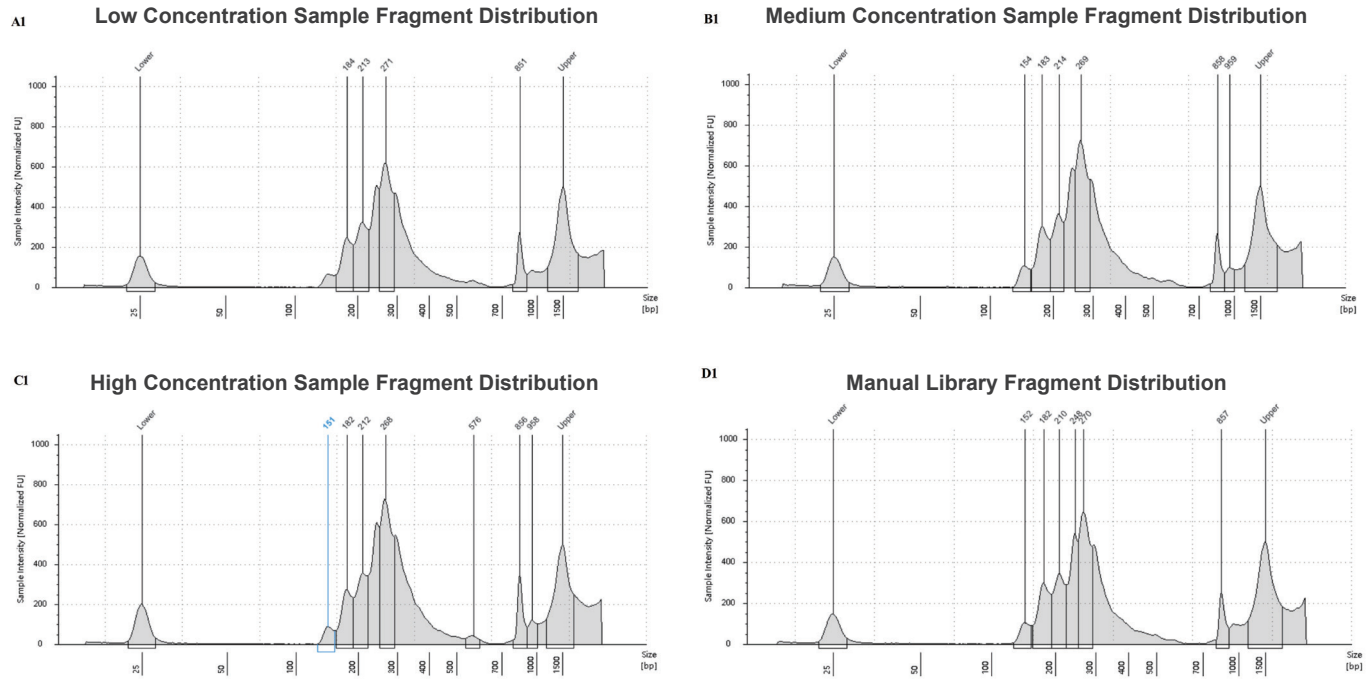


Figure 4 Library Fragment Length

❷ Sequencing Result

The successfully constructed library is pooled in Auto-NGS 200 with 96 mix 1, and the mass of each sample is 60 ng for normalization and then prepared for sequencing. Sequencing is done in MiniSeq System platform, and the sequencing results are shown in Figure 5.

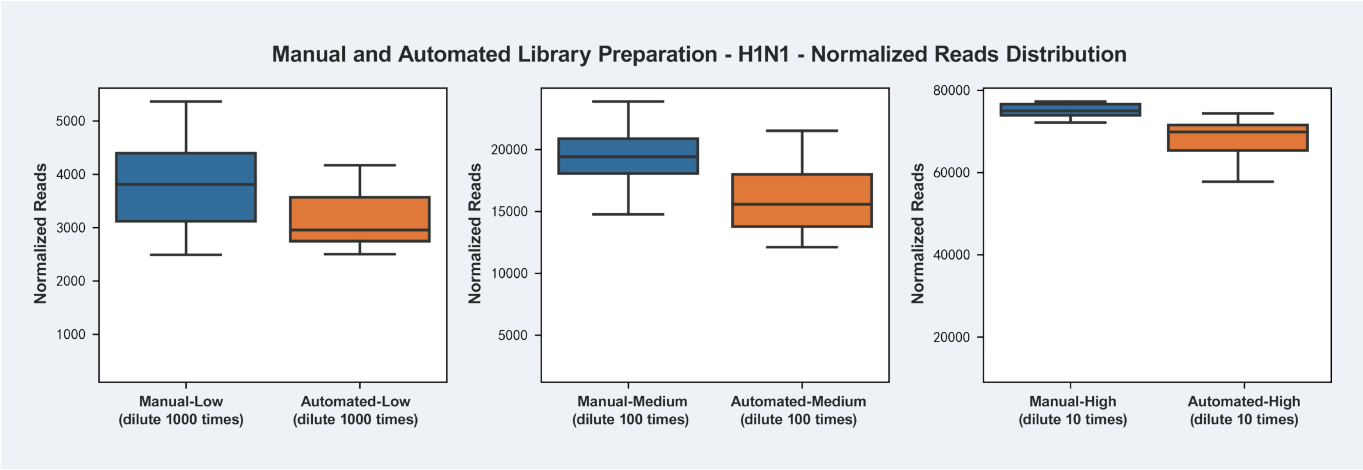


Figure 5 Normalized Reads Distribution for Auto-NGS 200 Library Preparation and Manual Library Preparation

3.2 Detection Limit Test

Experimental solution: Select two types of DNA viruses, RNA viruses, bacteria, and fungi, for a total of 8 samples. Mix nucleic acids of the same sample and concentration into one tube. According to the amount of nucleic acid, divide into 14 μl per well. Use manual-automated library preparation for the detection limit nucleic acid and compare the detection differences between the two methods. Transfer the nucleic acid products from cultures of DNA virus 1 (4000), RNA virus 1 (1000), DNA virus 2 (2000), RNA virus 2 (500), fungus 1 (10000), bacterium 1 (10000), fungus 2 (10000), and bacterium 2 (15000) into PCR plates according to the form in Table 2. The same applies to manual library preparation, comparing whether the sequencing results can be detected.

Automated library preparation layout	1	2	3	4	5	6	7	8	9	10	11	12
A	DNA virus 1 4*10^3	DNA virus 2 2*10^3	DNA virus 2 2*10^3	Fungus 1 10^4	Fungus 1 10^4	Bacterium 1 10^4	Bacterium 1 10^4	Bacterium 2 1.5*10^4	Bacterium 2 1.5*10^4	Bacterium 2 1.5*10^4	Fungus 2 10^4	Fungus 2 10^4
B	DNA virus 1 4*10^3	DNA virus 2 2*10^3	DNA virus 2 2*10^3	Fungus 1 10^4	Fungus 1 10^4	Bacterium 1 10^4	Bacterium 1 10^4	Bacterium 2 1.5*10^4	Bacterium 2 1.5*10^4	Bacterium 2 1.5*10^4	Fungus 2 10^4	Fungus 2 10^4
C	DNA virus 1 4*10^3	DNA virus 2 2*10^3	RNA virus 2 500	Fungus 1 10^4	Fungus 1 10^4	Bacterium 1 10^4	Bacterium 1 10^4	Bacterium 2 1.5*10^4	Bacterium 2 1.5*10^4	Bacterium 2 1.5*10^4	Fungus 2 10^4	Fungus 2 10^4
D	RNA virus 1 1000	DNA virus 2 2*10^3	RNA virus 2 500	Fungus 1 10^4	Fungus 1 10^4	Bacterium 1 10^4	Bacterium 1 10^4	Bacterium 2 1.5*10^4	Bacterium 2 1.5*10^4	Fungus 2 10^4	Fungus 2 10^4	Fungus 2 10^4
E	RNA virus 1 1000	DNA virus 2 2*10^3	RNA virus 2 500	Fungus 1 10^4	Fungus 1 10^4	Bacterium 1 10^4	Bacterium 1 10^4	Bacterium 2 1.5*10^4	Bacterium 2 1.5*10^4	Fungus 2 10^4	Fungus 2 10^4	Fungus 2 10^4
F	RNA virus 1 1000	DNA virus 2 2*10^3	RNA virus 2 500	Fungus 1 10^4	Fungus 1 10^4	Bacterium 1 10^4	Bacterium 1 10^4	Bacterium 2 1.5*10^4	Bacterium 2 1.5*10^4	Fungus 2 10^4	Fungus 2 10^4	Fungus 2 10^4
G	DNA virus 2 2*10^3	DNA virus 2 2*10^3	RNA virus 2 500	Fungus 1 10^4	Fungus 1 10^4	Bacterium 1 10^4	Bacterium 1 10^4	Bacterium 2 1.5*10^4	Bacterium 2 1.5*10^4	Fungus 2 10^4	Fungus 2 10^4	NTC1
H	DNA virus 2 2*10^3	DNA virus 2 2*10^3	RNA virus 2 500	Fungus 1 10^4	Fungus 1 10^4	Bacterium 1 10^4	Bacterium 1 10^4	Bacterium 2 1.5*10^4	Bacterium 2 1.5*10^4	Fungus 2 10^4	Fungus 2 10^4	NTC2

Note: The number below the sample represents the nucleic acid concentration of the bacteria / fungi / virus, in ng/μL.

Table 2 Nucleic Acid Layout of Auto-NGS 200 Library Preparation

❶ Library Concentration

The library concentration detection method is the same as 3.1.1, as shown in Figure 6. The library concentration of Auto-NGS 200 is slightly higher than that of the manual library preparation.

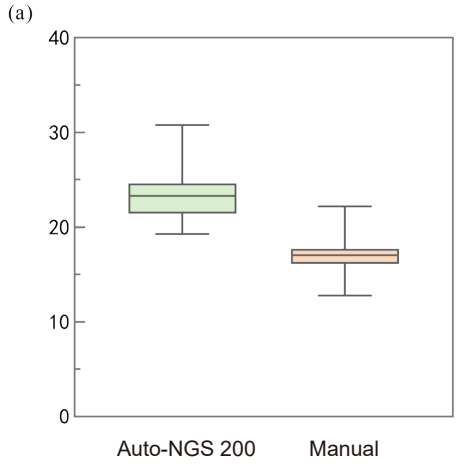


Figure 6 Detection Limit Concentration for Auto-NGS 200 and Manual Library Preparation

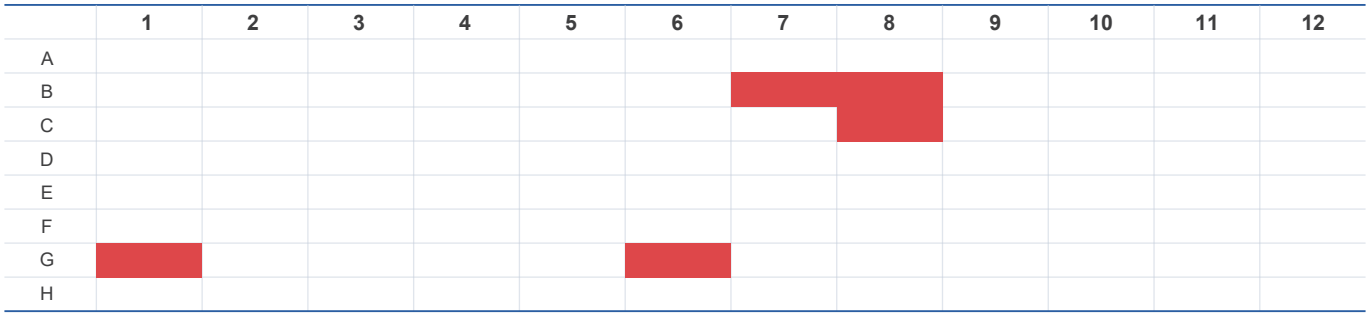
❷ Sequencing Result

Cultures given in the table	Detection limit analysis (>=20) considered detected: detected quantity / total number of samples	Automated / manual efficiency
RNA virus 1 (1000)	1000 can be detected (3/3)	0.87
DNA virus 2 (2000)	2000 manual (12/12), automated (11/12), with missed detection in automation	0.82
RNA virus 2 (500)	500 manual (3/6), automated (4/6), all missed detections	0.74
Fungus 1 (10000)	10000 can be detected (16/16)	0.79
Bacterium 1 (10000)	10000 can be detected (16/16)	0.84
Fungus 2 (10000)	10000 can be detected (19/19)	0.91
Bacterium 2 (15000)	15000 can be detected (19/19)	0.88
DNA virus 1 (4000)	4000 can be detected (3/3)	0.90

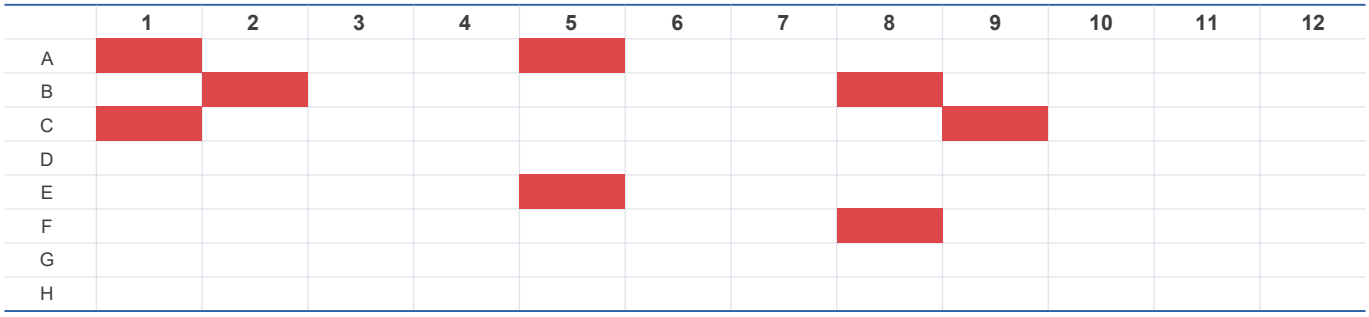
Table 3 Detection Results of Automated and Manual Library Preparation

3.3 Contamination Test

As shown in Figure 7, arrange the samples and NTC in a plum blossom pile pattern in 96-well PCR plates, and determine the experimental contamination by normalizing the reads value of NTC. Manual and instrument synchronization. Experimental environment: Working in Auto-NGS 200 standard negative pressure laboratory; the manual library preparation is completed in the clean bench.



(a) Contamination Situation of Manual Library Preparation



(b) Contamination Situation of Automated Library Preparation

Figure 7 Contamination Situation

Summary

4.1 Linearity Test Conclusion

According to the linearity test sequencing data, the final normalized reads value corresponds to the gradient of sample concentration. The library concentration of Auto-NGS 200 is slightly higher than that of manual library preparation, and there is no significant difference between the CV (coefficient of variation) of the automated library and that of the manual library. The normalized reads value of Auto-NGS 200 for H1N1 is about 80-85% of that of manual library preparation; the library concentration of instrument is slightly higher than that of manual concentration, especially when the dilution gradient is 10; the repeatability of manual library preparation is not as good as that of instrument library preparation, and the coefficient of variation (CV) is relatively high.

4.2 Detection Limit Test Conclusion

According to the culture types in the table, it is considered that a normalized reads value ≥20 indicates detection, as shown in Table 1. The automated library preparation of DNA virus 2 has missed detections, and RNA virus 2 has also missed detections. Other pathogen detection situation: there was some contamination in this experiment, and the samples detected more than just the target pathogen in the culture. According to the pathogen detection spectrum analysis, some pathogens with consistent spectra were also included in the statistics, mainly including rhinovirus, influenza B virus, Burkholderia cepacia complex and other contaminants.

4.3 Contamination Test Conclusion

The overall contamination of automated library preparation is not much different from that of manual library preparation. Automated library preparation is mainly contaminated by pathogens such as Candida albicans and onions, while manual library preparation is mainly contaminated by rhinovirus and nontuberculosis mycobacteria, etc. T2P2-ZDH-NTC-47 sample is heavily contaminated; from the layout, manual NTC has almost no H1N1 contamination, while automated NTC has almost no H1N1 contamination.

NTC detection spectrum: rhinovirus, Escherichia coli, Candida albicans detections of the highest frequency, and in the layout of the wider distribution, suspected that the laboratory environment with these pathogens contamination; the contamination of rhinovirus, Haemophilus influenzae, Fusobacterium nucleatum in manually library preparation has significantly increased.

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