राष्ट्रीय पशु जैव प्रौद्योगिकी संस्थान (एन आई ऐ बी)
(जैव प्रौद्योगिकी विभाग, विज्ञान एवं प्रौद्योगिकी मंत्रालय, भारत सरकार का स्वायत्त संस्थान)
National Institute of Animal Biotechnology
(An Autonomous Institute of the Department of Biotechnology, Ministry of Science & Technology, Govt. of India)
NIAB National Institute of Animal Biotechnology

October 28, 2021

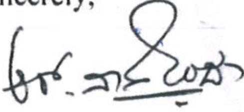
Dr. Rachana Tripathi
CEO
Huwel Lifesciences Pvt. Ltd.
Hyderabad

Dear Dr. Tripathi,

I am hereby enclosing the report of the comparison of your viral nucleic acid extraction kit against another, commercially availed kit, as well as your molecular transport medium.

Basically, in a series of experiments, we compared the performance of your kits in extracting DNA from laboratory preparations of adenovirus, a DNA virus, and further in the detection and quantification of the viral DNA, to a commercial kit. The results show (a) that the transport medium is likely to inactivate all biological material in a very short period of time, and (b) that your kits were non-inferior to the commercial kit for nucleic acid extraction as well as quantifiable limits of detection of a DNA virus through real-time PCR.

Sincerely,



(Nagendra Hegde)
Scientist – H

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Comparative study to determine the efficiency of viral nucleic acid extraction kits and validation of molecular transport medium for virus inactivation

Introduction

The analysis of biological samples for various nucleic acid-based diagnostic purposes requires the isolation of genetic material. Commercially, different kits are available for the extraction of DNA and RNA. Occasionally, for a sample with unidentified pathogen, or a sample with limited volume/ availability it becomes critical to extract genome of high purity for downstream applications. In such cases, the use of a single kit that allows extraction of both DNA and RNA is cost and time effective, especially in a diagnostic setting. Here we have compared the Huwel nucleic acid extraction kit with the commercially available Nucleospin® virus from Macherey-Nagel (MN). We have also tested the inactivation of the virus in Huwel's molecular transport medium (MTM).

Materials and methods

Cells

Human embryonic kidney (HEK)-293IQ cells (Microbix Biosystems Inc., Canada) were grown in minimum essential medium (Thermo Fisher Scientific, Cat no: 41500-034) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Cat no: 10270-106) in the presence of 10,000 units/ml of penicillin and 10,000 µg/ml of streptomycin and 25 µg/ml of Amphotericin B (Antibiotic-Antimycotic) (Thermo Fisher Scientific, Cat no: 15240-062).

Virus

Recombinant human adenovirus 5 (Ad5) generated and available as a part of an ongoing project was propagated in 293IQ cells to prepare a virus stock. The virus was titrated in 293IQ cells and $TCID_{50}$/ml was determined as per standard protocols and using the Reed and Muench method.

Nucleic acid extraction

The adenoviral DNA was extracted using the nucleic acid extraction kits from Huwel Lifesciences and Macherey-Nagel (MN) according to the manufacturers' recommendations. Brief step-wise protocols are shown in Fig 1.

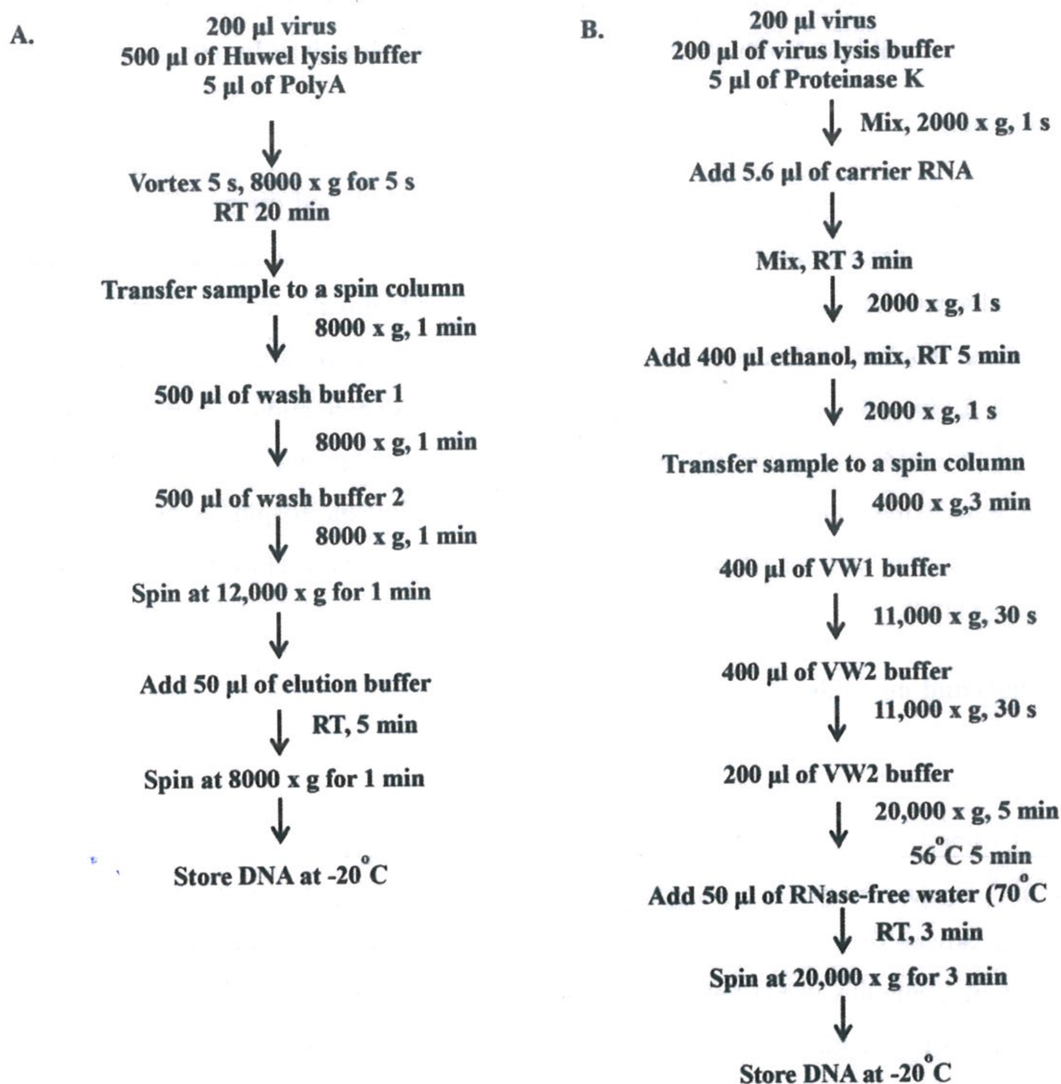

Fig 1. The steps involved in nucleic extraction using the kit from Huwel Lifesciences (A) and Macherey-Nagel (B) are shown. In both methods, the spin column was transferred to a fresh collection tube at each step. DNA was eluted in a fresh microfuge tube.

Quantitative real-time PCR

To quantify the viral DNA extracted using the kits, a qRT-PCR assay was performed using the Quantiplus® ADENO RT-PCR kit. Briefly, 10 µl of the extracted DNA (neat or diluted as needed) or provided DNA standards or nuclease-free water were used as templates separately in

reaction mixtures containing 15 µl of Huwel ADENO ready mix and 1 µl of Huwel IC-A (internal control-A) mix. The cycling conditions are shown in Table 1. The plates were read in FAM and VIC channels.

Limit of detection

The DNA extracted using the kits and the number of gene copies was determined. The DNA was the serially diluted 10-fold and then used as a template for qPCR.

Table 1. Cycling conditions for qPCR

Steps	**No. of cycles**	**Temperature (°C)**	**Time**
Initial denaturation	1	95	15 min
PCR cycle 1	10	90	30 sec
		54	30 sec
		60	30 sec
PCR cycle 2	30	90	30 sec
		56	30 sec
		60	60 sec

Virus inactivation assay

To test inactivation of the virus in molecular transport medium (MTM), 0.5 ml of Ad5 virus was incubated in 1.5 ml of MTM or MEM for 10, 20 and 30 min and then added to 293IQ cells and incubated for 18 h. The next day the cells were observed for cytopathic changes. As a control, 0.5 ml of the virus was directly added on to 293IQ cells. The next day and on every 3rd day subsequently, the medium was changed, and the flasks were examined for presence of cells till day 7.

Results and discussion

The Ad5 viral DNA extracted using the kits from Huwel Lifesciences and MN was subjected to qRT-PCR to test the efficiency of extraction. Post the qPCR run, the data was first analyzed in VIC channel to ensure amplification of the IC in all the samples. Next, the amplification of Ad5

gene was assessed using FAM channel. The slope of standards and the efficiency were ensured to be ranging from -3.1 to -3.6 and 90 to 110% respectively. In the negative control samples, where nuclease-free water was used as template, absence of amplification was ensured. The resulting data was then used to determine the copies of Ad5 virus genome.

Table 2.

S. No.	**Sample**	**Copies/ml**	**Average log copies/ml**
1.	H1	5.003E+08	8.854
2.	H2	9.488E+08	
3.	H3	5.186E+08	
4.	H4	8.401E+08	
5.	H5	8.086E+08	
6.	H6	6.742E+08	
7.	M1	3.799E+08	8.859
8.	M2	7.190E+08	
9.	M3	8.436E+08	
10.	M4	7.897E+08	
11.	M5	9.134E+08	
12.	M6	7.811E+08	

H1- H6 denote the 6 replicates of DNA samples extracted using the nucleic acid extraction kit from Huwel and M1-M6 denote the 6 replicates of DNA samples extracted using the MN kit.

The following formula was used to calculate the no. of copies/ml.

$$\text{Copies/mL} = \frac{\text{Copies/}\mu\text{l x Elution volume (}\mu\text{l)}}{\text{Sample volume (mL)}}$$

We found that the copies of Ad5 extracted using the kits were comparable.

For the limit of detection assay, DNA was freshly extracted from Ad5 using kits from Huwel and MN and the number of DNA copies were determined. The DNA was then serially diluted 10-fold

from 10^{-1} to 10^{-8}. From the diluted DNA, 10 μl was used as template for qPCR. Duplicate of each sample was used. The data is summarized in Tables 3.1 and 3.2.

Table 3.1

S. No.	Sample	Average copies/ml	Average log copies/ml
1.	H	9.97E+08	7.999
2.	M	2.70E+08	8.431

The copies of Ad5 genome in undiluted DNA are shown.

Table 3.2

S. No.	Sample	Average copies/ml	Average log copies/ml
1.	H 10^{-1}	4.1697E+03	3.6201
2.	H 10^{-2}	7.3681E+02	2.8673
3.	H 10^{-3}	6.4664E+01	1.8106
4.	H 10^{-4}	2.06330E+00	0.3145
5.	H 10^{-5}	8.3662E-01	-0.0774
6.	H 10^{-6}	0	-
7.	H 10^{-7}	0	-
8.	H 10^{-8}	0	-
9.	M 10^{-1}	9.2774 E+03	3.9674
10.	M 10^{-2}	1.6289 E+03	3.2119
11.	M 10^{-3}	1.853 E+02	2.2679
12.	M 10^{-4}	2.199 E+01	1.3422
13.	M 10^{-5}	7.047 E-01	-0.1519
14.	M 10^{-6}	0	-
15.	M 10^{-7}	0	-
16.	M 10^{-8}	0	-

The copies of Ad5 genome in serial dilutions are shown. The values shown are average of two replicate samples.

From our limit of detection assay, the lowest Ad5 genome copy detectable within a linear range was 2 copies/ml.

For the virus inactivation assay, the control cells showed normal morphology whereas, cytopathic changes were observed in cells where Ad5 alone or Ad5 incubated in MEM was added. We noted immediate lysis of the cells when virus incubated in MTM was added to the cells (Fig. 1). No cells could be recovered even after one week of culturing.

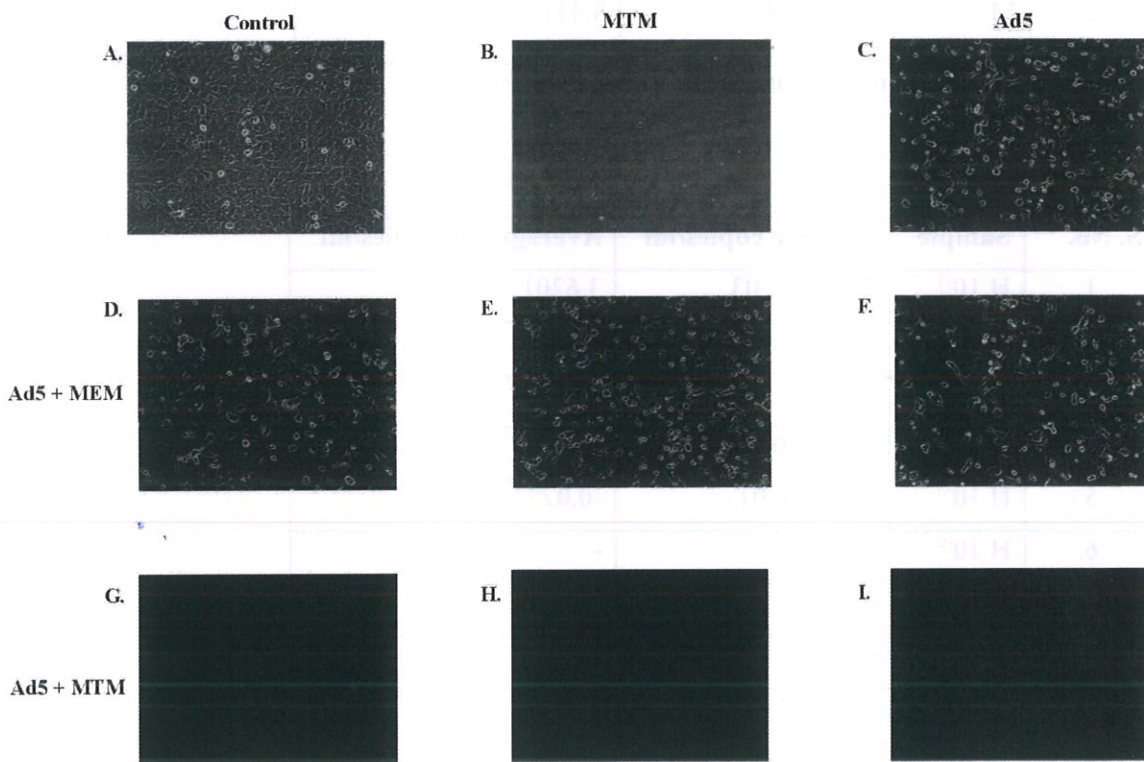

Fig 1. The effect of MTM on Ad5 and 293 cells is shown. **(A)** Untreated 293IQ cells. **(B)** Cells treated with MTM. **(C)** Cells infected with Ad5. Panels D, E and F show 293IQ cells treated with Ad5 incubated with MEM for 10, 20 and 30 min respectively. Panels G, H and I show 293IQ cells treated with Ad5 incubated with MTM for 10, 20 and 30 min respectively.

Conclusion

Our experimental studies show that the DNA extraction performed using the Huwel Nucleic Acid Extraction kit was comparable to extraction using the Nucleospin® virus kit from MN. The

difference in the copy number observed could be due to the slight variation in the pipetting of sample or presence of virus trapped in cell debris (a centrifugation step was included to pellet down any visible debris prior to aliquoting the virus for extraction procedure). Using the Quantiplus® ADENO RT-PCR kit we were able to detect up to 2 copies/ml of the Ad5 DNA. The molecular transport medium was found to be highly detrimental to the viability of cells since cell lysis was observed immediately upon addition on to cells.

References

1. Bustin *et al*. MIQE précis: Practical implementation of minimum standard guidelines for fluorescence based quantitative real-time PCR experiments. BMC Molecular Biology 2010, 11:74.