Application Note

Evaluation of Genomic DNA isolation with AcroPrep™ Advance 96-Well Long Tip Filter Plate for Nucleic Acid Binding

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1. Introduction

The advent of next generation sequencing techniques has enabled collection of unprecedented amounts of sequencing data in short spans of time. This has greatly contributed to our understanding of genomic and gene structure across many species and has also allowed us to gain much information about candidate genes for genetic disorders, cancer causing somatic mutations, and misexpression due to epigenetic changes. Validation of candidate genes can take place by genetically engineered disease models created in mammalian cell culture or whole animal models.

More of the workflows involved in these studies are carried out in high-throughput fashion aided by the use of multi-well DNA binding filter plates that allow larger numbers of genomic DNA samples to be obtained in parallel.

Here we demonstrate the use of Pall AcroPrep Advance 96-Well Long Tip Filter Plates for Nucleic Acid Binding (Pall NAB plate) for the isolation of high quality genomic DNA from human embryonic kidney cell line HEK293 (HEK293) and Chinese hamster ovary cell line CHO (CHO). As researchers may store cultured cell samples frozen prior to isolating and purifying gDNA, experiments were carried out with freshly prepared cells and with cells that were stored frozen prior to gDNA isolation. Use of frozen cells resulted in a reduced DNA isolation efficiency from frozen samples and increased occurrence of clogged wells, especially at higher cell densities, with both Pall NAB plates and commercially available plates. We present protocol suggestions that reduce clogging and improve genomic DNA isolation efficiency from frozen or fresh cells. Genomic DNA isolated with the Pall NAB plates using the presented protocol is suitable for next generation sequencing applications.

2. Materials and Methods

2.1 Centrifugation

Except where noted, all centrifugation steps were carried out in an Allegra◆ X-22R centrifuge (Beckman Coulter) with the chamber temperature set at room temperature (21 °C) using the S2096 rotor for plates and the SX4250 rotor for all other centrifugations.

2.2 Cell Culture

Human HEK293 embryonic kidney cells (ATCC◆ CRL-1573™) were grown in Corning◆ CellBIND◆ Surface 175 cm^2 cell culture flasks or CellSTACK◆ cell culture chambers (Sigma-Aldrich) in high glucose Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific), and were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were harvested using TrypLE◆ Select dissociation reagent (Thermo Fisher Scientific)◆, centrifuged in a Sorvall◆ ST 40 Centrifuge with a TX-750 swinging bucket rotor (Thermo Fisher Scientific) at 700 x g, and resuspended in complete media.

CHO-S suspension Cells (Thermo Fisher Scientific) were maintained in CD FortiCHO◆ medium (Thermo Fisher Scientific) with added GlutaMAX-1 (Thermo Fisher Scientific), HT supplement (Thermo Fisher Scientific), and Puromycin (Thermo Fisher Scientific) in VWR◆ sterile polycarbonate Erlenmeyer flasks (VWR) on an orbital shaker platform rotating at 135 rpm in a humidified atmosphere of 8% CO_2 in air.

Cell concentration and viability were determined using a Vi-CELL◆ XR Cell Counter (Beckman Coulter). After appropriate dilution in PBS, cells were transferred to a MASTERBLOCK◆ 96-well polypropylene storage plate with 1 mL well volume (Greiner Bio-One) at densities ranging from 0.5 to 5 x 10^6 cells/well. Cells then were pelleted by centrifugation for 5 min at 300 x g, after which the supernatant was removed by careful aspiration. Cells were used fresh or frozen after storage at -20 °C.

2.3 Genomic DNA Purification

HEK293 cell pellets (fresh or frozen) were resuspended in 200 µL/well PBS after which 20 µL/well proteinase K and 200 µL/well lysis Buffer AL (Qiagen◆) were added. The plate was covered with Axygen◆ AxySeal PCR-SP-S sealing film (Corning). The closed-cell polyethylene foam packaging pad that comes with the Pall NAB plates was placed on top of the sealing film, followed by an inverted empty 96-well plate. The stacked plates were then vigorously shaken up and down for 15 s to obtain a homogeneous lysate. The lysate was collected at the bottom of wells by a brief centrifugation pulse at 1,100 x g. Incubation for 10 min at 56 °C in a water bath followed to allow Proteinase K digestion. During this period, the plates were occasionally shaken to mix the samples.

Prior to loading the samples on the filter plate, sample viscosity was reduced through addition to each sample of either 200 µL/well 96 – 100% ethanol (Lysate Mixture 1) or 400 µL of a 1:1 mixture of buffer AL and 96 – 100% ethanol (Lysate Mixture 2). Mixing and sample collection were accomplished as described above after which the samples were transferred to a 96-well filter plate (Pall NAB plate or commercial as indicated). Except where expressly noted, this process was carried out expeditiously following the Proteinase K digestion allowing the storage plate holding the samples to remain warm to the touch up until transfer of samples to the Pall NAB plate. In the “cold” protocol, the plate was allowed to cool to room temperature by leaving it on the bench for an extended period of time.

Genomic DNA was allowed to bind to the filter plate media through vacuum filtration at 67 kPa (20 in Hg) in a plate manifold (Pall), and filtrate was captured in a 2.2 mL deep-well plate (VWR). The top of the filter plate was covered with a sealing film backing (shiny side down) to maintain constant vacuum pressure by closing off empty wells. In instances where not all wells emptied completely, the filter plate, sealed with an AirPore Tape Sheet (Qiagen), was placed on a MASTERBLOCK 96-well polypropylene storage plate with 0.5 mL well volume (Greiner Bio-One) and centrifuged for four minutes at up to 1,500 x g.

Two successive washes with 500 µL/well volumes were carried out to remove unwanted contaminants. Buffer AW1 (Qiagen) was used for the first wash and buffer AW2 (Qiagen) for the second. Wash buffer was removed by vacuum filtration at 67 kPa (20 in Hg) or by two minutes centrifugation at up to 1,500 x g. Prior to elution, the plate was dried to completion by centrifugation for 15 minutes at up to 1,500 x g. Elution of genomic DNA into a MASTERBLOCK 96-well polypropylene storage plate with 0.5 mL well volume (Greiner Bio-One) was accomplished through addition of 200 µL/well of Buffer AE preheated to 60 °C, followed by a 1 min incubation at room temperature (15 – 25 °C) and centrifugation at up to 1,500 x g for 4 min.

2.3 Evaluation of gDNA samples

2.3.1 Concentration, Yield and Quality

Genomic DNA concentration and yield were determined fluorimetrically by the Invitrogen◆ Quant-iT◆ PicoGreen◆ dsDNA Assay Kit (Thermo Fischer Scientific).

2.3.2 Next Generation Sequencing

Whole exome sequencing (Standard Human WES Express) was performed on two gDNA samples. The process utilized transposase-based sample preparation followed by hybrid capture using a 38Mb whole exome design. Sequencing took place on the Illumina HiSeq 4000 system with paired 76 bp reads. Prior to library construction, suitability of the samples for library construction was evaluated by real-time qPCR using the KAPA hgDNA Quantification and QC kit according to manufacturer's protocol. The results were used to generate a Q score indicative of gDNA quality and suitability for preparation of next generation sequencing libraries.

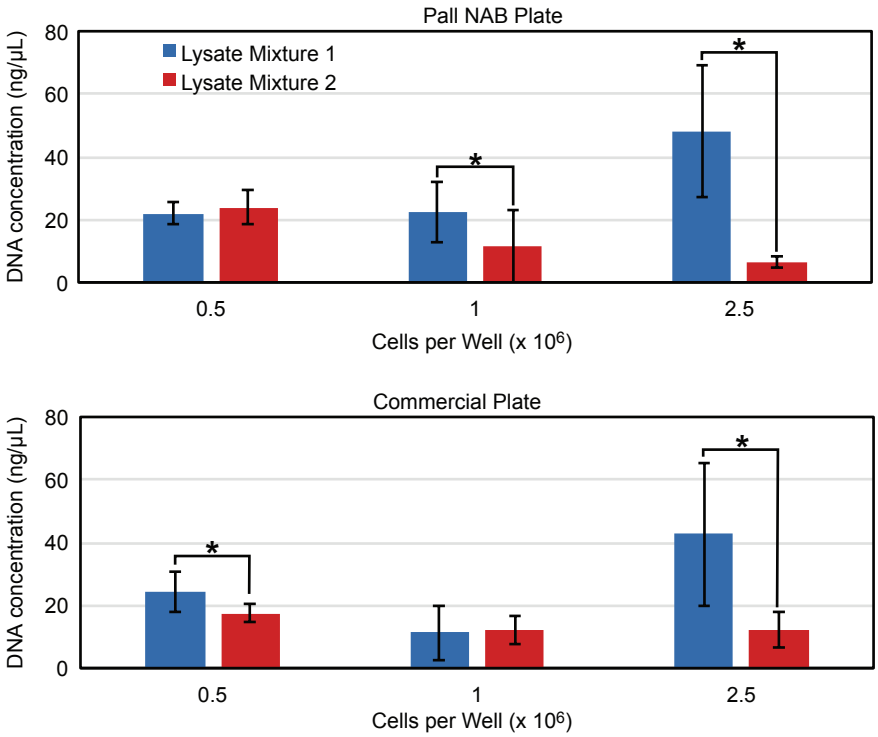
3. Results and Discussion

3.1 Genomic DNA Isolation from frozen cells

Genomic DNA was isolated from cultured HEK293 cells frozen prior to use at cell densities ranging from 0.5 to 5 x 10^6 cells/well (Figure 1). Prior to loading the lysates on the Pall NAB plate (left panel) or a commercially available plate (right panel) either ethanol alone (Lysate Mixture 1; blue bars) or an equal volume to volume mixture of ethanol and lysis buffer (Lysate Mixture 2; red bars) was added to the lysates to reduce sample viscosity. Although in all cases cell lysis appeared complete as judged by the clarity of the lysates, wells of both plate types clogged either directly upon filtration of the lysates or during subsequent washes, especially at higher cell densities. Attempts to rescue genomic DNA collection from the obstructed wells through filtration by centrifugation up to 1,500 x g instead of filtration by vacuum were only partially successful. Genomic DNA isolation yield proved highly variable for the frozen samples with both plates, and no genomic DNA was obtained from HEK293 cells at a density of 5 x 10^6 cells/well with both plate types (data not shown). With both plate types, genomic DNA yields with Lysate Mixture 2 were significantly lower than those obtained with Lysate Mixture 1 at the highest cell density and where indicated (see Figure 1). Elution in these experiments took place with elution buffer at room temperature.

Figure 1

Genomic DNA Isolation from frozen HEK293 Cells



Genomic DNA was isolated from frozen HEK293 cells with the Pall NAB plate (top panel) and a commercially available plate (bottom panel) using two different lysate mixtures (Lysate Mixtures 1 and 2, blue and red bars, respectively). Genomic DNA Isolation proved highly variable and failed at 5 x 10^6 cells per well with both plate types (data not shown). Bars indicate an average of 12 samples. Error bars indicate standard deviation. Stars indicate significant differences between treatment groups as determined by t-test with a P value < 0.05.

3.1.2 Genomic DNA Isolation from fresh and frozen cells with improved yields

In subsequent experiments, the following procedural modifications were implemented with the aim of improving genomic DNA efficiency at higher cell densities:

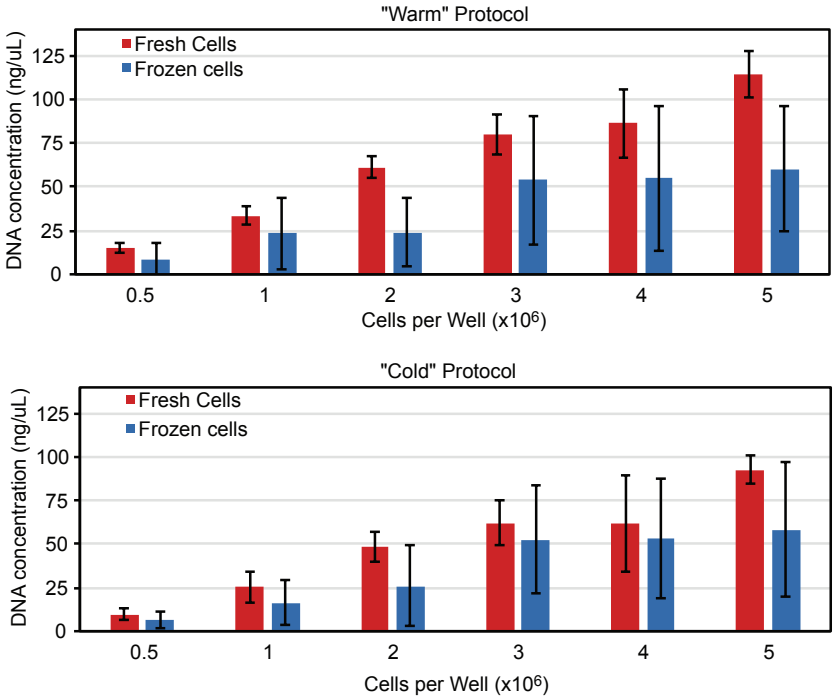
a) Vacuum filtration efficiency was improved by covering the top of the filter plate with a sealing film backing (shiny side down) to reduce loss of vacuum through empty wells thereby better maintaining vacuum pressure in wells that need to empty.

b) Elution was improved by preheating the elution buffer to 60 °C.

Figure 2 shows the results from an experiment in which genomic DNA was isolated from frozen (blue bars) and freshly harvested (red bars) HEK293 cells. In addition, the influence of the sample temperature during loading on the filter plate was evaluated. This was accomplished by either completing the isolation procedure expeditiously (“Warm” Protocol; top panel) upon completion of the Proteinase K treatment at 56 °C or by allowing the plates to cool to room temperature instead (“Cold” Protocol; bottom panel).

Figure 2

Genomic DNA isolation from frozen (blue bars) and freshly harvested (red bars) HEK293.



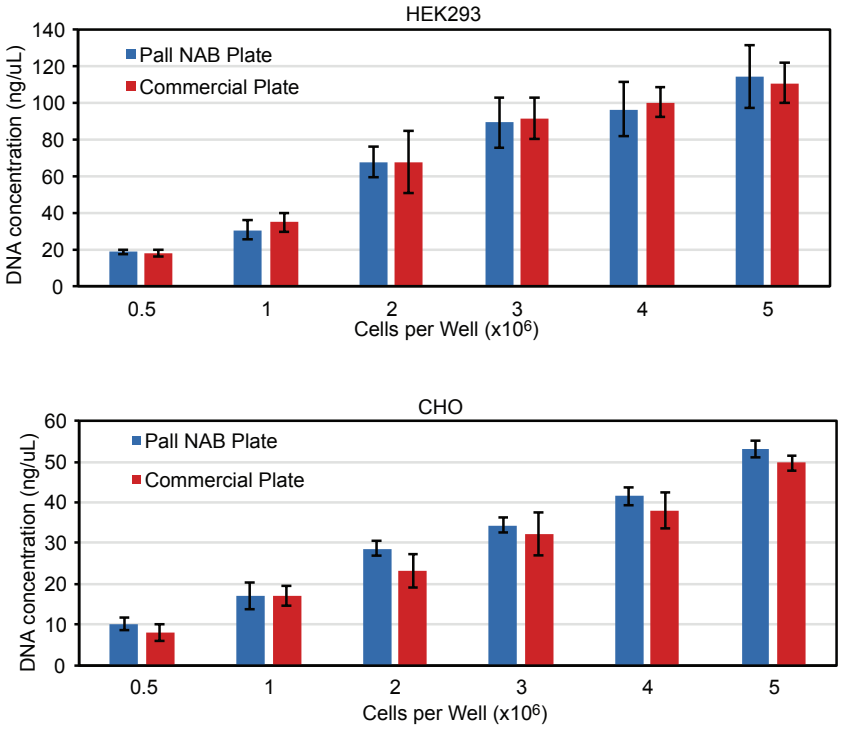
Upon completion of the Proteinase K treatment at 56 °C, plates were allowed to cool to room temperature ("Cold" Protocol; bottom panel) or the isolation procedure was completed expeditiously ("Warm" Protocol; top panel). Use of frozen cells severely impacts gDNA concentration and yield and does not benefit from expeditious processing as observed with fresh cells. Bars indicate an average of 8 samples. Error bars indicate standard deviation.

The sample temperature greatly influenced the gDNA isolation efficiency. Expeditious completion of the isolation procedure allowed the lysate containing plates to remain warm to the touch. When using fresh cells as substrate, expeditious completion of the isolation procedures ("warm" protocol) improved genomic DNA isolation. In contrast, a severely reduced gDNA concentration and yield was observed under both conditions when using frozen cells without discernable improvement when following the "warm" protocol. Altogether, the implemented protocol modifications did allow gDNA isolation at cell densities of up to 5 x 10^6 cells/well for both frozen and fresh cells.

Figure 3 shows results from genomic DNA isolation experiments with fresh HEK293 cells and CHO cells. The DNA isolation efficiency with the Pall NAB plate (blue bars) and a commercially available plate (red bars) were near identical and results proved highly repeatable (data not shown). The lower yield observed with the CHO cells compared to the HEK293 cells correlates with the ploidity of the cell lines. Whereas the CHO cell line misses the full chromosomal complement, the HEK293 cell line is pseudotriploid, and a given HEK293 cell therefore is expected to have a higher genomic DNA content than a given CHO cell.

Figure 3

Genomic DNA isolation from freshly harvested HEK293 and CHO cells.



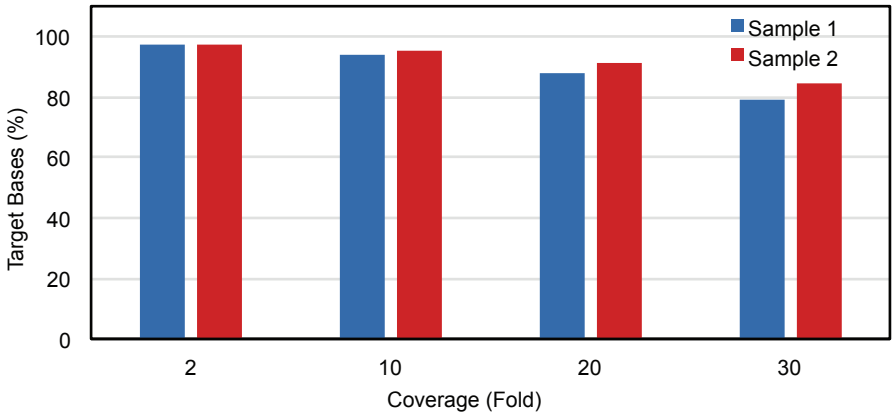
Genomic DNA isolation from freshly harvested HEK293 and CHO cells (top and bottom panels, respectively) with Lysate Mixture 1 is very similar for the Pall NAB plate (blue bars) and the commercial plate (red bars). Bars indicate an average of 8 samples. Error bars indicate standard deviation.

3.1.3 QPCR Analysis and Next Generation Sequencing

Two HEK293 cell genomic DNA samples were subjected to whole exome sequencing. Prior to sequencing, a quality assessment of the samples prior to NGS library construction was performed in a real-time QPCR assay using the KAPA hgDNA Quantification and QC Kit according to manufacturer's protocol. The ratio between two amplification products of increasing size was used to generate a Q score between 0 (poor) and 1 (excellent) to indicate gDNA quality. Both samples were found to be of excellent quality with Q scores of 0.98. Whole exome sequencing was performed at the Standard WES Express protocol, which uses a ligation-based sample preparation, followed by hybrid capture. Whole exome sequencing took place on an Illumina HiSeq 4000 sequencing system with 76 bp paired reads. The process typically yields 20X or greater coverage at 80% of the targeted regions. As shown in Table 1, the libraries covered a considerably higher percentage of the targeted region (88% and 91%, respectively) under these conditions, illustrating the excellent quality of the DNA prepared with the Pall NAB plates.

Figure 4

Whole exome sequencing results of two HEK293 gDNA samples performed with Illumina HiSeq 4000 system with paired 76 bp reads.



Library Property	Sample 1	Sample 2
Total Reads	110,399,118	137,101,484
Mean Read Length	76	76
Reads Aligned in Pairs	108,907,852	135,202,658
PF HQ Aligned Reads	93,162,447	115,873,239
PF Bases Aligned (Paired)	8,269,655,164	10,268,182,641
PF HQ Aligned Bases	7,048,886,513	8,766,072,986
PF HQ Aligned Q20 Bases	6,890,864,336	8,571,445,410

Table shows whole exome sequencing library statistics. Graph shows target base coverage at stated read coverage (fold) and above.

4. Summary

- Pall AcroPrep Advance 96-well Filter Plates for Nucleic Acid Binding allow isolation and purification of high quality genomic DNA from freshly prepared or previously stored frozen cultured mammalian cells.
- Protocol modifications are presented that improve genomic DNA isolation and purification from both fresh and previously stored frozen cultured mammalian cells.
- Genomic DNA isolated with Pall AcroPrep Advance 96-well Filter Plates for Nucleic Acid Binding is excellent for use in downstream applications as real-time qPCR and next generation sequencing library construction.

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