BIOTECH SUPPORT GROUP

100,000+ PCRs Possible from 10 ml Blood

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Abstract

A requirement of massively parallel SNP analysis is to have high quality DNA suitable for target amplifications, and prepared economically from practical blood volumes. **ProPrep™ Genomic** is a blood DNA purification system based upon the unique protein binding reagent, **ProCipitate™**. Data is presented demonstrating as little as 1-2 ng of template DNA being sufficient for a strong PCR amplicon signal. This means that theoretically 100,000+ PCR reactions, or multiples of that for multiplexed SNPs, can be obtained from one, 10 ml whole blood sample. The flexible **ProPrep™** system permits the user to customize a multiple PCR or SNP strategy without regard to collecting impractical quantities of whole blood from any one individual. **ProPrep™ Genomic** can be also be scaled to accommodate different starting volumes. The protocol can be completed in approximately 1 hour with quantitative yields starting directly from whole blood.

Similar DNA quality is achieved through an adaptation for direct lysis of whole blood, bypassing all centrifugal requirements, including the initial red cell lysis and the alcohol precipitation. Consequently, the **ProPrep™ Genomic 96** protocol is extremely fast and readily compatible with automation using 96-well filtration.

Introduction

ProPrep™ Genomic and Genomic 96 are complete DNA purification systems based upon **ProCipitate™**, a unique protein aggregating reagent developed from patented solid-phase polyelectrolytes[1]. **ProCipitate™** is characteristic of phenol/chloroform separation but is non-hazardous and has the additional benefits of solid-phase suspensions; that is the adaptability to filtration and automation. It is routinely used and cited in protocols for improving the consistency of yields and the improvement in sequence and PCR quality of low to high molecular weight DNA, including plasmids, BACs and Genomic DNA[2,3,4,5,6,7].

An important measure of DNA purity is the suitability as template for PCR amplifications. For massively parallel SNP studies, there is a requirement for many PCR reactions from one sample. If the sample size is limited or if economics dictate small samples, it becomes desirable to produce as many PCR reactions as possible from the available DNA. The purpose of the investigation described herein was to ascertain the minimal template quantity required for PCR. This was done by first purifying the DNA using the **ProPrep™ Genomic** & **Genomic 96** protocols, serially diluting the purified DNA, and then finally using the serially diluted DNA as template for PCR. Theoretical calculations were made to determine the number of PCR reactions possible at these template concentrations.

ProPrep™ Genomic Protocol
Starting Volume 1 Ml Whole Blood

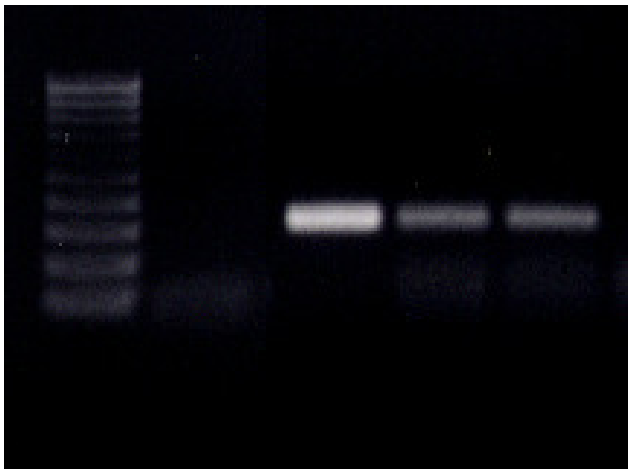
1. Add 3 ml SL1 solution to 1 ml of whole blood to selectively lyse red cells. Mix by inversion. Allow to stand for 10 minutes at room temperature. Mix again by inversion.
2. Centrifuge for 10 minutes at 2000 x G.
3. Aspirate supernatant, leaving behind small volume of supernatant and pellet.
4. Add 400 µl of GL2 and mix by re-pipetting to lyse buffy coat cells. Transfer the lysate to a new centrifuge tube. Allow to stand for 5 minutes.
5. Shake **ProCipitate**™ well to resuspend solid-phase. Using wide bore pipette tip, add 1 ml of **ProCipitate**™, vortex, and allow to stand for 5 minutes.
6. Centrifuge for 6 minutes at 10,000 x G.
7. Transfer the supernatant to a new centrifuge tube. Note - if the supernatant has suspended particles in it, centrifuge for 1 minute at 10,000 X G.
8. Add 1 ml of Isopropanol. Mix by inversion about 25 times.
9. Centrifuge for 5 minutes at 2000 X G. Carefµlly decant the supernatant while recovering the DNA pellet. Invert the tube onto an adsorbent paper towel for 15 minutes.
10. Add 1000 µl of TE3 to the pellet, mix by tapping the tube. Incubate for 20 minutes at 65°C. DNA is now suitable for amplification or other molecular biology procedures.

Total Experiment Time

Buffer	Purpose	Time (min)
SL1	Red blood cell lysis – Removes Hemoglobin, retains white cells	10
Gl2	White Cell lysis Solution – Disrupts Nucleosome	5
ProCipitate™	Removes Protein Contaminants	5
Isopropanol	DNA Precipitation	15
TE3	DNA Resolubilization	20

Total time for the experiment regardless of the sample size including centrifugation and DNA resolubilization is approximately 1 hour, 20 minutes.

PCR Results



Human Genomic DNA purified using **ProPrep**™ **Genomic** producing 280 bp amplicons from Human HLA-DRß primers at 32 cycles, used at the following template quantities:

Lane 1, 100 – 1000 bp Ladder
Lane 2, Negative Control
Lane 3, 260 ng total DNA template
Lane 4, 26 ng total DNA template
Lane 5, 2.6 ng total DNA template

Linearity Experiment

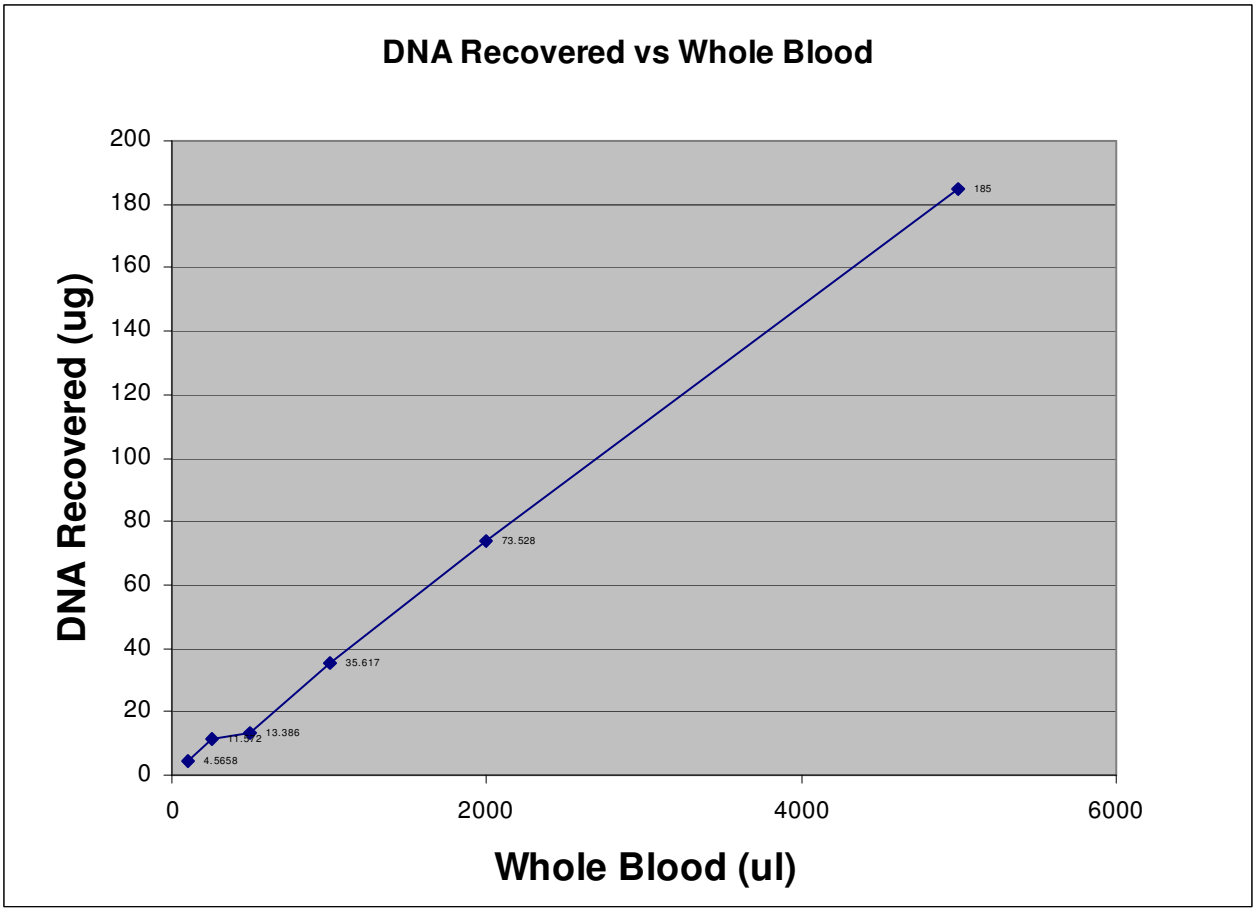
The aforementioned protocol was used as a basis for studying the linearity of DNA yields starting with different blood volumes. The protocol shown below was used for all the different volumes of blood and adjusted accordingly. The buffer volumes were adjusted proportional to the starting blood volume, however the centrifuge times were the same for all the experiments.

Whole Blood (µl)	SL1 Buffer	Gl2 Buffer	ProCipitate	Isopropanol	TE3 Buffer
100	300µl	40µl	100µl	100µl	1000µl
250	750µl	100µl	250µl	250µl	1000µl
500	1500µl	200µl	500µl	500µl	1000µl
1000	3000µl	400µl	1000µl	1000µl	1000µl
2000	6000µl	800µl	2000µl	2000µl	1000µl
5000	15000µl	2000µl	5000µl	5000µl	2000µl

All the samples were resolubilized in TE3. OD 260/280 readings were done on all the samples and the following results were obtained.

Whole Blood (µl)	OD 260/280	Total DNA (ug)
100	2.09	4.6
250	2.05	11.6
500	2.28	13.4
1000	2.15	35.6
2000	1.97	73.5
5000	2.00	185.0

The results obtained above show that there is linearity from sample to sample with the range of whole blood from 100µl to 5ml. The following graph illustrates the linearity of DNA recovered verses the starting Whole blood volume.



Results

Based on the chart shown above it can be seen that the amount of DNA recovered from different volumes of Whole Blood is consistent and linear within 5-10% variance. It shows that the results can be obtained using the same protocol hence the protocol can be scaled according to the sample size.

PCR Suitability Experiments

From 1 ml Whole Blood

1ml of Whole Blood Sample yields 35.6 µg total DNA. To ascertain the suitability of the template DNA for PCR reactions, the following dilutions and concentrations were used:

ProPrep™ DNA solution (µl)	Volume of water added (µl)	Total Volume (µl)	DNA conc. (ng/µl)	Volume used for PCR reaction (µl)	Absolute DNA amount used for PCR (ng)	Number of Possible PCR Reactions
A. 1000	-	1000	35.6	10	**356**	**100**
B. 100 from A	900	1000	3.56	10	35.6	1,000
C. 100 from B	900	1000	0.356	10	**3.56**	**10,000**
D. 500 from C	500	1000	0.178	10	**1.78**	**20,000**

Samples A, C & D were subjected to a PCR reaction.

PCR Suitability Experiments

From 5 ml Whole Blood

5ml of Whole Blood Sample yields 185 µg total DNA. To ascertain the suitability of the template DNA for PCR reactions, the following dilutions and concentrations were used:

ProPrep™ DNA solution (µl)	Volume of water added (µl)	Total Volume (µl)	DNA conc. (ng/µl)	Volume used for PCR reaction (µl)	Absolute DNA amount used for PCR (ng)	Number of Possible PCR Reactions
A. 2000	-	2000	92.5	10	925	200
B. 100 from A	900	1000	9.25	10	**92.5**	**2,000**
C. 100 from B	900	1000	0.925	10	9.25	20,000
D. 400 from C	600	1000	0.308	10	**3.08**	**50,000**
E. 100 from C	900	1000	0.093	10	**0.93**	**200,000**

Samples B, D & E were subjected to a PCR reaction.

PCR Conditions

The purified DNA (10 µl) was amplified using the human HLA-DRß primer pair as supplied by Lifecodes, Inc., and the REDTaq™ Genomic DNA Polymerase SuperPak™ Kit from Sigma as follows:

Amount (µl)	Component	Final Concentration
63	Water	-
10	10x PCR Buffer	1x
2	10mM dNTP mix	200µM of each dNTP
5	Forward Primer	0.1 – 0.5 µM
5	Reverse Primer	0.1 – 0.5 µM
5	REDTaq DNA Polymerase	0.05 Units/µl
10	Template DNA	(varies)
100	**Total Reaction Volume**	

Mix gently by vortex and samples are ready for PCR.

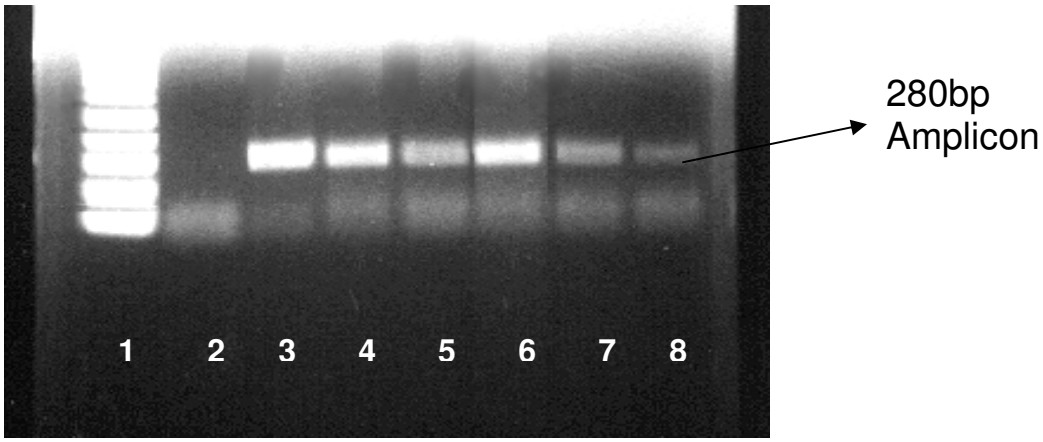
The following cycling program was used:

Initial Denaturation	30sec	95°C
3 step cycle		
Denaturation	1 min	95°C
Annealing	1 min	60°C
Extension	1 min	72°C
Cycles	32	
Final Extension	10 min	72°C

After the DNA was amplified its presence was detected by electrophoresis in 1.0% agarose gel containing ethidium bromide. 10 µl of the amplified DNA was added to 2 µl of DNA loading buffer. 10 µl of that mixture was loaded onto the gel. The gel was run for 45 minutes at 100 volts.

The size of the amplicon generated is 280 base pairs.

PCR Results



Lane 1: 100 – 1000 bp Ladder
Lane 2: Negative Control
Lane 3: 356 ng DNA (Sample A from 1 ml Table)
Lane 4: 3.56 ng DNA (Sample C from 1 ml Table)
Lane 5: 1.78 ng DNA (Sample D from 1 ml Table)
Lane 6: 92.5 ng DNA (Sample B from 5 ml Table)
Lane 7: 3.08 ng DNA (Sample D from 5 ml Table)
Lane 8: 0.93 ng DNA (Sample E from 5 ml Table)

Since PCR reactions worked at 1.0 ng template, we can calculate the number of PCR reactions that can be done from 1ml of this individual's Blood. There will be variances in DNA quantity obtained from blood, but these results are indicative of performance: 1ml of Blood yielded 35,617ng DNA and using 1 ng as template per PCR reaction, 35,617 PCR reactions are possible.

ProPrep™ Genomic 96 Protocol
Starting Volume 50 µl Whole Blood

In an effort to shorten the protocol and eliminate centrifugation, the following protocol and results are demonstrated in a 96 well format.

1. In a 96 deep well plate, to 50 µl of whole blood, add 100 µl of GL1D buffer to lyse all the cells. Tape seal the plate and vortex for 30 seconds. Incubate 10 minutes at 65 C and vortex again briefly.
2. Shake **ProCipitate**™ well to resuspend solid-phase. Using wide bore pipette tips, add 250 µl of **ProCipitate**™ to each well, mix by pipetting up and down 8-10 times to insure that each sample is homogeneous. Allow to stand for 5 minutes.
3. Transfer each sample to the corresponding well of the 96-well filter, and vacuum to collect filtrate, about 10-15 minutes. The collected supernatant contains the DNA.

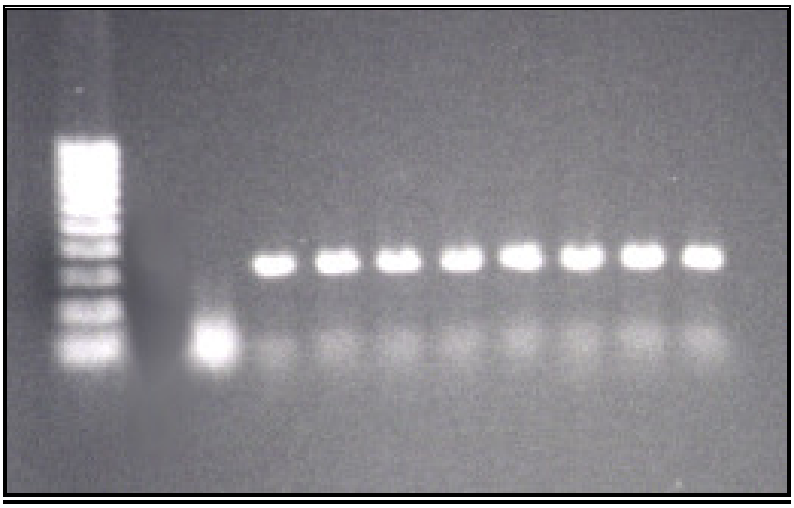
Dilution and PCR

The volume recovered after filtration was approximately 250 µl. A 1:50 dilution was made and PCR was performed from 10 µl aliquots, as previously described. Thus the number of possible PCR reactions from the final volume is 1,250. Furthermore, the initial concentration of DNA from the whole blood sample was calculated to be 25.4 µg/ml, based on purification from ProPrep™ Genomic and quantified by $A_{260/280}$.

Therefore,
50 µl of the whole blood sample contained ≈ 1,270 ng DNA;
Final diluted volume = 12,500 µl, which contained 1,270 ng DNA or
0.1 ng/µl;
10 µl or approximately 1 ng was used as input to PCR.

After the DNA was amplified its presence was detected by electrophoresis in 1.0% agarose gel containing ethidium bromide. 10 µl of the amplified DNA was added to 2 µl of DNA loading buffer. 10 µl of that mixture was loaded onto the gel. The gel was run for 45 minutes at 100 volts.

PCR Results from ProPrep™ Genomic 96



Lane 1: 100-1000 bp Ladder
Lane 2: Negative Control
Lanes 3-10: PCR Amplicons from 1 ng template DNA purified from whole blood, randomly selected from 96 wells

Conclusion

These experiments demonstrate that the DNA yield from **ProPrep™ Genomic** is near quantitative, yields are linear with starting sample volume, and the DNA recovered from all the samples was consistent with virtually no protein contamination as measured spectrophotometrically.

ProPrep™ Genomic can be scaled to accommodate different sample sizes and does not require any hazardous solvents. The whole protocol can be completed in just over 1 hour including DNA re-solubilization after alcohol precipitation.

For direct lysis of whole blood, the **ProPrep™ Genomic 96** protocol is readily compatible with automation using 96-well filtration. There is no need for any centrifugation; it eliminates the centrifugal requirement of initial red cell lysis and alcohol precipitation. Good PCR amplicon signals are obtained from as little as 1 ng template DNA. It is therefore theoretically possible to achieve 1,250 PCR reactions from 50 µl of whole blood, or by extrapolation, 250,000 PCR reactions from 10 ml whole blood, using this protocol.

These flexible **ProPrep™** systems permit the user to customize a massive PCR or SNP strategy without regard to collecting impractical quantities of whole blood from any one individual. The isolated DNA is of the highest quality, and PCR can be achieved from as little as 1-2 ng of template DNA. This means that an estimated 100,000 to 300,000 PCR reactions can be obtained from one, 10 ml whole blood sample. For moderate situations, the process allows an economical way to scale down the starting sample volume or work in multiwell formats with an automation compatible protocol.

References

1. Composition and utility patents for ProCipitate™ and related technologies are covered under U.S. Patents Numbers 5,294,681, 5,453,493 & 5,658,779 and other patents pending.
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