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Genotyping by Sequencing (GBS) library protocols

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We use this protocol and it's working

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Abstract

Genotyping by sequencing (GBS) is a restriction enzyme based targeted approach developed to reduce the genome complexity and discover genetic markers when *a priori* sequence information is unavailable. Sufficient coverage at each locus is essential to distinguish heterozygous from homozygous sites accurately. The number of GBS samples able to be pooled in one sequencing lane is limited by the number of restriction sites present in the genome and the read depth required at each site per sample for accurate calling of single-nucleotide polymorphisms. Loci bias was observed using a slight modification of the Elshire et al. method: some restriction enzyme sites were represented in higher proportions while others were poorly represented or absent. This bias could be due to the quality of genomic DNA, the endonuclease and ligase reaction efficiency, the distance between restriction sites, the preferential amplification of small library restriction fragments, or bias towards cluster formation of small amplicons during the sequencing process. To overcome these issues, we have developed a GBS method based on randomly tagging genomic DNA (rtGBS). By randomly landing on the genome, we can, with less bias, find restriction sites that are far apart, and undetected by the standard GBS (stdGBS) method. The details about how each approach performs can be found in the citation shown below.
This protocol explains how to prepare each type of GBS library, with comments on expected results and controls needed to confirm each step worked.

Attachments

PDF
Hilario, Barron, Den...
1.2MB

XLSX
RADsites test_EHilar...
76KB

Guidelines

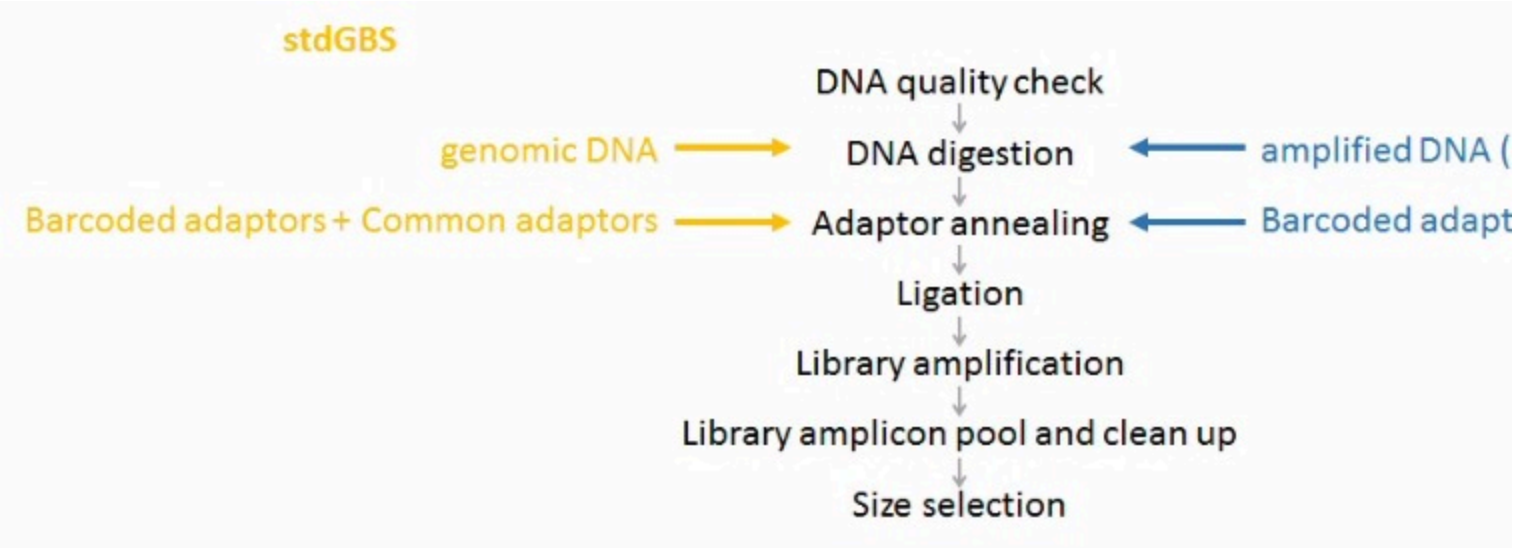
This protocol explains how to prepare GBS libraries from intact (standard GBS (stdGBS)) and from amplified DNA (random tagged GBS (rtGBS)).

The stdGBS protocol described below differs from Elshire et al. in the following steps:

- One microgram of genomic DNA of each plant was used for the restriction digestion.
- The restriction enzyme was selected based on the RADsites test_iPlant.xlsx (see file uploaded).
- Adaptors are annealed according to Ko et al. (Ko W-Y, David R M, Akashi H (2003) J Mol Evol (2003) 57:562–573 DOI: 10.1007/s00239-003-2510-x).
- The adaptor ligation step was done after digestion, without drying out the DNA/adaptor mixture.
- A high fidelity enzyme was used for amplification (AccuPrime Taq DNA polymerase High Fidelity, Life Technologies).
- The amplification of the library was done on each individual plant before pooling.
- The GBS adaptors and primers were designed by **Deena Bioinformatics**.
- All oligonucleotides were ordered from BioSearch Technologies. The common adaptors and PCR primers were ordered at 200 nmol scale, cartridge purified, dry pellets, in tubes. The barcoded adaptors were ordered normalised at 5 nmol, purified by desalting, dry pellets, in plates.

The rtGBS protocol is ilustrated with the following diagram:

The stdGBS and rtGBS methods share most of the steps described below, with some modifications: the source of the starting material, and the omission of the common barcoded adaptor in rtGBS:


stdGBS
DNA quality check
genomic DNA
DNA digestion
amplified DNA (
Barcoded adaptors + Common adaptors
Adaptor annealing
Barcoded adapt
Ligation
Library amplification
Library amplicon pool and clean up
Size selection

Before start

NOTES

1. Dissolve the barcoded adaptor oligonucleotides (plus and minus strand) in 1X TE pH 7.5 to a final concentration of 20 pmol/µL. Dissolve the common adaptor oligonucleotides to 500 pmol/µL. Assign one person to look after these stocks to prevent cross contamination between wells and tubes.
2. Include digestion and ligation controls processed in parallel to your experiment to confirm those steps worked. For restriction enzymes that cut frequently, Lambda DNA and/or pBluescript are good controls. Do an *in silico* digestion to evaluate the desired enzyme or enzyme combination.
3. If you are planning a stdGBS experiment with a rare restriction enzyme, consider a double digest GBS approach (Poland JA, Brown PJ, Sorrells ME, Jannink J-L (2012) Development of high-density genetic maps for barley and wheat using a novel two-enzyme genotyping-by-sequencing approach. PLoS ONE 7: e32253.)
4. You need **high molecular DNA fragments >20 kbp**, without RNA, regardless if you are performing a stdGBS or rtGBS experiment.
5. For stdGBS normalize your genomic DNA samples to the same concentration value, for example 100 ng/µL, to speed up the pipetting. Aim for a rather concentrated DNA solution to avoid carrying too much liquid into the restriction digestion reaction, especially if the DNA has been dissolved in TE buffer. Up to 20 µL of DNA in TE buffer can be safely used in a restriction digestion, in a final volume of 50 µL.
6. Please note the number of cycles in the PCR profile was optimized for the BamH I adaptor set used for a kiwifruit pilot study. You need to optimize the number of cycles for your own plant/restriction enzyme combination. Evaluate 12-20 or 25 cycles, in increments of 2. Analyze your amplified libraries in a Fragment Analyzer, or a BioAnalyzer.
7. Size selection can be done by gel extraction, but is labor intensive. Run the amplicons in a 3% Agarose gel. Make this gel mixture with 0.5 g Standard Agarose, and 2.5 g High resolution Agarose, in 100 mL 1X TAE. Run the gel at 140 V for 30 min. Stain the gel with SybrSafe, and cut the desired block under blue light.
8. The rtGBS protocol requires a modified common oligonucletide. When ordering your barcoded oligonucleotides, include the GBS-C-N oligo: CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNNN, at 200 nmol, cartridge purified, delivered as a dry pellet.
9. When **pipetting dense solutions**, like the ligation or PCR master mixes, prime the pipette tips by drawing and expelling the liquid slowly, one time. Multichannel pipettes (and robotic stations) allow you to select the speed of this operation. Also, if the required volume is 47.5 µL, set the pipette to 46.7 µL, and don't dip the tips too far into the solution. The amount of carry over solution outside the tip will compensate for the "missing" µL in the settings.

MATERIALS

Consumables

- 1 mL deep well plate (NUNC cat# 260252) or 2 mL deep well plate (NUNC cat# 278752)
- 1.5 mL screw-capped tubes, sterile
- Falcon tubes, 15 mL
- Disposable reservoirs

- Aluminium tape (NUNC cat# 232698, or similar). For PCR reactions and long term storage
- Clear packaging tape (48 mm wide, for example: Snell). For temporary plate sealing
- Cutter (retractable blade, general purpose)
- PCR plates (Thermo-Fast AB-0600)
- FrameStar® 96 Well Semi Skirted PCR (Cut Corner A12, 4titute cat# 4ti-0770/C). These plates are used for the Fragment Analyzer only
- 2 L plastic box (21 × 7.5 × 14.4 cm). Mark on the side of the box the water level at 1.5 L and always fill it up to this mark. By doing this small step you will be saving 30-60 min at the cool down annealing steps since small changes in water volume translate to a long/short wait.
- Paper towels, low lint

Equipment and kits

- Water bath at 65°C
- Bench top centrifuge with plate rotor (max speed 4000 rpm)
- Bench top microcentrifuge (for 1.5 mL tubes)
- Vortex with plate adaptor
- **Fragment Analyzer**, High Sensitivity genomic DNA kit (1000 reactions, DNF-488) and High Sensitivity NGS (1000 reactions, DNF-474)
- Qubit and High Sensitivity dsDNA kit
- Horizontal gel electrophoresis unit with power supply
- Image capture equipment (for example, GelDoc, LSA3000, etc)
- Multichannel pipettes (1-10 µL, 5-200 µL, 50-1000 µL) or liquid handling robotic station with appropriate multichannel tools and tips, for example Biomek series, Hamilton, Integra's Viaflo, etc
- PCR machine
- Timer

Reagents

- Sterile deionised water, 100 mL
- Restriction enzyme set from NEB, Invitrogen, Fermentas, etc.
- Lambda DNA (Life Technologies cat# 25250010)
- Uncut pBluescript or any other cloning vector like pUC19 often included as control in competent cells kits. A BAC clone could also be used as control, especially for rare cutting restriction enzymes.
- 1X TE pH 7.5 (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) sterile, 100 mL
- Blue TE buffer (1X TE pH 7.5, 0.0025% xylene cyanol FF), 50 mL
- 10 mM Tris-HCl pH 8, sterile, 50 mL
- 25 mM $MgCl_2$ sterile, 2 mL
- T4 DNA ligase and 5X T4 DNA ligase buffer (Life technologies)
- 20 mg/mL Dextran Blue (dissolved in sterile deionised water, Sigma Aldrich cat# D5751), 1 mL
- 3 M sodium acetate pH 5.2, 50 mL
- Isopropanol

- 100% absolute ethanol, 100 mL
- 70% ethanol (freshly made), 100 mL
- AccuPrime Taq DNA polymerase High Fidelity 5 U/µL and 10X AccuPrime High Fidelity Buffer I and II (Invitrogen 12346094)
- Mineral oil (Sigma-Aldrich cat # M5904-500ML)
- Platinum *Pfx* DNA polymerase 2.5 U/µL (Invitrogen 11708039)
- 1.2 M Trehalose, dissolved in sterile deionized water and filtered through 0.2 or 0.4 µm
- 10X *Pfx* reaction buffer homemade: 200 mM Tris-HCl, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, pH 8, sterile. ***Note**: I have not tried the 10X buffer and enhancer solutions supplied with the Platinum Pfx DNA polymerase (Invitrogen 11708039). It should work, but do a preliminary test.*
- 50 mM $MgSO_4$ (use the stock provided with either DNA polymerase)
- 2 mM dNTP (dilution prepared in deionized sterile water from 100 mM stocks)
- 1% agarose gel in 1X TAE buffer, 100 mL, plus 1 L of 1X TAE running buffer
- 1 kb plus DNA ladder (Invitrogen) or similar
- SybrSafe (Invitrogen)
- Ampure XP magnetic beads (Beckman Coulter) and appropriate magnet stand
- 85% ethanol, 50 mL freshly made

ACKNOWLEDGEMENTS

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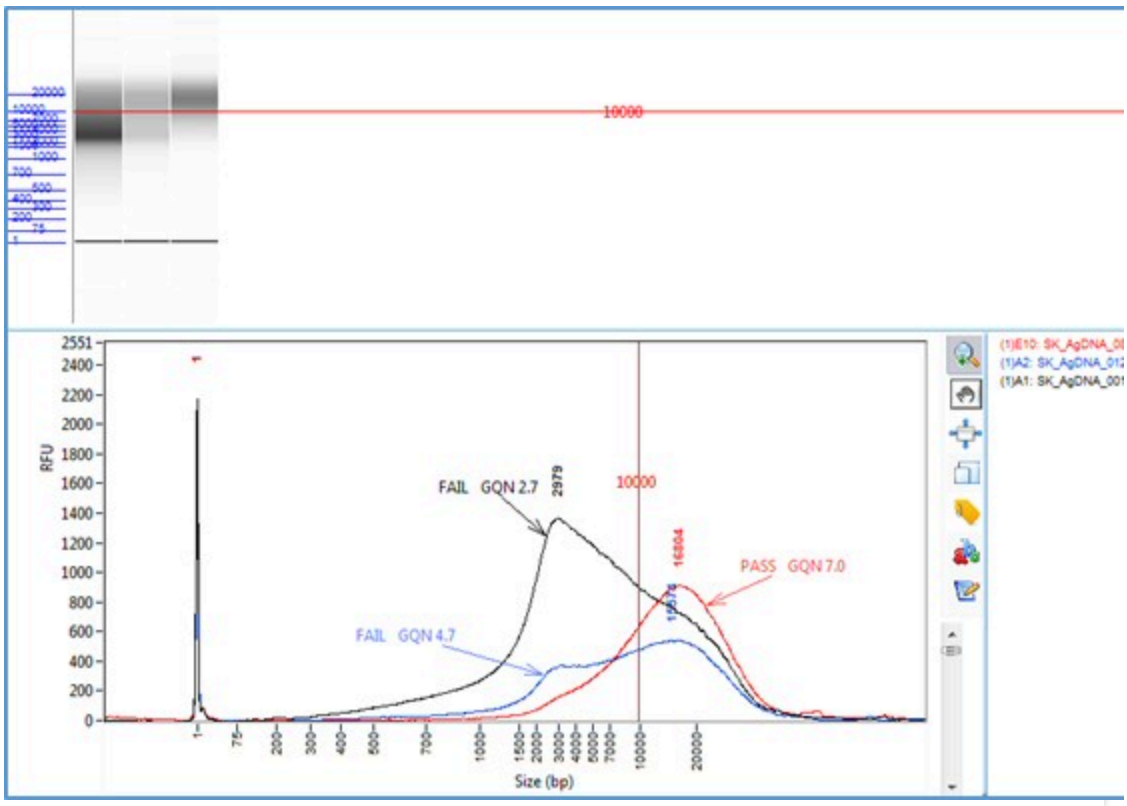
DNA quality check

1 Prepare a dilution plate of your gDNA sample. Aim for 1-5 ng/µL. Use Blue TE buffer.

2 Analyze 2 µL of each sample in the Fragment Analyzer (DNF-488 HS genomic DNA). Use FrameStar® 96 Well Semi Skirted PCR to set up your sample plate. In well G12 include 1-5 ng of lambda DNA control.

Expected result



3 Open the outfile in Prosize 2.0. The default GQN cut off value should be 10,000 bp. Analyse your data accordingly, and discard any samples with a GQN value of <7.

DNA digestion

4 Set up the DNA restriction enzyme digestions in a 1 mL deep well plate:

	DNA digestion plate	
		One reaction, µL
	Deionised sterile water	x
	10X NEB reaction buffer	5
	100X BSA (if needed)	0.5
	Restriction Enzyme 20 U/µL (NEB)	1
	DNA	x
	Final volume	50

Note

The source and ammount of DNA can be either intact genomic DNA (stdGBS, 1 µg) or random tagged DNA (rtGBS 0.5-1 µg).

5 Dispense the restriction enzyme mix manually.

6 Transfer the DNA solution from the source plate into the DNA digestion plate. Seal the plate with packaging tape, vortex and spin down briefly.

7 Incubate the digestions at 37°C for 3 hours.

Note

NEB provides high fidelity versions of many common restriction enzymes that require shorter incubation time for complete DNA digestion. These high fidelity enzymes have been optimized to prevent star activity. Check if your selected enzyme is prone to this unwanted activity. The suggested incubation time is recommended for digesting intact genomic DNA. For rtGBS your sample is less complex and a shorter incubation time (1 h) could be used instead.

8 Spin down and store at -20°C until ready for the next step.

Note

There is no need to inactivate the restriction enzyme. The barcoded adaptors designed by Deena Bioinformatics do not recreate the restriction enzyme site and are safely annealed and ligated without a clean up step after digestion.

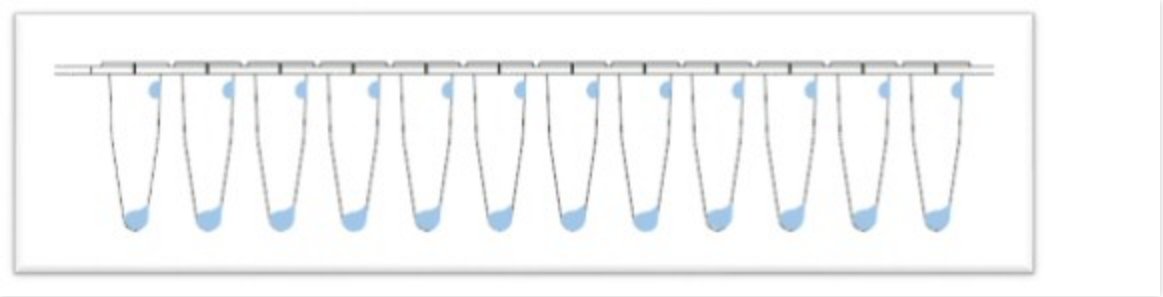
Adaptor annealing

9 In a new PCR plate, aliquot the Barcode adaptors to have a final concentration of 10 pmol/µL of each oligonucleotide:

	Restriction Enzyme (RE) Barcode adaptor pair plate	
		One reaction, µL
	Barcode (+) strand 20 pmol/µL (GREp_1)	10
	Barcode (-) strand 20 pmol/µL (GREn_1)	10
	Total volume	20

10 Seal the plate with aluminium tape. Mix by vortexing and spin down briefly.

11 Add 1.6 µL of 25 mM $MgCl_2$ to the inner side of each well of the Barcode adaptor pair plate with a multichannel pipette:



12 Seal the plate with aluminium tape.

13 The Common adaptors are annealed in 1.5 mL screw-capped centrifuge tubes, to a final concentration of 10 pmol/µL of each oligonucleotide, according to the following table:

	Common adaptor pair tube	
		One reaction, µL
	Common adaptor 500 pmol/µL (GC)	1
	Common Restriction Enzyme (RE) adaptor 500 pmol/µL (GCRE)	1
	1X TE pH 7.5	48
	Total volume	50

14 Mix by vortexing and spin down briefly.

15 Add 4 µL of 25 mM $MgCl_2$ to the top inner side of the Common adaptor pair tube as shown above for the barcoded adapter pair plate. Close the tube.

16 Incubate the Barcode adaptor pair plate and Common adaptor pair tube at 65°C in the water bath for 5 min. If you go over 5 min it's not an issue.

17 Spin down briefly. Mix gently by vortexing and spin down again.

18 Incubate at 65°C for 5 min.

19 Remove about 1.5 L of water from the water bath and place it in the large plastic box.

20 Transfer the Barcode adaptor pair plate and the Common adaptor pair tube to the plastic box and cool down to room temperature (~23°C) for about 2 h. Do not close the plastic box.

Note

There are many ways of annealing adaptors. This is a very gentle one. But it can also be set up in a PCR machine with a gentle cooling down slope, resembling the method described here. If you know of a quicker way of that has worked for you in the past, please follow it and share your experience with the protocols.io community. Another good reason to do it the gentle and slow way is because it gives you time to do other things while the adaptors are annealing, for example, the restriction digestion could be done while waiting.

21 Spin down briefly.

22 Add 60 µL of 1X TE pH 7.5 to each well of the Barcode adaptor pair plate with a multichannel pipette. Seal the plate and mix gently by vortexing. The final volume is 80 µL at 2.5 pmol/µL.

23 Add 50 µL of 1X TE pH 7.5 to the Common adaptor pair tube. Mix contents by tapping the end of the tube. The final volume is 100 µL at 5 pmol/µL.

Annealed stdGBS adaptor plate

24 In a new PCR plate Add 7 µL of 1X TE pH 7.5 to each well.

25 Aliquot 1 µL of the annealed Common adaptor mix to each well.

26 Aliquot 2 µL of annealed Barcoded adaptor pair mix to its corresponding well. Seal the plate, mix and spin down briefly.

27 This is now the Annealed stdGBS adaptor plate at 0.5 pmol/µL of annealed GREp_1 and GREn_1 and 0.5 pmol/µL annealed GC and GCRE adaptors. For short-term storage (< 1 week), keep at 4°C; otherwise, store at -20°C.

rtGBS adaptor annealing

28 In rtGBS the common adaptor is incorporated during the PEP-PCR step. Perform steps 9-12 and 16-22 described above. The annealed barcoded adaptor pair plate concentration is 2.5 pmol/µL each (step 19). Make a 1:5 dilution: 4 µL of each annealed barcoded adaptor pair plus 16 µL pf 1X TE pH 7.5, final volume 20 µL. The dilution plate is now 0.5 pmol/µL of annealed GREp_1 and GREn_1.

29 Aliquot 2 µL from the annealed stdGBS adaptor plate (step 27) or the annealed barceded pair plate for rtGBS (step 28) into their corresponding well of the DNA digestion plate. Seal the plate, spin down briefly, vortex gently, and spin down again.

Ligation

30 Prepare a T4 DNA ligase master mix according to the following table. Include at least 4 extra reactions:

	Ligation plate	
		One reaction, µL
	Deionised sterile water	2
	5X T4 DNA Ligase buffer (Life Technologies)	14
	T4 DNA Ligase 1 U/µL (Life Technologies)	2
	DNA RE digested + annealed GBS adaptors	52
	Final volume	70

Note

The T4 DNA ligase buffer contains a high concentration of polyethylene glycol which assist the enzyme during ligation. It can be difficult to resuspend. Vortex the tube thoroughly and carefully pipette in and out about 1/3 of the total volume. Make sure there is no PEG at the bottom of the tube (it might look like a gooey pellet).

The buffer also contains ATP, which degrades after many cycles of freeze/thaw. If in doubt, order a new buffer stock. The strong smell of this buffer is due to the reducing agent DTT. If your buffer doesn't smell it might be an indication of how old it is and you should also consider buying a new stock.

31 Dispense 19 µL of the T4 DNA master mix to each well of the plate containing the digested DNA and annealed GBS adaptors. Seal the plate, mix by gently vortexing and spin down briefly.

32 Incubate the ligation reactions at 4°C (refrigerator) overnight.

Note

If you have access to a 16°C incubator, you could use this instead of a refrigerator. This is a cohesive end type of ligation and probably happens rather quickly while cooling down in the refrigerator, but leaving it overnight increase the chances of fully ligating all the constructs.

33 Spin down at 2000 rpm for 5 min at room temperature.

34 Prepare the precipitation master mix, 100 reactions in a 15 mL Falcon tube:

	Precipitation mastermix	
	Dextran Blue 20 mg/mL	100 µL
	1X TE pH 7.5	2900 µL

35 Vortex thoroughly.

36 Dispense 30 µL of the precipitation master mix to each well with a multichannel pipette. Seal the plate and vortex 3-5 times.

37 Dispense 10 µL 3 M sodium acetate pH 5.2 with a multichannel pipette. Seal the plate and vortex 3-5 times.

38 Add 200 µL 100% absolute ethanol with a multichannel pipette. Seal the plate and mix gently by vortexing. Incubate at -20°C for at least 2 h, or overnight.

39 Spin down at 3000 rpm for 25 min at room temperature.

40 Discard supernatant and blot the plate over 2 paper towels for 5-10 sec to remove all liquid.

41 Air dry the plate at room temperature for 10 min or until all traces of liquid have evaporated.

Note

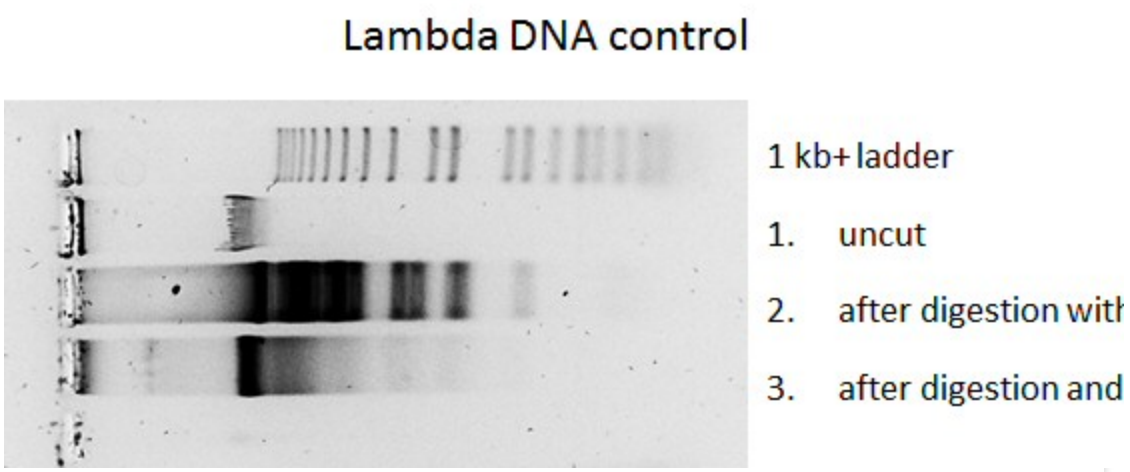
To determine if the ethanol has evaporated completely, weight the plate at t= 0 and check every 10 or 15 min until there is no change in the weight value.

42 Add 100 µL of 1X TE pH 7.5 with a multichannel pipette. Seal the plate, vortex thoroughly and spin down briefly. Let the DNA dissolve completely at 4°C overnight.

43 For long-term storage, keep at -20°C.

44 Run the restriction enzyme digestion and ligation controls in a 1% agarose gel, 1X TAE, against a DNA ladder, 30 min at 140 V. Stain with SyberSafe (3 µL stock in 50 mL 1X TAE buffer, in the dark with gentle shaking) and take a picture.

Expected result



Library amplification

45 Dissolve PCR primers PPA and PPB in 1X TE pH 7.5 to have a stock concentration of 1 mM. Make a 1:100 in 1X TE pH 7.5 to have a working solution of 10 pmol/µL.

46 Make a PCR master mix for the total number of libraries that need to be amplified, according to the table below. Include at least 4 extra reactions in your master mix.

	Amplified library plate	
		One reaction, µL
	Deionised sterile water	37.8
	10X AccuPrime High Fidelity Buffer I	5
	PPA 10 pmol/µL	1
	PPB 10 pmol/µL	1
	AccuPrime Taq DNA polymerase High Fidelity 5 U/µL	0.2
	GBS library plate (corresponding well)	5
	Total volume	50

47 Aliquot 45 µL of the PCR master mix in each well of a new PCR plate labelled as 'your initals_experiment details_GBS_RE_lib amp_plate number'. For example: SK_KF_rtGBS_BstEII_lib amp_P1.

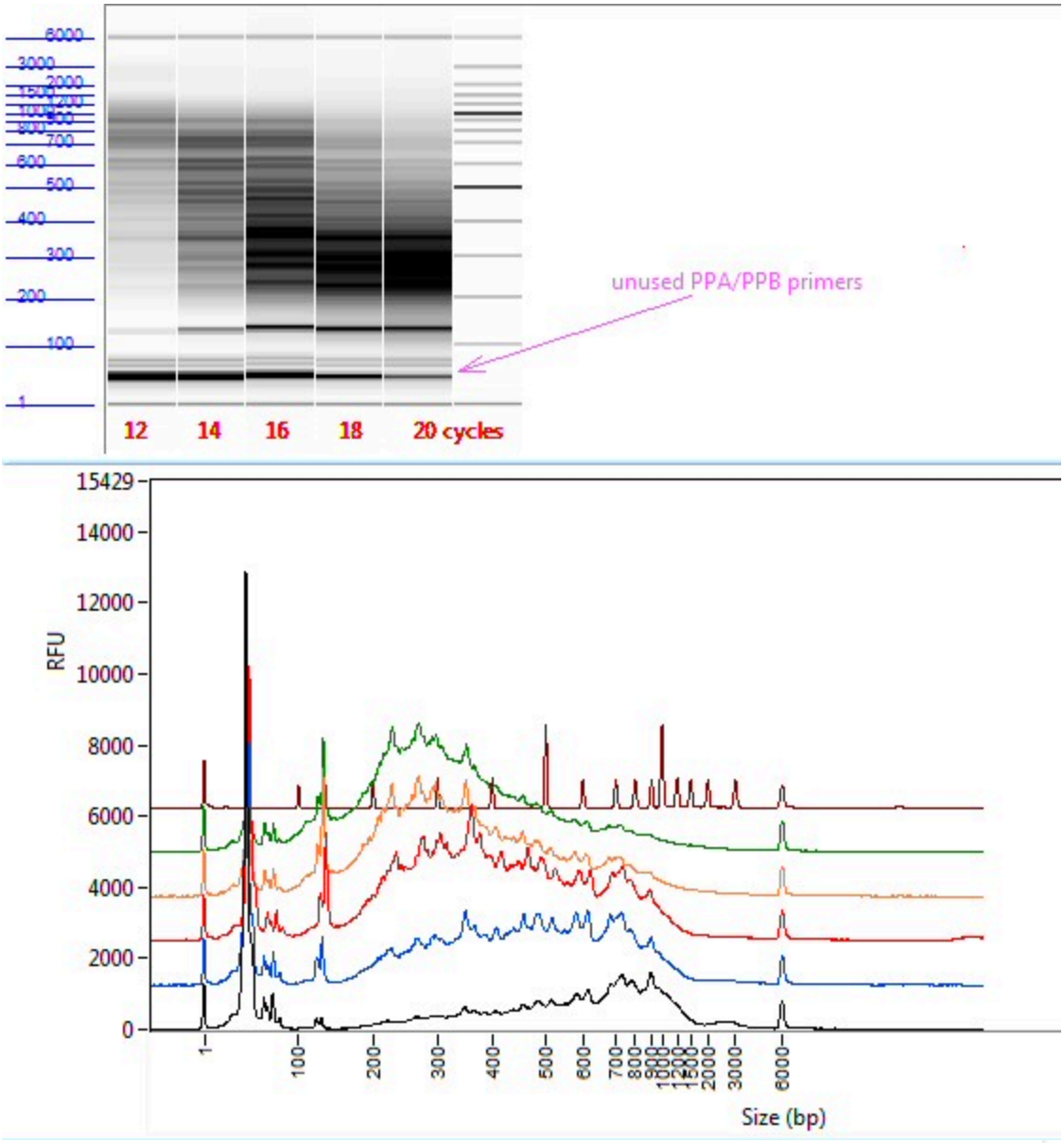
48 Transfer 5 µL of each GBS library plate well (step 43) to its corresponding location in the GBS amplified library plate (step 47). Seal the plate with aluminium tape and mix gently by vortexing. Spin down the plate briefly.

49 Run the following PCR profile:

72°C, 5 min → 94°C, 1 min→ (94°C, 30 sec → 65°C, 30 sec → 68°C, 30 sec) x 25 cycles → 68°C, 5 min → stop, leave at room temperature.

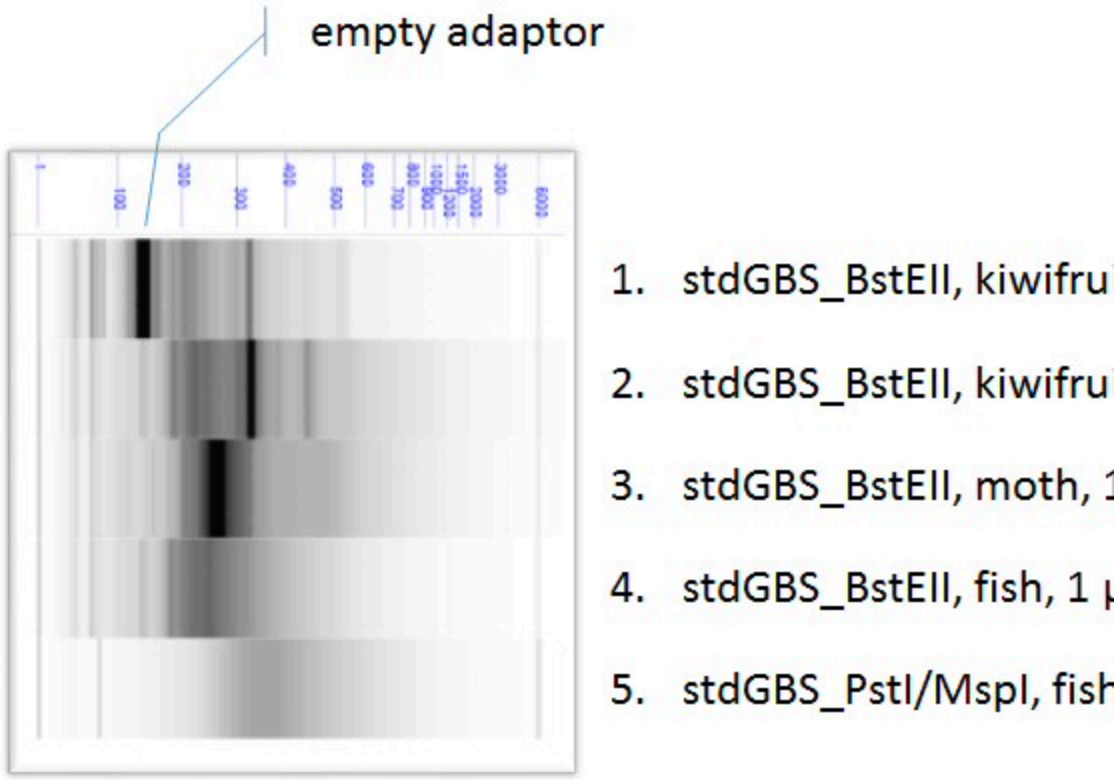
Note

Perform a cycle test for your experiment to determine the best range of amplicon size and minimize the production of PCR duplicates.



50 Analyze 2 µL of each GBS amplified library in the Fragment Analyzer (DNF-474 HS NGS kit) to confirm that every library was successfully prepared and amplified. Use FrameStar® 96 well semi skirted PCR plates for preparing the sample plate.

Expected result



Expected result

Expected result

Library pools and clean up

51 To prepare the GBS amplified library pool: Transfer the contents of the GBS amplified library plate (step 48) into a reservoir using a multichannel pipette and then transfer to a 15 mL Falcon centrifuge tube. Measure the final volume.

Note

The number of samples to pool will depend on how much coverage can be obtained per sequencing lane. The RADsites calculation sheet uploaded for this protocol can give you an estimate of expected coverage given the genome size, GC content, and number of samples pooled. A discussion with your bioinformatics colleagues will help you decide the pooling strategy.

If you have more than 1 plate of amplicons to pool but you would like less than 96 samples per lane and you are working with a 96-barcoded adaptor set, pool your samples with a window set by the number of samples per lane and move across the total number of plates. Alternative, a second barcode added to the GC/GCRE adaptor for stdGBS. Consult with **Deena Bioinformatics** on how to include this second barcode.

52 Purify the GBS amplified library pool by precipitating the pooled reactions as follows:

53 Add 1/10 volume of 3 M Na Acetate pH 5.2, mix and add 2 volumes of 100% ethanol. Vortex thoroughly and store at -20°C for at least 2 h or overnight.

Note

The library pool contain some dextran blue from the GBS library plate template used for the amplification. Two hours at -20°C should be enough to precipitate the DNA, but it can be left overnight too.

54 Spin down at 3000 rpm for 25 min at room temperature

55 Discard the supernatant. The pellet might be slighlty blue and fluffy. Wash the pellet with 2-4 mL of 70% Ethanol, spin down as before

56 Remove all ethanol and air dry the pellet at room temperature for ~ 30 min or until no traces of liquid are visible

57 Resuspend the pellet in 100-200 µL of 1X TE pH 7.5, transfer the pooled library to a screw capped tube and store at 4°C.

58 Perform a size selection with Ampure XP magnetic beads and quality check step.

Library pool size selection: 0.6x/0.2x cut example

59 Depeding on the sequencing read length available (100, 125, 150 or 250 b), select the correct dual size selection cut. The instructions provided below are for a read length dataset of 100 bases. Beckman Coulter explains in detail how to achieve other cuts: **SPRIselect User Guide**

60 Resuspend beads in hybrydization oven rotisserie at 37°C, moderate speed, for about 1 hour.

61 Prepare fresh 85% ethanol

62 Adjust your DNA sample to final volume of 100 µL with 1X TE pH 7.5, mix and spin down briefly.

63 Add 60 µL of resuspended beads to DNA sample, resuspend by pipetting 10 times

64 Incubate at room temperature for 1 min

65 Place tube on magnet

66 Incubate at room temperature 2 min

67 Transfer supernatant to new tube

68 Discard beads (containing the unwanted large fragments)

69 Add 20 µL beads, resuspend by pipetting 20 times

70 Incubate at room temperature 1 min

71 Place tube on magnet

72 Incubate at room temperature 2 min

73 Discard supernatant (contains the unwanted small fragments), leave tube on magnet

74 Add 180 µL of 85% ethanol

75 Incubate at room temperature for 30 sec

Note

Do not go overtime.

76 Discard the ethanol

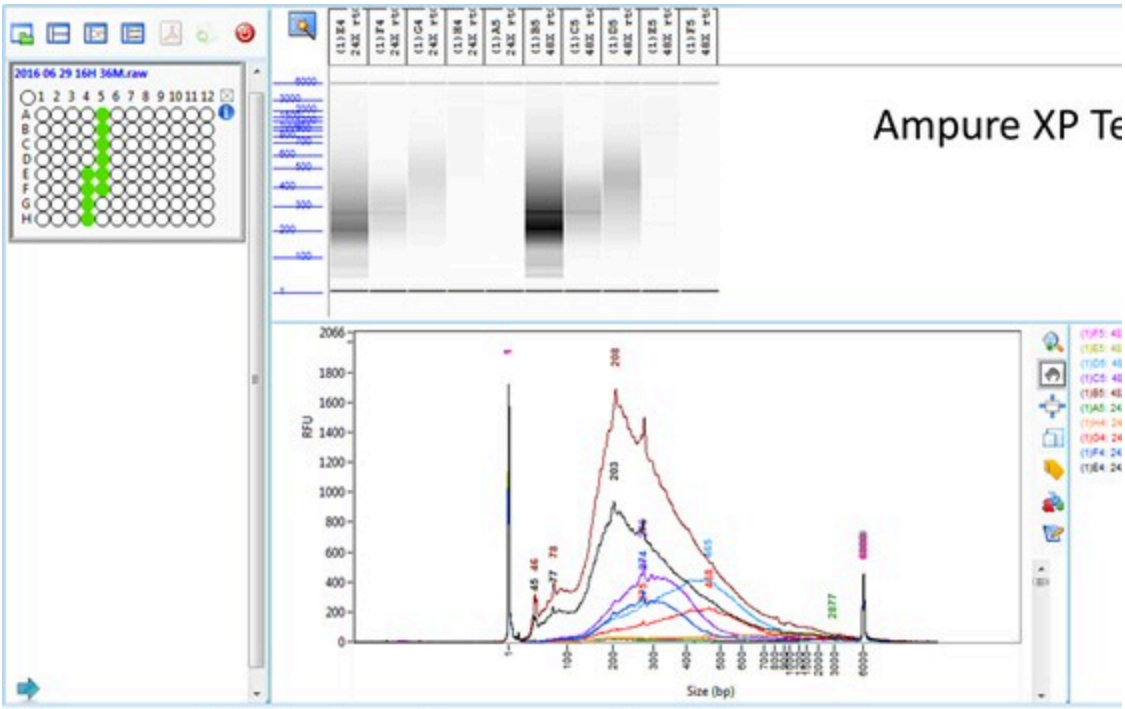
77 Add >20 µL of TE buffer. Resuspend by tapping the bottom of the tube gently

78 Let elute at room temperature for at least 10 min, the longer the better

79 Collect eluted DNA by placing the tube on the magnet as described in steps 65-66.

80 Analyze 2 µL in the Fragment Analyzer (DNF-474 HS NGS kit, Use FrameStar® 96 Well Semi Skirted PCR to set up your sample plate). There shouldn't be any bands at ~129 bp, which correspond to the empty ligated barcoded and common adaptors, in stdGBS only. In rtGBS there shouldn't be any empty adaptors. If any bands appear at this range might be due to a very small DNA restriction fragment tagged by PEP-PCR. These should be removed as well. The expected results image shows several bead ratios tested on a rtGBS library pool.

Expected result



rtGBS_BstEII pool (recovery, %)

	Smear range (bp)				
	100-2000	200-450	300-600	500-2000	1000-2000
unsized	91.1	55.1	29.3	8.2	1.1
0.8/0.2	97.0	74.3	41.9	5.7	1.0
0.6/0.2	96.3	52.1	57.8	24.1	2.2
0.4/0.2	86.6	27.7	30.6	43.8	12.9
0.3/0.15	78.6	40.6	24.2	14.2	7.3

rtGBS_BstEII pool (yield, ng)

From ~ 330 ng starting sample	Smear range (bp)			
	100-2000	200-450	300-600	
0.8/0.2	31.85	24.39	13.90	
0.6/0.2	33.88	18.47	20.35	
0.4/0.2	6.27	2.01	2.21	
0.3/0.15	1.83	0.94	0.57	

81 Store the sized library pool in a screwcap tube, at 4°C until ready to be sent to the sequencing provider.

Note

Most sequecing providers require a 10 nM solution of the size selected pooled amplified libraries, in TE buffer. Aim for at least twice that concentration and send only half of the sample to be sequenced. The remaining half is your back up in case something goes wrong in transit or if the sequencing run needs to be repeated.

rtGBS: PEP-PCR

82 Make a Master Mix according to the following table, with at least 4 extra reactions:

PEP-PCR	
	One reaction, µL
Deionised sterile water	41.8
10X AccuPrime High Fidelity Buffer II	5
GBS-C-N, 10 pmol/µL	1
AccuPrime Taq DNA polymerase High Fidelity 5 U/µL	0.2
genomic DNA (1-5 ng/µL)	2
Total volume	50

83 Read note regarding pipetting dense solutions. Aliquot 48 µL of master mix per well. Transfer the gDNA solution to its corresponding well. Seal the plate with aluminium tape and vortex vigorously. Spin down briefly.

84 Add 20 µL of sterile mineral oil to each well with a multichannel pipette, seal the plate with a new aluminium tape, and amplify as follows:

94°C, 2 min → **(94°C, 40 sec** → **30°C, 2 min** → **48°C, 4 min (set ramp at +0.1°C/sec)** → **68°C, 1 min) x 50 cycles** → **68°C, 7 min** → **stop** and leave at room temperature (running time: 10 h)

Note

The annealing step is done at two temperatures: 30 and 48°C, with a very gentle ramp that will increase 0.1°C per second.

85 Mineral oil should prevent evaporation but check all wells, especially the ones at the edge of the plate. If less than half of the total volume of the reaction has evaporated, add enough 1X TE pH 7.5 buffer to bring back the volume to 50 µL. Repeat the PEP-PCR reaction if more than half of the liquid has evaporated.

86 For short term storage, keep plate at 4°C, otherwise store at -20°C.

rtGBS: TD-PCR

87 Make a Master Mix according to the following table, with at least 4 extra reactions:

TD-PCR		
	One reaction, µL	Final concentration
Deionised sterile water	6.25	
1.2 M Trehalose	25	0.6 M

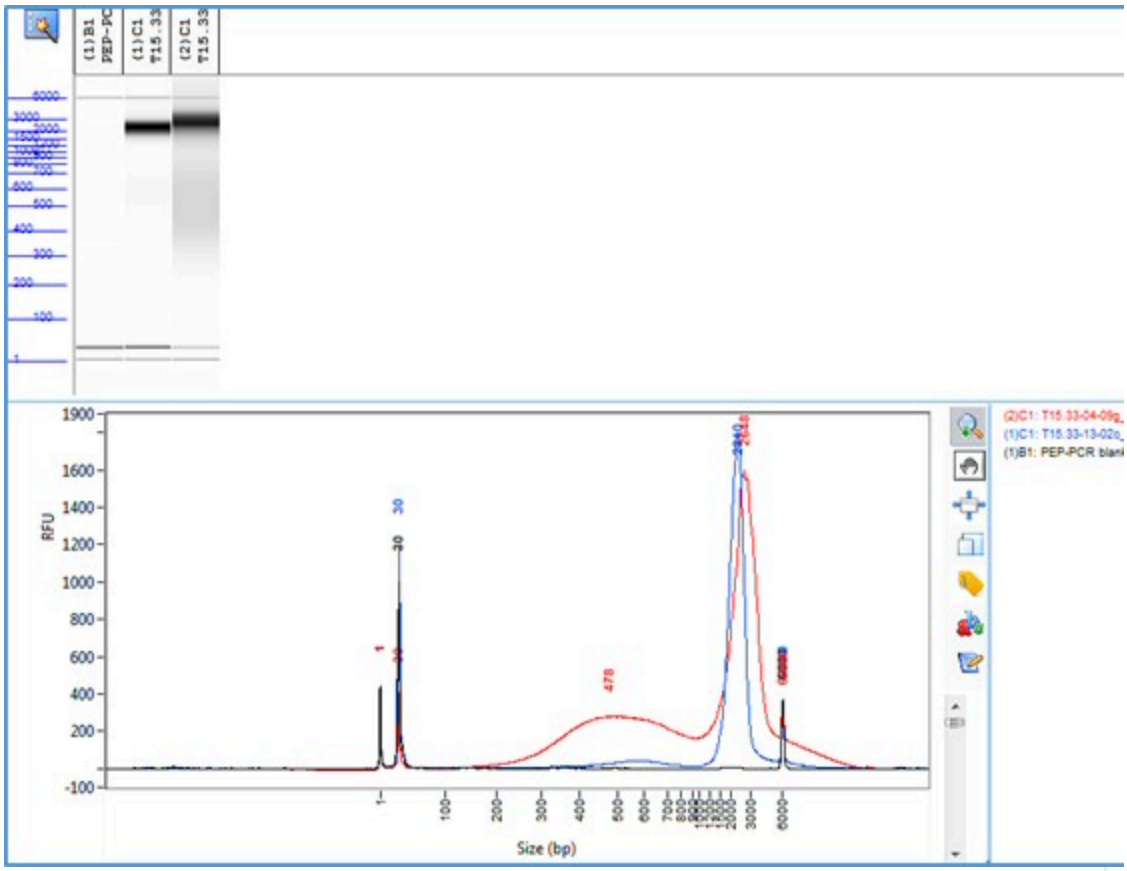
	10X *Pfx* Reaction Buffer (homemade)	10	2X
	50 mM $MgSO_4$	0.5	0.5 mM
	2 mM dNTP	5	0.2 mM
	GBS-C, 10 pmol/µL	1	10 pmoles
	Platinum *Pfx*, 2.5 U/µL	0.25	0.625 Units
	PEP-PCR reaction, undiluted	2	
	Total volume	50	

88 This is a dense solution. Aliquot 48 µL of master mix per well with a multichannel pipette. Transfer the gDNA solution to its corresponding well. Seal the plate with aluminium tape and vortex vigorously. Spin down briefly and amplify as follows:

94°C, 2 min → (94°C, 30 sec → Touchdown 68°C-58°C, 30 sec at -0.5°C/cycle → 68°C, 1 min) x 20 cycles → (94°C, 30 sec → 58°C, 30 sec → 68°C, 1 min) x 10 cycles → 68°C, 7 min → stop and leave at room temperature

89 Analyze 2 µL of each sample in the Fragment Analyzer (DNF-474 HS NGS). Use FrameStar® 96 Well Semi Skirted PCR to set up your sample plate. Well G12 contains the PEP-PCR blank. This reaction should not give any product. Expected results are shown here:

Expected result



Note

If no amplification is observed, check if enough template from the PEP-PCR was added to the TD-PCR, this could be a pipetting error only. But if no amplification is observed after checking this step, try 5-30 ng genomic DNA as template for the PEP-PCR step.
The PEP-PCR blank amplified after TD-PCR should yield no product. If some amplification is observed, a component of either PCR mix might be contaminated. Start again from PEP-PCR step.

90 Add 5 µL of 3 M sodium acetate pH 5.2 to each well of a deep well plate with a multichannel pipette.

91 Transfer the contents of the TD-PCR plate to their corresponding wells of the deep well plate and seal it with packaging tape.

92 Spin down the plate briefly, vortex gently and spin down again

93 Remove the tape and add 35 µL isopropanol with a multichannel pipette. Seal the plate, vortex thoroughly, and spin down briefly.

Note

If isopropanol is not available, add 100 uL of 100% absolute ethanol.

94 Incubate the plate at -20°C for at least 2 h or overnight.

95 Spin down the plate at 3000 rpm for 25 min at room temperature.

96 Discard supernatant by flipping the plate over an empty plastic container and blot for 5 sec over several layers of paper towels. Flip back to the upright position.

97 Add 100 µL of 70% ethanol. Dry the top of the plate with a paper towel and seal. Let it sit on the bench for ~ 10 min.

98 Spin down as above.

99 Discard supernatant as above.

100 Air dry on the bench for 0.5-1 h or at 37°C for 10 min or until all traces of ethanol have evaporated.

Note

See note in step 41

101 Add 30 µL 10 mM Tris-HCl pH 8 to each well with a multichannel pipette, seal the plate and let it resuspend overnight at 4°C.

102 Proceed to the DNA digestion step.

go to step #4