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Library Preparation for Illumina Sequencing of Microsatellite Loci

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We use this protocol and it's working

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Disclaimer

Abstract

Through several decades, microsatellite length polymorphisms have been considered the most reliable and reproducible genetic markers for population genetic studies. As a PCR-based method using taxon-specific primers, microsatellite loci can be amplified even from low-concentration, degraded or contaminated DNA samples. However, known issues with the interpretation of microsatellite length data include band shifts, stuttering, length homoplasy, null alleles, paralogs or off-target amplicons and missing information on allele dosage in polyploids. Comparing the actual sequences at and around microsatellite loci, instead of just their length according to capillary electrophoresis, may alleviate some of these difficulties. Since multiple microsatellite loci are needed to achieve representativeness of the marker system across the genome, but multiple PCRs for large numbers of samples soon become too cost- and time-intensive, we combine multiple locus-specific PCR reactions in one or several multiplex PCRs. PCR products are subsequently prepared for Illumina sequencing, including the integration of sample-specific combinatorial sequence tags.

This protocol is based on the BeGenDiv Amplicon dual indexing protocol (general structure; in turn based on Illumina's 16S Metagenomic Sequencing Library Preparation protocol), Lepais et al. 2020 for multiplex SSR amplification, and Information from the Illumina and UC Davis Genome Center websites about size selection with magnetic beads.

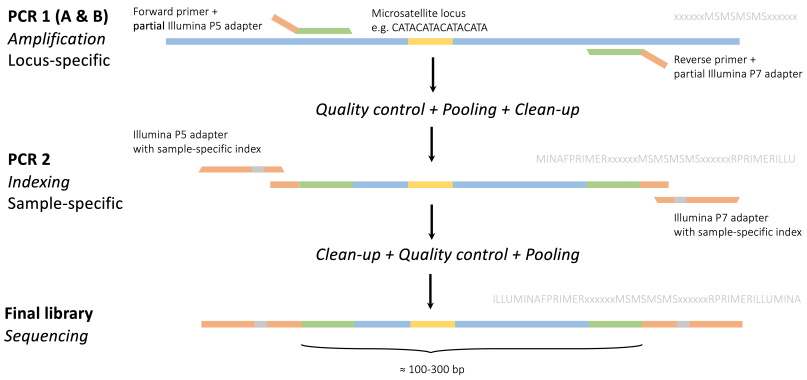

Figure 1. Schematic overview of the process described and format of DNA products obtained with this protocol.

Image Attribution

K. Reichel, modified from L. Drautz.

Materials

Reagents

- Locus-specific primers with partial Illumina tail (see below)
- Sample-specific Illumina primers
- gDNA of sufficient quality / quantity to serve as template
- high-precision polymerase, e.g. Herculase (Agilent), and its buffer
- dNTPs
- DMSO
- magnetic beads, e.g. CleanNA beads (GC biotech)
- Ultra-pure Water
- TE buffer
- Ethanol

Consumables

- 96 well plates or PCR tubes

Equipment

- Pipettes and tips
- Thermocycler
- Gel electrophoresis equipment & chemicals – see SOP on gel electrophoresis
- Qubit & chemicals
- Tape Station / Fragment analyser (automated gel electrophoresis) & chemicals
- optionally: Plate reader (FLUOstar OPTIMA), qPCR

Locus-specific primer design

For this protocol to work, locus-specific primers have to be extended by Illumina overhangs on their 5′ ends (i.e. new primer sequence always starts with the Illumina overhang). These are:

F primer (5′>3′): TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
R primer (5′>3′): GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

Between the Illumina overhang and the locus-specific primer, 0-4 unspecific bases may be added during primer synthesis, to increase the diversity of the bases being read synchronously. However, for small numbers of samples and diverse target loci, this may be unnecessary.

Troubleshooting

Before start

If you have not previously used the primers / primer pools or polymerase you will work with, first test their compatibility on some samples for which you have a lot of material / gDNA. Testing primer pools in silico with software such as PrimerPooler (Brown et al. 2017) or MultiPLX (Kaplinski et al. 2005) has so far proved uninformative, but may serve as inspiration for suitable pools.

PCR 1 – Locus-specific (multiplex) PCR

1 For each sample and pool, plus a negative control containing no template, run a PCR with the following protocol:

	Volume [µl]	Reagent	Stock concentration
	12.9	dd H2O	
	4.0	Polymerase buffer	5x
	0.4	dNTP mix	25 mM each
	0.3	HiFi Polymerase (Herculase)	5 U/µl
	1.6	Primer mix (F+R)	10 pm/µl each
	0.8	Template DNA	~ 10 ng/µl
	20.0	*Total volume*	
	Programme	95°C 2' 30'', [95°C 30'', Ta°C 30'', 72°C 30'']30, 72°C 2', 4°C ∞	

Table 1: PCR 1A conditions.

The annealing temperature **Ta** should be set according to the properties of your primers. If working on PCR plates, make sure to use the exact same order of samples on each plate, to ease pooling later on.

2 Check your PCR products on a 1-1.5% agarose gel (see Fig. 2).

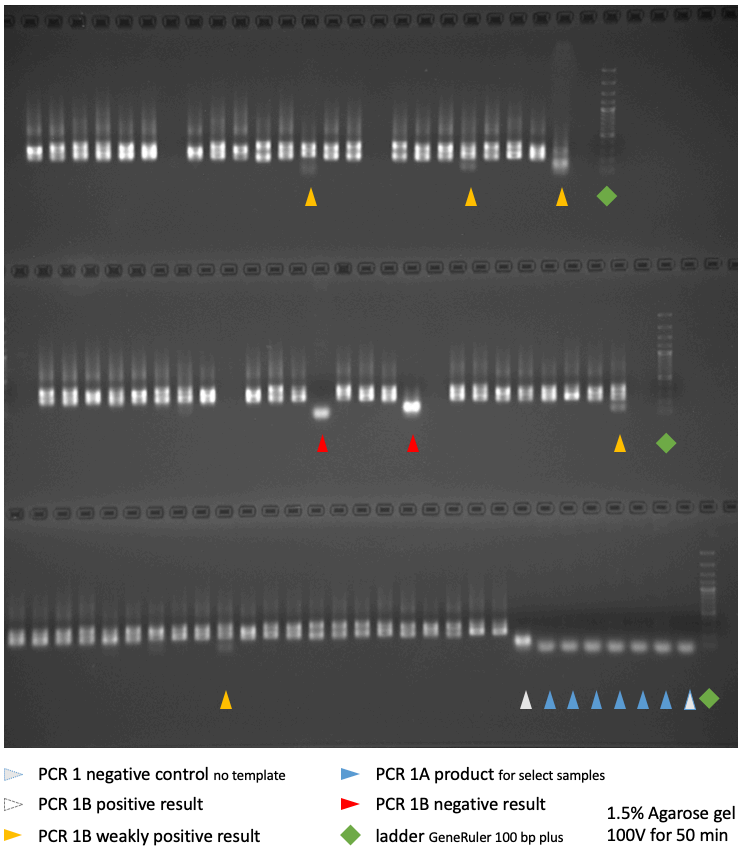

Figure 2. Examples of PCR 1B results for one multiplex PCR reaction. Result after PCR 1A and negative controls for comparison.

3 If necessary, supplement the first PCR (PCR 1A, step 1) with a second round of PCR, adding only new (diluted) polymerase to the samples (PCR 1B):

	Volume [µl]	Reagent	Stock concentration
	18.0	Product of PCR 1A	
	1.8	dd H2O	
	0.2	HiFi Polymerase (Herculase)	5 U/µl
	20.0	*Total volume*	
	Programme	95°C 2' 30'', [95°C 30'', Ta°C 30'', 72°C 30'']30, 72°C 2', 4°C ∞	

	Volume [µl]	Reagent	Stock concentration

Table 2: PCR 1B conditions.

All settings of the original PCR programme (step 1) are kept identical.

4 Check your PCR products on an agarose gel again (see step 2).

Equimolar locus pooling

5 For each sample and pool, determine the DNA concentration of the PCR product using fluorometric quantification (Qubit). When working with many samples, either use a plate reader or select samples which appear to have very high, very low and “typical” product concentrations according to the agarose gel (step 4).

6 Optionally check peculiar / “aberrant” PCR products against negative and positive (“typical”) controls in an automated electrophoresis system (Tape Station or Fragment Analyzer).

7 Calculate the amount of PCR products to transfer for each sample from each primer pool in PCR 1, so that the amount of DNA per pool (or, more correctly still, the product per primer pair in each pool) are the same.

8 Transfer the PCR product amounts calculated in step 7 into one new tube per sample / one well per sample on a new plate.

Bead clean-up 1

9 Freshly prepare at least 400 µl * no. of samples of 70% ethanol for washing.

10 Bring magnetic beads to room temperature and vortex thoroughly, then follow the size selection protocol (single-sided clean-up):

10.1 Add beads in a **0.8** : 1.0 (beads : PCR product) ratio to the pooled PCR products and mix by pipetting up & down several times.

	A	B	C	D	E	F	G	H
	Sample [µl]	10	15	20	25	30	40	50

	A	B	C	D	E	F	G	H
	Beads [µl]	8	12	16	20	24	32	40
	Total [µl]	18	27	36	45	54	72	90

Table 3: Bead clean-up sample and bead volumes.

10.2 Incubate at room temperature for 5 min (without shaking).

10.3 Place tubes / plate on magnetic rack for 2-5 min; beads should aggregate in a pellet close to the magnet, solution should be clear.

10.4 While still on the magnetic rack, carefully remove and discard the supernatant without disturbing the bead pellet (have NO beads in the pipet!).

10.5 Add 200 µl of 70% Ethanol (from step 9) to each sample and incubate for 10 s.

10.6 Remove and discard supernatant.

10.7 Repeat the two previous steps.

10.8 Air dry the bead pellets (room temperature or incubate at 37°C) for approx. 10 min with opened lids (do not overdry the beads!).

10.9 Add the same amount of 1x TE buffer to each sample as corresponds to its original volume after step 8.

10.10 Remove samples from magnetic rack, vortex gently and incubate for 5 min at room temperature.

10.11 Place tubes / plate on magnetic rack for 2 min; beads should aggregate in a pellet close to the magnet.

10.12 Transfer the supernatant (about 90-95% of the original volume) for each sample into a new tube per sample / one well per sample on a new plate.

11 Optionally check the outcome (band removal, uniformity of sample concentration) on a gel and/or automated electrophoresis system. If necessary, adjust the bead volume ratios (more beads ≈ less removal).

PCR 2 – Sample-specific PCR

12 For each sample, plus a negative control containing no template, run a PCR with the following protocol, **giving each sample a unique combination of P5/P7 primers**:

	Volume [µl]	Reagent	Stock concentration
	to total	dd H2O	
	4.0	Polymerase buffer	5x
	0.2	dNTP mix	25 mM each
	0.2	HiFi Polymerase (Herculase)	5 U/µl
	0.5	Index-Primer P5	10 pm/µl
	0.5	Index-Primer P7	10 pm/µl
	0.8	DMSO	
	2-10	solution of PCR product	~ 10 ng/µl
	20.0	*Total volume*	
	Programme	95°C 2', [95°C 20'', 52°C 30'', 72°C 30'']8, 72°C 3', 4°C ∞	

Table 4: PCR 2 conditions.

The volume of the PCR product depends on the concentration after clean-up (step 11).

Bead clean-up 2

13 Perform clean-up (step 10).

14 Repeat the previous step, re-suspending the DNA in 17 µl 1x TE at step 10.9.

Library check and equimolar sample pooling

15 Check all PCR products, including the negative control and a subset of templates, on a gel (see step 2).

16 Compare template and cleaned product for a selection of samples on an automated electrophoresis system. There should be a clear length shift between template and products, although indexing doesn't work perfectly and some un-indexed fragments will remain.

17 Depending on the uniformity of concentrations and indexing success as estimated through steps 15 and 16, choose one of three options:

concentrations / indexing ~ uniform:
A – pool PCR products from all samples with the same volume

indexing ~ uniform:
B – get fluorometric measurements from all samples (plate reader) and pool equimolarly based on these values

else:
C – perform qPCR on all samples (BeGenDiv) and pool equimolarly based on these values

If available, a fourth alternative is to test-sequence the library and then re-pool the samples according to the sequencing output for each sample.

18 Hand over your pooled library to the sequencing facility.

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