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A large genome center's improvements to the Illumina sequencing system

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Supplementary Table 1. Summary table of modifications and improvements

step	modification	benefit	Supplementary Protocol
Fragmentation	AFA	Greater proportion of DNA in desired size range.	1
Post-fragmentation	Double size selection	Fewer chimeric templates	5
Ligation	i) ultrapure ligase; ii) ubiquitous use of PE oligos	i) higher yield of adapter-ligated template; ii) convenience and flexibility	2; 3
PE size selection	Thin gel slice	Improved robustness	4
Gel extraction	Cold dissolution of gel slices	Decrease in GC bias	4
PCR	i) optimised template quantity; ii) optimised PCR conditions; iii) SPRI beads iv) PCR-free sequencing v) direct sequencing of short amplicons	i) cleaner libraries, fewer PCR duplicates; ii) improved yield, fewer cycles of amplification; iii) fewer adapter dimers; iv) removal of duplicates; v) reduced amplification bias	6; 7; 8; 9; 10

Quantification	qPCR assay	Accurate prediction of cluster density	11
Denaturation	Modified hybridisation buffers	Counteracts pipetting errors and allows sequencing of dilute templates	12
Amplification	QC step	Allows verification of cluster amplification and density	13

Supplementary Protocol 1: Adaptive Focused Acoustics (Covaris Incorporated)

To fragment samples to 200bp +/-

Duty Cycle: 5%
Intensity:10
Cycles per burst: 200

For 4.5µg: 12 min
For 2.25µg: 18 min
For 1.12µg: 22 min

Supplementary Protocol 2: Paired End oligos

Paired end libraries are compatible with both paired and single end flowcells, and so we generally make all of our libraries with paired end oligos, as this gives us more flexibility and, a library that was first run single ended can be rerun as paired end without repeating library prep.

Supplementary Protocol 3: Gel extraction / Paired end size selection

Taking a 2mm gel slice for PE libraries greatly improves the robustness of the sample prep. Care must be taken to cut horizontally so as not to increase the size range of fragments. DNA is then extracted as for a single end library, using a chaotropic buffer and spin column. It is essential to dissolve gel slices in chaotropic buffer at room temperature rather than by heating. This typically takes 5-10 minutes with frequent mixing.

Supplementary Protocol 4: Ligation

As with the standard protocol, ligations with ultrapure T4 ligase (Enzymatics Incorporated) are performed at room temperature for 15 minutes, in a total volume of 50µl. Reactions contain 10µl end-repaired, A-tailed DNA sample, 1x DNA ligase buffer, 10µl Illumina adapter oligo mix and 3 units of ultrapure DNA ligase. Following ligation, reaction products are purified with a spin column.

Supplementary Protocol 5: Double size selection

Blunt-ended ligation of two or more short fragments can occur during the ligation step, as a result of incomplete A-tailing. If the resulting chimeric fragment happens to fall within the desired size range, it will be excised and extracted along with the fragments which are genuinely that size, after the gel size selection step. To avoid this, after fragmentation and

purification of sample DNA, the entire column eluate is run in 4 wells of an 2% agarose gel, alongside a 100bp ladder. For a 500bp library, a horizontal slice of the intended fragment size +/- 100bp is taken (narrower than this for shorter insert libraries). Thus chimeric fragments will be 800bp or longer and will be removed in the regular size selection step that follows.

Supplementary Protocol 6: Template quantity

We use 3ng DNA and 14 cycles of PCR amplification for single end libraries, 10ng DNA and 18 cycles for high complexity libraries, and 25ng DNA and 12 cycles for lower complexity samples. These quantities give the optimal compromise between clean libraries and a low frequency of duplicate sequences.

Supplementary Protocol 7: PCR yield

Using 2 units Platinum Pfx polymerase (Invitrogen) in the supplied buffer at 1x concentration, with 2mM $MgSO_4$, 400nM dNTPs, 1μM primers the PCR yield can be increased up to 10-fold over the standard amplification conditions.

Supplementary Protocol 8: PCR cleanup

Rather than clean PCR products with a spin column, we use AMPure beads (Agencourt BioSciences Corporation), following the manufacturer's protocol.

Supplementary Protocol 9: Sequencing without PCR

This protocol is available from the corresponding author upon request (djt@sanger.ac.uk).

Supplementary Protocol 10: Direct sequencing of short amplicons

This is achieved by attaching a tail on the amplicon-specific primers that will allow amplicons to hybridise directly to the flowcell after PCR. Sequencing is performed using the tailless specific primers at a concentration of 500nM.

[AATGATACGGCGACCACCGAGATCTACACT] [specific primer 1]
[CAAGCAGAAGACGGCATACGAGAT] [specific primer 2]

Supplementary Protocol 11: Quantitative PCR

c_qPCR_v2.1	AATGATACGGCGACCACCGAGATC
PE_qPCR_v2.2	CAAGCAGAAGACGGCATACGAGATC
DLP	[6FAM]CCCTACACGACGCTCTTCCGATCT[TAMRA]

PCR primers c_qPCR_v2.1 and PE_qPCR_v2.2 were desalted, whereas DLP was HLPC purified.

All dilutions are performed in water or 10mM Tris pH8.5 (buffer EB), with 0.1% Tween-20 added, and in low-bind tubes, to avoid degradation of DNA, and adhesion to the walls of the tubes. It is essential to avoid buffers containing EDTA.

Standards are chosen to be of a similar insert size range as the unknown, and are libraries that have been sequenced previously – i.e. for which cluster number and loading concentration are known. Standards and unknown libraries are run in duplicate / triplicate. Standards are diluted to 100pM, 10pM and 1pM, based on e.g. the Agilent Bioanalyzer 2100 concentration. Unknown samples are diluted to 10pM, also based on their Bioanalyzer concentration, allowing a 10x inaccuracy on either side.

qPCRs are carried out in a total volume of 25µl. Final concentrations are given in parentheses.

2.5µl	10x Platinum taq buffer (Invitrogen)
0.75µl	50mM $MgCl_2$ (→ 1.5mM)
2.5µl	template DNA
0.625µl	10µM DLP (→ 250nM)
0.5µl	50x Rox (Invitrogen; → 1x)
0.75µl	c_qPCR_v2.1 (→ 300nM)
0.75µl	PE_qPCR_v2.2 (→ 300nM)
2µl	2.5mM dNTPs (→ 200µM)
0.1µl	Platinum Taq (Invitrogen; → 0.04 units / µl)
14.525µl	H_2O

Cycling conditions:

94C for 2 min

94C 15sec	
62C 15sec	x 40
72C 32sec	

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Supplementary Protocol 12: Modified hybridisation buffers

For subnanomolar libraries (must be at least 24pM), prepare neutralization buffer:

1.5M sodium chloride
0.15M sodium citrate
0.1% Tween-20
Adjust pH to 4.6 with HCl

Filter with 0.2µm vacuum filter

Add 1M Tris-HCl pH7.3 to give a final Tris concentration of 5mM. Mix by shaking. Prepare 0.2N NaOH, by diluting supplied 2NNaOH with supplied UltraPure water. Prepare Tris-Hyb buffer by adding 60µl 1M Tris-HCl pH7.3 to new 12ml bottle of Illumina Hybridisation Buffer, to give a final Tris concentration of 5mM. Mix thoroughly by shaking.

Pipette 5µl low concentration library into 200µl tube, add an equal volume of 0.2N NaOH, vortex, and incubate at room temperature for 5 minutes.

Add 20µl neutralization buffer.

Add appropriate volume of Tris-Hyb buffer to give final loading concentration. pH can be checked with Universal Indicator paper. It should be 7-8.5. If higher, adjust with 0.1M HCl, but we have never found this to be necessary.

For routine denaturation, 0.2N NaOH and Tris-Hyb buffer are still easier to work with than the supplied 2N NaOH and Hybridisation Buffer, as pipetting inconsistencies are minimized.

Supplementary Protocol 13: Amplification QC

Immediately following amplification, the double stranded clusters can be stained by manually flowing a filtered solution containing 0.1M Tris-HCl pH8.0, 0.1mM sodium ascorbate (Sigma) through the flowcell on the cluster station (75µl per channel over 5 minutes), followed by 0.1M Tris-HCl pH8.0, 0.1mM sodium ascorbate and 2x SybrGreen I (Invitrogen; 150µl per channel over 10 minutes). Clusters can then be visualised on a fluorescence microscope and the cluster density assessed. Afterwards, we return the flowcell to the cluster station and flush through with storage buffer (reagent #12, 150µl per channel over 10 minutes), before storage or linearization & blocking.

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