

time PCR on the Illumina Eco instrument or by conventional PCR. The reasoning was that by qPCR, female should have slightly more PLP1 (chrX) signal than male. By conventional PCR, we were able to check amplicon size and uniqueness. Both tests yielded data consistent with the interpretation that all eight assays performed well.

- 5 **PCR reaction set up:** For each female or male PCR reaction, a 250 µl master mix was made on ice containing:
- 100 µl of water
 - 25 µl of 10X STD Taq buffer
 - 25 µl of 25 mM MgCl₂
 - 10 • 25 µl of 60 ng/µl sheared gDNA (female and male were the same concentration by Qubit)
 - 12.5 µl DMSO
 - 12.5 µl 10 mM dNTPs
 - 6.25 µl EvaGreen dye (Biotum)
 - 15 • 5 µl ROX dye (Invitrogen)
 - Mix well and add 2.5 µl Taq DNA polymerase.

Table 7: Cq values from PLP1 qPCR assay validation

	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8
	chrX	chrX	chrX	chrX	chrX	chrX	chrX	chr9
F	23.27371	24.01229		24.19554	22.3413	22.10594	22.65759	22.31407
	23.33794	22.49233	23.87286	22.53665	22.49127	22.19686	22.73586	22.0446
	22.33889	22.40355	23.68538	22.50178	22.42961	22.23099	22.83415	22.32891
M	24.00953	23.56869		23.2648	23.51487	23.11367	23.65077	22.15477
	23.63462	23.48547	23.95081	23.18794	23.25046	22.93675	23.66965	21.23208
	23.77216	23.16852	24.16562	23.21774	22.93373	22.95635	23.61168	22.16391
F	22.98351	22.96939	23.77912	22.51921	22.42073	22.17793	22.74253	22.2292
M	23.80544	23.40756	24.05821	23.22349	23.23302	23.00226	23.64403	21.85026
M-F	0.8	0.4	0.3	0.7	0.8	0.8	0.9	-0.4

- For the experiment, 24 µl of mix was aliquoted into two sets of 8 strip tubes (female or male) and added 6 µl of primer mix that contained 10 µM of the forward and
- 20 reverse primers from each assay. After mixing, three identical 5 µl amounts were aliquoted into columns of a 48-well Eco PCR plate (triplicate female on top in columns, triplicate male samples in columns on the bottom). The instrument was set to monitor SYBR and ROX and to cycle to 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec for 40 cycles. A JPG image of the amplification traces for assay 6 is shown in Figure 9.
- 25 The copy difference between the female and male samples was clear. All of the “Cq”

RESULTS

To interpret the experimental data, the agarose gels that are shown in Figure 14 were examined. Under the cycling conditions used with the primers used (etc.) it was observed that assay sets 3, 5 and 6 yielded PCR products consistent with the assay amplicon (top gel) or the post-processed PLP1 to adaptor amplicons (bottom gel). The more successful assay sets corresponded to:

- Probe 4 with assay 3
- Probe 2 with assay 5
- Probe 3 with assay 4

The qPCR Cq values are shown in Table 16 below. Assays 1 and 2 failed gel analysis. Successful assays are shown in assays 3, 5 and 4. To derive % processing values, Cq's were converted to absolute values (In "Excel speak", Abs value = $\text{power}(10, \log_{10}(1/2) * Cq + 10)$). The quotient of post-processed over capture only was then expressed as a percentage. This measurement assumed that the amplification efficiency of the all amplicons was the same and conforms to an idealized standard curve (probably reasonably accurate). Assuming this was correct, then approximately 10% of captured material appears to be processed.

Table 16: qPCR analysis of post-capture processing sensitivity

	probe 1	probe 1	probe 4	probe 4	probe 2	probe 3
	assay 1	assay 2	assay 3	assay 2	assay 5	assay 4
capture	23	21	25	21	25	26
post-process	16	23	29	22	28	29
% processed			7%		17%	10%

EXAMPLE 10

CONSTRUCTION OF EXPANDED CODE MALE AND FEMALE gDNA LIBRARIES

PURPOSE

Build set of 16 coded male and female gDNA libraries that will be used to test multiple capture parameters in a single MiSeq sequencing run.

METHODS

Step 1: gDNA. Repaired gDNA was prepared.

The reactions were wash twice with 200 µl of 70% EtOH/water (v/v), air dried, and resuspended in 100 µl TEz.

Step 4: qPCR. Make qPCR master mix containing:

- 175 µl of water
- 5 • 50 µl of 10X STD Taq buffer
- 50 µl of 25 mM MgCl₂
- 100 µl of ACA2 primer (10 µM)
- (50 µl of template – added later)
- 25 µl of DMSO
- 10 • 25 µl of 10 mM dNTPs
- 12.5 µl of Eva green
- 10 µl of ROX
- 5 µl of Taq DNA polymerase

9 µl was distributed into the 48 wells of an Illumina Eco qPCR plate. Two
15 serial dilutions of library calibration standard were made that are 10 pg/µl and 1 pg/µl.

The remainder of the plate was loaded with libraries as shown in the tables below.

Table 17: Sample key for 48 well assay plate 1

	1	2	3	4	5	6	7	8
A	10 pg/ul STD	1 pg/ul STD	F1	F2	F3	F4	F5	F6
B	10 pg/ul STD	1 pg/ul STD	F1	F2	F3	F4	F5	F6
C	F7	F8	F9	F10	F11	F12	F13	F14
D	F7	F8	F9	F10	F11	F12	F13	F14
E	F15	F16	M1	M2	M3	M4	M5	M6
F	F15	F16	M1	M2	M3	M4	M5	M6

The second plate had the layout shown in Table 18 below.

Table 18: Sample key for 48 well assay plate 2

	1	2	3	4	5	6	7	8
A	100 pg/ul STD	10 pg/ul STD	1 pg/ul STD	NTC	F15	M1	M2	M3
B	100 pg/ul STD	10 pg/ul STD	1 pg/ul STD	NTC	F15	M1	M2	M3
C	M1	M2	M3	M4	M5	M6	M7	M8
D	M1	M2	M3	M4	M5	M6	M7	M8
E	M9	M10	M11	M12	M13	M14	M15	M16
F	M9	M10	M11	M12	M13	M14	M15	M16

20 Ligation efficiency was measured by the following cycling program:

- 72°C – 2min
- 94°C – 30 sec, 60°C – 30 sec, and 72°C – 60 sec; 40 cycles

For qPCR analysis, genomic DNA from sample F at 10 ng/μl (2 μl is added to 8 μl of PCR mix to give a final volume and concentration of 10 μl and 2 ng/μl, respectively) was used as control. Purified processed material from the F and S samples was diluted to 0.01 ng/μl = 10 pg/μl and 2 μl was added to each 8 μl PCR reaction to give a final concentration of 2 pg/μl. The results are shown in Table 41.

Table 41. qPCR results

Cqs	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8
gDNA	23	22	25	22	22	23	23	19
F-lib	13	13	13	13	13	20	12	35
S-lib	14	14	13	13	14	20	13	N/A
	Assay 9	Assay 10	Assay 11	Assay 12	Assay 13	Assay 14	Assay 15	Assay 16
gDNA	21	21	22	22	22	21	21	21
F-lib	11	13	13	16	18	17	14	13
S-lib	11	13	14	17	17	16	13	12
Abs	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8
gDNA	16	24	4	17	22	15	8	146
F-lib	12150	13296	8874	16739	13738	79	22495	0
S-lib	6142	7704	10410	10093	6425	132	14648	
	Assay 9	Assay 10	Assay 11	Assay 12	Assay 13	Assay 14	Assay 15	Assay 16
gDNA	60	39	18	30	17	43	49	37
F-lib	45447	11671	11034	1288	440	853	6373	16448
S-lib	68935	12477	7634	678	1077	1203	10171	19305
Adj	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8
gDNA	16	24	4	17	22	15	8	146
F-lib	12150333	13296288	8874203	16739230	13737864	79482	22495321	2
S-lib	6141637	7703565	10410406	10093396	6424647	131876	14648060	0
	Assay 9	Assay 10	Assay 11	Assay 12	Assay 13	Assay 14	Assay 15	Assay 16
gDNA	60	39	18	30	17	43	49	37
F-lib	45447322	11670722	11034289	1288163	440236	852919	6373326	16447972
S-lib	68934509	12476684	7634492	677562	1077493	1203428	10170993	19304903
Adj	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8
F-lib	778820	562525	2393851	987542	627702	5247	2657954	0
S-lib	393671	325914	2808248	595466	293551	8706	1730754	0
	Assay 9	Assay 10	Assay 11	Assay 12	Assay 13	Assay 14	Assay 15	Assay 16
F-lib	757399	300823	627284	42799	25665	19716	130461	439562
S-lib	1148823	321597	434010	22512	62817	27819	208198	515912

The qPCR data indicated that the library-free technology is very effective at retrieving the targeted genomic regions and at leaving off-target regions behind (*e.g.*, Assays 6, 8). The fold purifications, often >500,000-fold, were directly comparable to data from earlier experiments generated with libraries as disclosed elsewhere herein.

Part II – Leave-one-out analysis: The enzymatic requirements for complex processing were evaluated: the design of experiment is shown in Table 42 below.