Multiplexed Direct Genomic Selection (MDiGS)-Gurnett Lab (Washington University in St. Louis)

Illumina Multiplexed Library Prep modified from Ramos, E. *et al.* Population-based rare variant detection via pooled exome or custom hybridization capture with or without individual indexing. *BMC Genomics* **13**, 683 (2012).
Biotinylated BAC Capture modified from Bashiardes, S. *et al.* Direct genomic selection. *Nat Methods* **2**, 63-9 (2005).
All pre-capture reactions are for 48 samples. Volumes can be scaled up to 96 samples or post capture pools of 48 can be combined for sequencing. Using 150 ng of genomic DNA, multiplex libraries are typically suitable for up to 10 captures.

Sonication

Start with DNA samples diluted to 50 ng/ul in H2O (Qubit)In an AB-1900 plate (Thermo-Fast), add 50 ul Ampure XP bead solution with 100 ul H2O to each well
Replace media with 106 ul 1x TE and add to each well:
Add 45 ul 5M NaCl
Add 6 ul DNA (300 ng total) (Final volume = 157 ul per well)
Cover wells with strip caps (Bio-Rad TCS0803) and sonicate on a Covaris E210
7mm vertical offset, 10% duty cycle, intensity = 5, 500 cycles per burst, 10 minutes per well
For wells with air pockets, mark wells, centrifuge and repeat 2 minutes per well

Purify and concentrate DNA

Pulse centrifuge 500 rpm and remove strip caps
Add 45 ul 50% PEG 3350 to each well
Cover with film (AB-0558-Thermo Scientific) and vortex > 10 seconds
Incubate 5 minutes at room temp
Pulse centrifuge (500 rpm) and place plate on magnet 2-3 minutes (until beads settle on well wall)
Wash each well 2x with 75 ul 70% EtOH
Dry 5 minutes, **MAKE SURE ALL WELLS ARE DRY BEFORE PROCEEDING**
Add 10 ul H2O to each well, cover with film and vortex 10 seconds
Incubate 2 minutes and pulse centrifuge

Agarose QC concentration and shearing
Remove 1 ul supernatant (bead separated) from 2 random wells, replace with 1 ul of H2O
Run 1 ul on an agarose gel to confirm shearing and DNA concentration **(Expect 300-800 bp)**

End Repair DNA using NEB reagents

100 ul T4 DNA Ligase Buffer with 10 mM dATP
50 ul T4 DNA Polymerase
50 ul T4 PNK
40 ul 10 mM dNTPs
10 ul Klenow (DNA Polymerase I, Large Fragment Klenow)
250 ul H2O
Aliquot 10 ul to each well (20 ul final volume) Cover with film, vortex and pulse spin

Incubate 30 min at 20C

PEG Cleanup

Add 20 ul of 25% PEG 3350/2.5M NaCl mix (2 ml 50% PEG + 2 ml 5M NaCl)
Cover and vortex for > 10 seconds
Incubate 5 min and pulse spin
Wash 2x with 75 ul 70% EtOH and dry 5 min
Add 10 ul H2O, cover and vortex 10 seconds
Incubate 2 min and pulse spin **(Store at 4C or proceed with A-Tailing. Do not freeze)**

Adenylate 3' ends

100 ul Klenow Buffer (NEB Buffer 2)
340 ul 1mM dATP
60 ul Klenow exo-
Aliquot 10 ul to each well
Pulse spin, vortex and pulse spin
Incubate 30 min, 37C

Add 20 ul of 25% PEG 3350/2.5M NaCl mix (2 mL 50% PEG + 2 mL 5M NaCl)
Cover and vortex for > 10 seconds
Incubate 5 min and pulse spin
Wash 2x with 75 ul 70% EtOH and dry 5 min
Resuspend in 8.75 ul water, cover and vortex 10 seconds
Incubate at room temp for 2 min and **PROCEED IMMEDIATELY TO LIGATION**

Ligate Adapters

Add 2.5 ul pre-annealed indexing adapters to each well (Ramos et al., 2012 BMC Genomics)
Make Buffer/H2O master mix for 50 samples:
625 ul 2x Ligation Buffer
62.5 ul T4 DNA Quick Ligase
Aliquot 13.75 ul to each well
Cover, vortex and pulse spin
Incubate 15 min at 20C
Add 2.75 ul 0.5M EDTA (Stop Solution), cover and vortex (27.75 ul final volume)

Remove unligated adapters

Add PEG/NaCl mix to achieve a final PEG concentration of 9.5% w/v
200 ul H2O
1,216 ul 50% PEG
1600 ul 5M NaCl
Aliquot 24.75 ul of PEG/NaCl mix to 27.75 ul of Stopped Ligation Reaction
Final Volume = 52.5 ul
Cover and vortex for > 10 seconds
Incubate 5 min and pulse spin
Wash 2x with 75 ul 70% EtOH and dry 5 min
Add 17 ul H2O, vortex and incubate 2 min at room temp

Spin and transfer 17 ul to fresh plate -> Qubit 1-2ul of each sample

Pool Multiplexed Library and Repeat PEG Purification

Aim to pool a total of 50 ng of DNA per individual in sets of 48.
If samples are less than 50 ng, do not titrate down the DNA from other samples to match the lower samples as this will increase the PCR duplication percentage throughout the entire data set.

Remove un-incorporated adapters prior to Pre-Capture PCR amplification
Repeat 9.5% PEG purification on pooled samples prior to Pre-Capture PCR using formula:

Final Volume (**FV**) =Pooled DNA volume * 1.82
50% PEG (**P**) = FV/5.26
5M NaCl (**N**) = FV/4
H2O (**H**) = **FV** – P- N- DNA

In Lo-Bind (Eppendort cat# 022431021) tube add:
50 ul AmpureXP beads + 100 ul H2O and replace supn't with Pooled Multiplexed DNA Library
Add **N** ul of 5M NaCl
Add **P** ul of 50% PEG and
Add **H** ul of H2O so that final concentration = 9.5% PEG and 1.25 M NaCl
Incubate 5 min, wash 2x with 500 ul 70% EtOH -> Elute in 50 ul H2O and Qbuit. (Expect ~40 ng/ul)
Transfer 1ug to a PCR tube and take up to 50 ul with H2O.
Run 2.5 ul on a gel to confirm adapter removal and check quality

Pre-Capture PCR

Add directly to multiplexed library elution in PCR tube:	
2.5 ul	Pre-Hyb Primer F (10 uM)
2.5 ul	Pre-Hyb Primer R (10 uM)
2.5 ul	10 mM dNTPs
55 ul	NEBNext High-Fidelity 2x PCR Master Mix
110 ul	Final Volume
Save 5 ul as Pre-PCR sample	

PCR Conditions

98C x 10 sec
6 Cycles:
98C x 20 sec
57C x 30 sec
72C x 30 sec
72C x 5 min
4C hold

Run 5 ul of Pre-PCR and 5 ul of Post-PCR on an agarose gel (100 ul of Post-PCR remaining)
Successful adapter ligation should give a brighter band for the Post-PCR compared to Pre-PCR product
Primer may be visible < 100, determine if adapter dimer is present (120 bp band)

If there is no adapter dimer:
Add 180 ul AmpureXP beads in stock solution to 100 ul Post-PCR product in Lo-Bind tube
Standard 5 min incubation/500 ul 70% EtOH washes (x2) and elute in 50 ul H2O
If there is adapter dimer (120 bp band):
Prepare 50 ul AmpureXP beads + 100 ul H2O in a Lo-Bind tube
Replace supernatant with 100 ul Post-PCR product
Add 45 ul 5M NaCl
Add 34 ul of 50% PEG (Final volume = 179 ul (9.5% PEG, 1.25M NaCl)
Standard 5 min incubation//500 ul 70% EtOH washes (x2) and elute in 25 ul H2O
Qubit 1 ul of purified sample (Expect 50-100 ng /ul)

Biotinylated BAC Hybridization

Biotinylate BAC (Nick Translation)

500 ng BAC
5 ul dNTP minus dTTP (0.2 uM each)
1 ul Biotin-16-dUTP (1mM)
1 ul Unlabeled dTTP (0.1mM)
Up to 45 ul with H2O
5 ul DNA Polymerase/DNase mix
Incubate 1 hr 15C
Add 5 ul 0.5M EDTA and 50 ul H2O
G-50 Sephadex purify and take up to 100 ul with H2O
Store at -20 or proceed with hybridization

BAC Capture

Combine in a 1.5 mL tube and lyophilize:
100 ng Biotinylated BACs at equimolar concentrations (Capture Region)
250 ng Non-Biotin sheared blocking (Non-Capture Region)*
5 ul Human Cot-1 DNA (1 ug/ul)
*Blocking BAC can be any non-biotin labeled BAC from outside the capture region that has been sheared to ~300-800 bp

Resuspend in 2.5 ul H2O and transfer to PCR tubes
Denature 95C - 5 min
Incubate 65C - 15 min
Add 2.5 ul 2x Hybridization and incubate 5 hrs at 65C

Combine in a 1.5 mL tube and lyophilize:
1 ug of Multiplexed Library (Use 1 ug regardless of sample size)
2.5 ul Blocking Oligo 1 (100 uM)
2.5 ul Blocking Oligo 2 (100 uM)
1 ul Post-Hyb R (100 uM)
Resuspend in 2.5 ul H2O and transfer to PCR tube
Denature 95C - 5 min
Incubate 65C - 15 min
Add 2.5 ul 2x Hybridization buffer and transfer total (5 ul) to BAC Hyb (10 ul final volume)
Add a drop of mineral oil to prevent evaporation and incubate 65C for 70hrs

Bind and Wash Hybridizations

Wash 100 ul Streptavidin beads with 200 ul Binding Buffer in Lo-Bind tube for each capture
Add 150 ul Binding Buffer to Hybridization and transfer to washed beads
Incubate 30 min at room temp, rocking
Wash 1x with Wash buffer 1, 15 min room temp rocking
Pre-warm Wash Buffer 2 to 65C
Wash 3x with Wash buffer 2, 15 min each at 65C
Elute in 50 ul 0.1M NaOH, 10 min 25C -> Transfer to Lo-Bind tube and add:
50 ul 1M Tris-HCl
180 ul AmpureXP beads
Incubate 5 min at room temp and wash with 2x 500 ul 70% EtOH
Elute in 44 ul H2O

Post Hyb PCR

Amplify captured products with Post-Hyb Linker primers:

1 ul	10mM dNTPs
50 ul	NEBNext Polymerase (2x)
2.5 ul	Post-Hyb primer F (10 uM)
2.5 ul	Post-Hyb primer R (10 uM)

Add 44 ul re-suspended capture product (100 ul final volume)

98C x 30 sec
16 Cycles
98C x 20 sec
57C x 30 sec
72C x 30 sec
72C x 5 min
4C hold

In 2 Lo-Bind tube, add 50 ul AmpureXP beads and 100 ul H2O
Replace supernatant with 100 ul Post-PCR Product and add:

50 ul	5M NaCl
39 ul	50% PEG
11 ul	H2O

Final volume = 200 ul (9.75% PEG and 1.25 M NaCl)
5 min hybridization and wash with 2x 500 ul 70% EtOH
Elute in 20 ul H2O

Qubit 1 ul of each using high sensitivity kit
Multiplexed DNA Library should be >30 ng/ul

Reagents

2x Hybridization Buffer: 1.5 M NaCl, 40 mM sodium phosphate buffer (pH 7.2), 10 mM EDTA (pH 8.0), 10x Denhardt's, 0.2% SDS

Streptavidin Bead Binding Buffer: 10 mM Tris-HCL (pH 7.5), 1 mM EDTA (pH 8) and 1 M NaCl

Wash Buffer 1: 1X SSC with 0.1% SDS

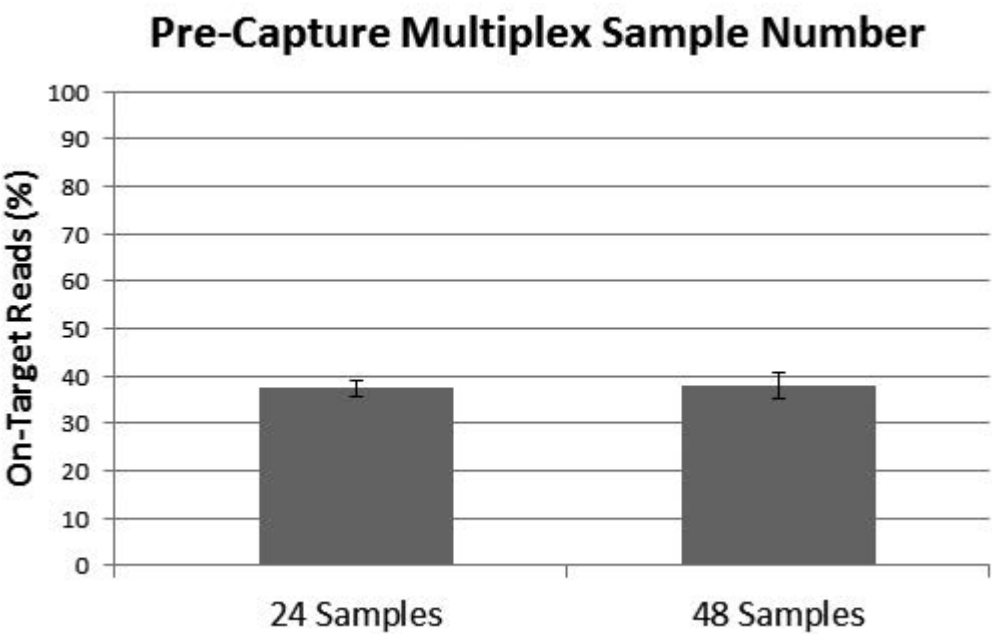
Wash Buffer 2: 0.1X SSC with 0.1% SDS

Elution Buffer: 0.1 M NaOH

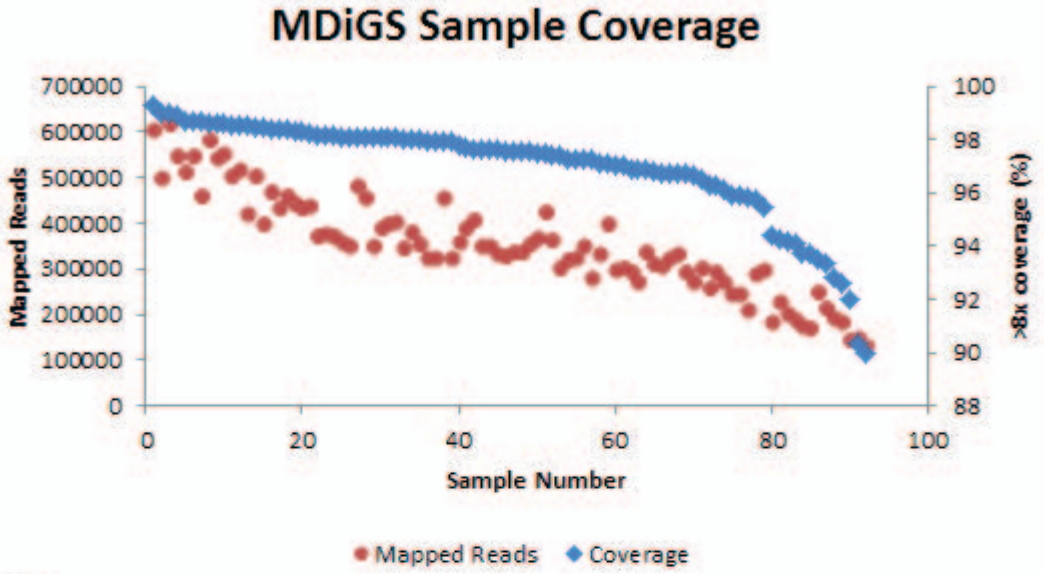
Supplier	Catalog Number	Reagent
NEB	MO203L	T4 DNA Polymerase
NEB	M0201L	T4 PNK
NEB	M0210L	Klenow
NEB	M0212L	Klenow (exo-)
NEB	M2200L	Quick Ligation Kit
NEB	M0541L	NEBNext High-Fidelity 2x PCR Master Mix
Invitrogen	112.05D	Dynabeads M-280 Streptavidin
Beckman Coulter	A63880	AmpureXP Beads
Sigma	P3640-500G	PEG3350
Roche	11093070910	Biotin-16-dUTP
Invitrogen	15279-011	Human Cot-1 DNA
Invitrogen	18160-010	Nick Translation System

PCR Primers and Blocking Oligo Sequences:
Indexing adapter sequences are described in Ramos, E. *et al. BMC Genomics* **13**, 683 (2012).

Pre-Hyb F	CAAGCAGAAGACGGCATACGAGAT
Pre-Hyb R	AATGATACGGCGACCACCGAGATC
Blocking Oligo 1	CAAGCAGAAGACGGCATACGAGATIIIIIIIGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Blocking Oligo 2	AGATCGGAAGAGCACACGTCTGAACTCCAGTCACIIIIIIITCTCGTATGCCGTCTTCTGCTTG
Post-Hyb F	CAAGCAGAAGACGGCATACGAGAT
Post-Hyb R	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT


Pre-Capture Multiplex Sample Number
On-Target Reads (%)
100
90
80
70
60
50
40
30
20
10
0
24 Samples
48 Samples

A


MDiGS Sample Coverage
Mapped Reads
>8x coverage (%)
Sample Number
Mapped Reads
Coverage

B

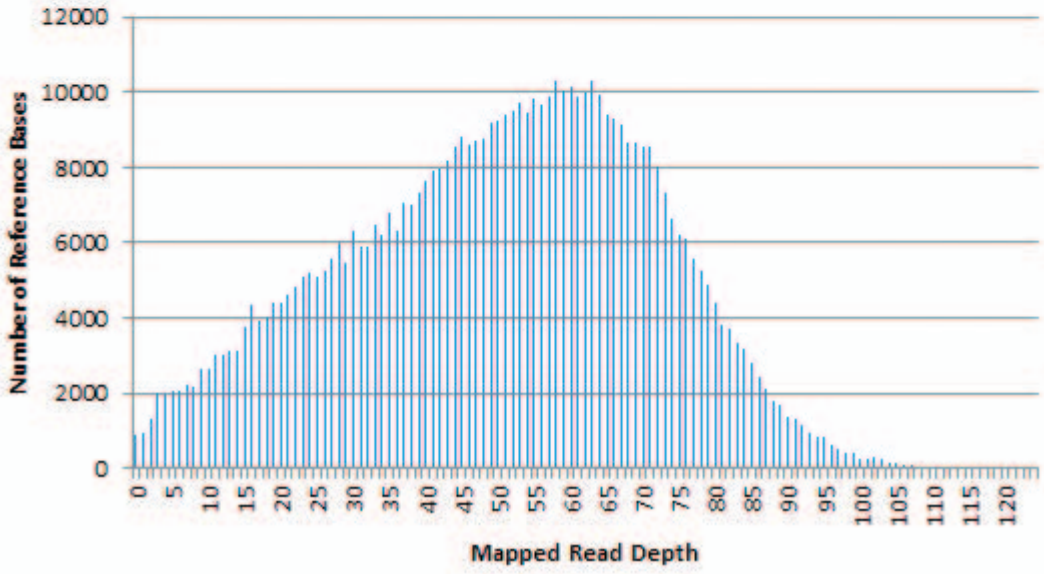
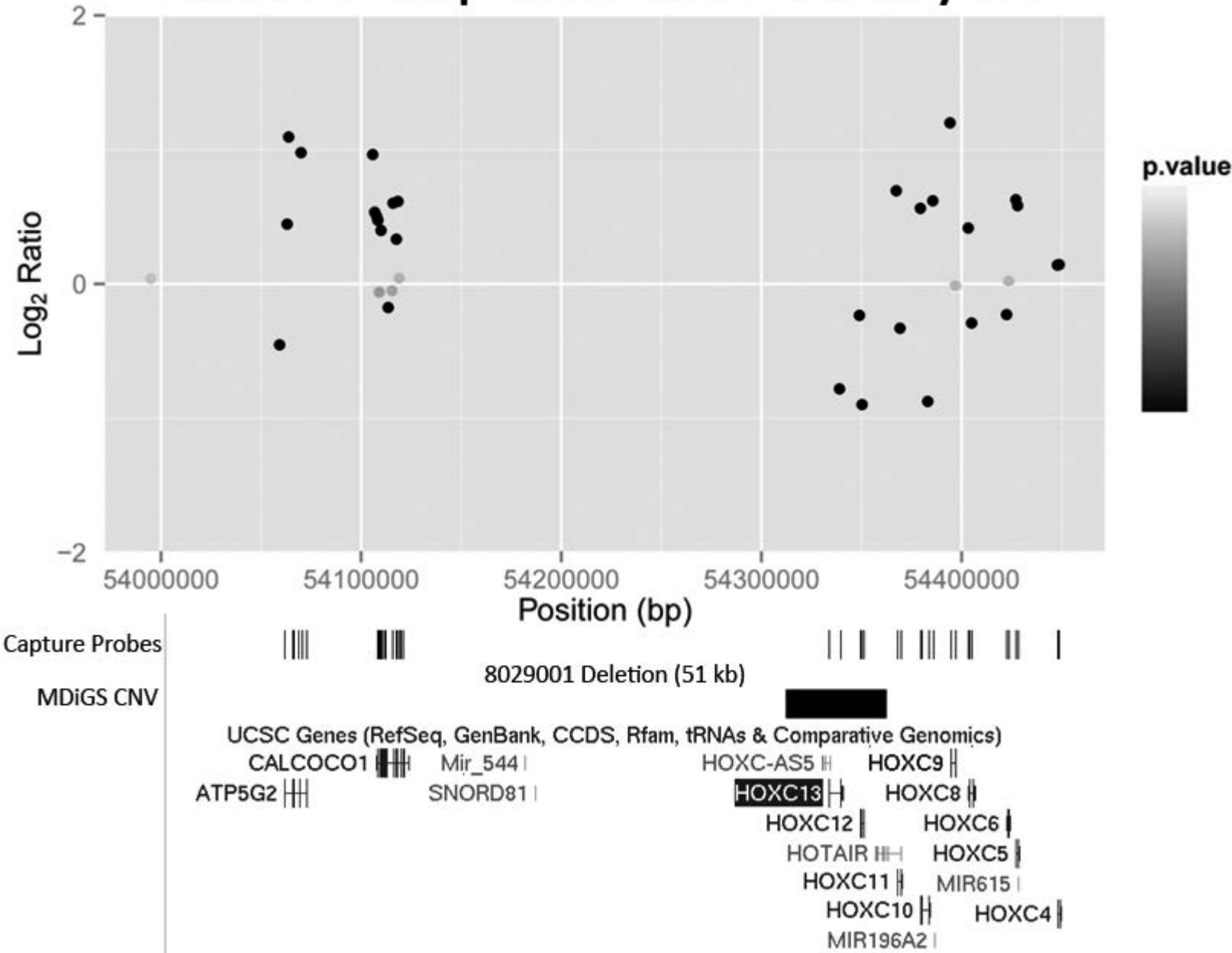

MDiGS Coverage Histogram
Number of Reference Bases
Mapped Read Depth

Table 1. Rare variants (MAF<0.01) in patients with lower limb malformations (n=168)

CHR	POS	rsID	dbSNP[a]	EVS[b]	GENE	REF	ALT	GVS	PP	AA
5	134367152	.	.	.	PITX1	CG	C	frameshift	.	.
12	54333343	.	.	.	HOXC13	C	A	missense	218/331	ALA/ASP
12	54338922	.	.	.	HOXC13	G	A	missense	292/331	ARG/HIS
12	54349241	rs189468720	.	.	HOXC12	C	G	missense	176/283	ASN/LYS
12	54350180	rs144078525	.	0.0015	HOXC12	G	A	missense	227/283	ALA/THR
12	54357753	rs76786961	.	.	HOTAIR	GT	T	ncRNA	.	.
12	54361101	rs192969443	.	.	HOTAIR	G	A	ncRNA	.	.
12	54367036	.	.	.	HOXC11	C	T	missense	4/305	SER/LEU
12	54367597	.	.	.	HOXC11	C	T	missense	191/305	SER/PHE
12	54369114	rs201917577	0.001	.	HOXC11	A	G	missense	278/305	ILE/VAL
12	54379510	rs149717368	0.001	0.0004	HOXC10	C	G	missense	156/343	SER/CYS
12	54379596	rs34108563	.	0.0004	HOXC10	G	A	missense	185/343	GLU/LYS
12	54403334	.	.	.	HOXC8	A	G	missense	89/243	TYR/CYS
17	59533867	rs117410176	0.001	0.0005	TBX4	G	A	missense	6/546	GLY/SER
17	59560350	rs199727670			TBX4	C	G	missense	371/546	PRO/ALA

[a]dbSNP=minor allele frequency in dbSNP; [b]EVS=minor allele frequency in all Exome Variant Server populations.

Exome Capture CNV Analysis



Supplementary Methods. MDiGS Method. Multiplexed Direct Genomic Selection (MDiGS) library prep and BAC hybridization protocol.

Supplementary Figure 1. MDiGS sample numbers. There was no difference in on-target read percentage for 24 and 48 samples multiplexed pre-capture.

Supplementary Figure 2. MDiGS coverage and histogram. (A) Individual sample read counts corresponding to coverage (≥8x) of target regions, all samples achieved ≥ 90% coverage. (B) Coverage histogram of mapped reads at coverage depths.

Supplementary Figure 3. Microdeletion detected by MDiGS cannot be identified using whole-exome sequence data. Copy number analysis of whole-exome data captured using non-contiguous probes failed to detect the novel 51 kb deletion in individual 8029001 that was detected by the MDiGS method (shown in Figure 7A).