# Expressed Exome Capture Sequencing (EecSeq): a method for cost-effective exome sequencing for all organisms with or without genomic resources

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Running title: EecSeq: exome capture for non-model species

# Abstract

Exome capture is an effective tool for surveying the genome for loci under selection. However, traditional methods require annotated genomic resources. Here, we present a method for creating cDNA probes from expressed mRNA, which are then used to enrich and capture genomic DNA for exon regions. This approach, called “EecSeq”, eliminates the need for costly probe design and synthesis. We tested EecSeq in the eastern oyster, *Crassostrea virginica*, using a controlled exposure experiment. Four adult oysters were heat shocked at 36° C for 1 hour along with four control oysters kept at 14° C. Stranded mRNA libraries were prepared for two individuals from each treatment and pooled. Half of the combined library was used for probe synthesis and half was sequenced to evaluate capture efficiency. Genomic DNA was extracted from all individuals, enriched via captured probes, and sequenced directly. We found that EecSeq had an average capture sensitivity of 86.8% across all known exons and had over 99.4% sensitivity for exons with detectable levels of expression in the mRNA library. For all mapped reads, over 47.9% mapped to exons and 37.0% mapped to expressed targets, which is similar to previously published exon capture studies. EecSeq displayed relatively even coverage within exons (i.e. minor "edge effects") and even coverage across exon GC content. We discovered 5,951 SNPs with a minimum average coverage of 80X, with 3,508 SNPs appearing in exonic regions. We show that EecSeq provides comparable, if not superior, specificity and capture efficiency compared to costly, traditional methods.



**Keywords:** exome capture, population genomics, selection

42 # Introduction

43 The invention of next-generation sequencing has made it possible to obtain massive amounts of
44 sequence data. These data have given insight into classical problems in evolutionary biology,
45 including the repeatability of evolution (e.g., Jones *et al.* 2012), the degree of convergent
46 evolution across distant taxa (e.g., Yeaman *et al.* 2016), and whether selection is driving changes
47 in existing genetic variation or new mutations (e.g., Reid *et al.* 2016). Despite this rapid progress,
48 it is still cost prohibitive to sequence dozens or hundreds of full genomes. This limits our ability
49 to study the genomic basis of local adaptation, which requires large sample sizes for statistical
50 power (De Mita *et al.* 2013; Lotterhos & Whitlock 2015; Hoban *et al.* 2016). This leads to an
51 inherent trade-off between sample size and genomic coverage, leading investigators to make
52 decisions about whether to sequence more individuals (for higher power and precision) versus
53 more of the genome (for making more accurate statements about the genetic basis of adaptation).

54 Reduced representation library preparation methods offer various kinds of random or targeted
55 genome reduction, but the available approaches have contrasting advantages and limitations.
56 RADseq uses restriction enzymes to randomly sample the genome and is appropriate for linkage
57 mapping and studying neutral processes like gene flow and drift (Puritz et al. 2014), but the data
58 can be limited for understanding the genetic basis of adaptation (Lowry *et al.* 2016, 2017;
59 Catchen *et al.* 2017; McKinney *et al.* 2017). To focus on coding regions, some investigators have
60 used RNAseq (De Wit *et al.* 2015); however, only about a dozen individuals can be sequenced
61 per lane because of log-fold differences in transcript abundance among loci. Additionally, allele-
62 specific expression limits the confidence in genotypes derived from RNAseq data (Pastinen

63 2010), especially in pooled samples. One increasingly popular option for increasing precision
64 with larger samples while still maintaining coverage of the entire genome is Pool-seq, which
65 sequences every individual to very low (1x) coverage and uses the data to calculate allele
66 frequency of the sample within the pool (Buerkle & Gompert 2013; Schlötterer *et al.* 2014;
67 Therkildsen & Palumbi 2017). Pool-seq is limited to only estimating allele frequency within
68 pools, which is a disadvantage because this data cannot be used to understand the fitness of
69 heterozygotes and some types of statistical analyses would be impossible to perform, such as
70 haplotype-based analyses (e.g. Fariello *et al.* 2013).

71 To overcome some of these limitations, many investigators have used capture approaches with
72 biotinylated probes (Jones & Good 2016). Capture approaches have the advantage of enriching
73 the data for sequences of interest - allowing for individual-level data and a large number of
74 individuals to be sequenced - but require the investigator to have genomic resources for probe
75 design and then to purchase the probes from a company. For non-model species, the development
76 of these resources takes time and a significant amount of bioinformatics expertise. In addition, for
77 a population-level genomic study with 100s of individuals, probes may cost several tens of
78 thousands of dollars, depending on how much sequence is captured. Overall, what is needed is a
79 cost-effective approach to subsample genomes for coding regions, without previously developed
80 genomic resources. Such an approach would allow for the assessment of rapid adaptation to
81 environmental disasters such as Deepwater Horizon Oil Spill (Lee *et al.* 2017).

82 Here, we present a novel, cost-effective method of exome capture that synthesizes probes in-situ
83 from expressed mRNA sequences. Expressed Exome Capture Sequencing (EecSeq) builds upon
84 existing approaches for in-situ probe synthesis that rely on restriction enzymes to sample the

85 genome or exome (Suchan *et al.* 2016; Schmid *et al.* 2017). To improve capture efficiency, we
86 developed a novel library preparation procedure that uses standardized procedures to synthesize
87 cDNA from expressed RNA (without template reduction via restriction digest) and then create
88 biotinylated probes from cDNA (see **Figure 1** for a conceptual diagram). The EecSeq design
89 includes custom RNA library adapters that offer several major advantages. The custom adapters
90 are fully compatible with duplex-specific nuclease normalization, which is included in the
91 protocol in order to reduce log fold differences in expression - resulting in more even coverage
92 across high- and low-expressed transcripts. The custom adapters also allow for probe sequencing
93 - before normalization if differential expression data is desired, or after normalization if probe
94 abundance data is desired. Moreover, the adapters are easily removed with a single enzymatic
95 treatment before biotinylation, preventing any interference during hybridization.

96 Our approach is cost-effective and does not require any prior genomic resources, making it a
97 good choice for studies seeking to understand adaptation in exomes. The approach, however, is
98 limited in the sense that the probes are designed from expressed RNA, and so investigators
99 should be careful to choose which tissues and life stages would be relevant. Here, we show
100 proof-of-concept of the approach in the eastern oyster (*Crassostrea virginica*), and find that the
101 performance of the approach is comparable, if not superior, to the performance of published
102 exome capture datasets where probes were designed from sequence data and purchased from a
103 company.

# 104 Methods

105 **Experimental overview**

106 Expressed exome capture sequencing (EecSeq) is designed with two specific goals: 1) to
107 eliminate the need for expensive exome capture probe design and synthesis and 2) to focus exon
108 enrichment of genes that are being expressed relevant to tissue(s) and condition(s) of interest. To
109 illustrate this conceptually, we exposed adult oysters to a stressor (extreme heat) that would
110 generate a predictable gene and protein expression profile (expression of heat shock proteins).
111 Having a predictable coverage profile in the probes allowed us to evaluate whether the genomic
112 DNA in these exons were captured by the probes. Note, however, that this experiment is not
113 specifically part of the EecSeq method and that the investigator can choose appropriate tissue(s)
114 and condition(s) of interest. The steps to probe synthesis and capture are visualized in Figure 1.

115 **Heat shock exposure, tissue collection, and nucleic acid extraction**

116 Eight adult *Crassostrea virginica* individuals were collected and acclimated to a flow-through
117 seawater system for 24 hours. After acclimation, individuals were randomly assigned to two
118 treatments, control and heat-shock (HS). HS individuals were placed a small aquaria filled with
119 36°C filtered seawater for one hour while control individuals were kept in an identical aquarium
120 filled with 14°C (ambient) filtered seawater. Immediately after the exposure period, all
121 individuals were shucked and mantle tissue was extracted and frozen in liquid nitrogen in
122 duplicate. DNA was extracted using the DNeasy kit (Qiagen) and RNA was extracted using TRI
123 Reagent Solution (Applied Biosystems) using included, standard protocols. DNA was visualized
124 on an agarose gel and quantified using the Qubit DNA Broad Range kit (Invitrogen). RNA was
125 visualized on an Agilent BioAnalyzer using the RNA 6000 Nano kit, and was quantified using
126 the Qubit High Sensitivity Assay Kit (Invitrogen).

127 **Expressed Exome Capture Sequencing**

128 A complete and updated EecSeq protocol can be found at (https://github.com/jpuritz/EecSeq).

129 *RNA Adapters*- Custom RNA adapters were used in this protocol. The RNA adapters were similar
130 to the Illumina TruSeq design, but include the SAlI restriction site at the 3' end of the "Universal
131 adapter" and at 5' end of the "Indexed adapter." The presence of this restriction site allows the
132 Illumina sequence to be removed before hybridization to prevent interference. Note that the
133 adapters used in this study had an erroneous deletion of a Thymine in position 58 of
134 "Universal_SAI1_Adapter" and in position 8 of all four indexed adapters (the corrected versions
135 are shown in Table 1, and erroneous version used in this study are shown in Supplemental Table
136 1). Adapters were annealed in equal parts in a solution of Tris-HCl (pH 8.0), NaCl, and EDTA,
137 heated to 97.5°C for 2.5 minutes, and then cooled at a rate of 3°C per minute until the solution
138 reaches a temperature of 21°C.

139 *mRNA Library Preparation and Normalization*- Probes were made from two (of four) control
140 individuals and two (of four) exposed individuals. The first step for this subset of individuals was
141 to prepare stranded mRNA libraries using the Kapa Stranded mRNA-Seq Kit (KAPA
142 Biosystems) with the following modifications: custom adapters were used, 4 micrograms of RNA
143 per individual were used as starting material, half volume reactions were used for all steps,
144 adapters were used at a final reaction concentration of 50 nM during ligation, and 12 cycles of
145 PCR were used for enrichment. Complete libraries were visualized on a BioAnalyzer using the
146 DNA 1000 kit, quantified using fluorometry, and then 125 ng of each library was taken and
147 pooled to single library of 500 ng.

148 To reduce the abundance of highly expressed transcripts in our final probe set, complete libraries
149 were normalized following Illumina's standard protocol for DSN normalization. First, the cDNA

150 library was heat denatured and slowly allowed to reanneal. Next, the library was treated with
151 duplex-specific nuclease (DSN), which will remove abundant DNA molecules that have properly
152 annealed. After DSN treatment, the library was SPRI purified and enriched via 12 cycles of PCR.
153 A subsample of probes was exposed to an additional 12 cycles of PCR to test for PCR artifacts in
154 probe synthesis. The normalized cDNA library was visualized on a BioAnalyzer using the DNA
155 1000 kit, quantified with a Qubit DNA Broad Range kit (Invitrogen), and then split into two
156 equal volume tubes, one to be saved for sequencing and one for probe synthesis. The DNS-
157 normalized libraries were sequenced on one half lane of HiSeq 4000 by GENEWIZ
158 (www.genewiz.com).

159 *Probe Synthesis*-To remove the sequencing adapters, the cDNA library was treated with 100 units
160 of SalI-HF restriction enzyme (New England Biolabs) in a total volume of 40 µl at 37°C for 16
161 hours. After digestion, the digested library was kept in the same tube, and 4.5 µl of 10X Mung
162 Bean Nuclease Buffer and 5 units of Mung Bean Nuclease (New England Biolabs) were added.
163 The reaction was then incubated at 30°C for 30 minutes. An SPRI cleanup using AMPure XP
164 (Agencourt) was completed with an initial ratio of 1.8X. After, visualization of the library on an
165 Agilent BioAnalyzer, a subsequent SPRI cleanup of 1.5X was completed to remove all digested
166 adapters. The clean, digested cDNA fragments were then biotin labeled using the DecaLabel
167 Biotin DNA labeling kit (Thermo Scientific) using the included protocol. The labeling reaction
168 was then cleaned using a 1.5X SPRI cleanup and fluorometrically quantified. To test the effects
169 of additional PCR cycles on probe effectiveness, 40 ng of the original, normalized cDNA library
170 was subjected to an additional 12 cycles of PCR, and then converted to probes as described
171 above.

172 *Genomic DNA Library Preparation*- Capture was performed on a standard genomic DNA library.
173 500 ng of genomic DNA from all eight individuals was sheared to a modal peak of 150 base pairs
174 using a Covaris M220 Focused-ultrasonicator. The sheared DNA was inserted directly into step
175 2.1 of the KAPA HyperPlus kit with the following modifications: half reaction volumes were
176 used, and a final adapter:insert molar ratio of 50:1 was used with custom TruSeq-style, barcoded
177 adapters (note: the adapters contained erroneous mismatches in the barcodes between the top and
178 bottom oligos; the original oligonucleotide sequences can be found in Supplemental Table 2 and
179 corrected versions in Supplemental Table 3). After adapter ligation, individuals were pooled into
180 one single library, and libraries were enriched with 6 cycles of PCR using primers that
181 complemented the Illumina P5 adapter and Indexed P7 (Supplemental Table 2). The final library
182 was quantified fluorometrically quantified and analyzed on an Agilent BioAnalyzer.

183 *Hybridization*- Three replicate captures were performed using the set of original probes and the
184 set of probes with 12 extra cycles of PCR. The hybridization protocol closely followed that of
185 Suchan *et al.* (2016). 500 ng of probes and 500 ng of genomic DNA library were hybridized
186 along with blocking oligonucleotides (Table 2) at a final concentration of 20 μM in a solution of
187 6X SSC, 5 mM EDTA, 0.1% SDS, 2X Denhardt's solution, and 500 ng $c_0$t-1 DNA. The
188 hybridization mixture was incubated at 95°C for 10 minutes, and then 65°C for 48 hours in a
189 thermocycler. The solution was gently vortexed every few hours.

190 *Exome Capture*- 40 μl of hybridization mixed was added to 200 μl of DynaBeads M-280
191 Streptavidin beads (Thermo Fisher Scientific). The beads and hybridization mixture were then
192 incubated for 30 min at room temperature. The mixture was then placed on a magnetic stand
193 until clear, and the supernatant was removed. This was followed by four bead washes under

194 slightly different conditions. First, the beads were washed with 200 µl 1X SSC and 0.1% SSC
195 solution, incubated at 65°C for 15 min, placed on the magnet stand, and the supernatant was
196 removed. Second, the beads were washed with 200 µl 1X SSC and 0.1% SSC solution incubated
197 at 65°C for 10 minutes, placed on the magnet stand, and the supernatant was removed. Third, the
198 beads were washed with 200 µl 0.5 SSX and 0.1% SDS solution, incubated at 65°C for 10
199 minutes, placed on the magnet stand, and the supernatant was removed. Finally, the beads were
200 washed with 200 µl 0.1X SSC and 0.1% SDS, incubated at 65°C for 10 minutes, placed on the
201 magnet stand, and the supernatant was removed. Lastly, DNA was eluted from the beads in 22 µl
202 of molecular grade water heated to 80°C for 10 minutes. The solution was placed on the magnet
203 and the supernatant was saved. The hybridized fragments were then enriched with 12 cycles of
204 PCR using the appropriate P5 and P7 PCR primers and cleaned with 1X AMPure XP with a final
205 elution in 10 mM Tris-HCl (pH 8.0). The six replicate captures, each containing 8 uniquely
206 barcoded individuals, were sequenced on one half lane (separate from the RNA libraries) on the
207 HiSeq 4000 platform by GENEWIZ (www.genewiz.com).

208 **Bioinformatic Analysis**

209 All bioinformatic code, including custom scripts and a script to repeat all analyses, can be found
210 at (https://github.com/jpuritz/EecSeq/tree/master/Bioinformatics)

211 *RNA libraries*- RNA reads were first trimmed for quality and custom adapter sequences were
212 searched for with Trimmomatic (Bolger *et al.* 2014) as implemented in the dDocent pipeline
213 (version 2.2.20; Puritz *et al.* 2014). Reads were then aligned to release 3.0 of the *Crassostrea*
214 *virginica* genome (Accession: GCA_002022765.4) using the program STAR (Dobin *et al.* 2013).
215 The genome index was created using NCBI gene annotations for splice junctions. Reads were

216 aligned in a two-step process, first using the splice junctions in the genome index, and then again
217 using both the splice junctions in the index and additional splice junctions found during the first
218 alignment. Alignment files from the four libraries were then merged with SAMtools (version 1.4;
219 Li *et al.* 2009) and filtered for MAPQ > 4, only primary alignments, and reads that were hard/soft
220 clipped at less than 75 bp. SAMtools (Li *et al.* 2009) and Bedtools (Quinlan 2014) were used to
221 calculate read and per bp coverage levels for exons, introns, and intergenic regions.

222 *EecSeq Libraries*- Raw reads were first trimmed using the standard methods in the dDocent
223 pipeline (version 2.2.20; Puritz *et al.* 2014). The DNA adapters contained erroneous mismatches
224 between the top and bottom oligos in the barcode (original oligonucleotide sequences can be
225 found in Supplemental Table 2 and corrected versions in Supplemental Table 3). These
226 differences prevented demultiplexing beyond the capture pool level, and also lead to potentially
227 erroneous base calls within the first 7 bp of sequencing. To remove these artifacts, the first 7 bp
228 of every forward read were clipped. Additionally, adapter sequences were searched for in the
229 paired-end sequences using custom scripts. After trimming, reads were aligned to the reference
230 genome using BWA (Li & Durbin 2009) with the mismatch parameter lowered from 4 to 3, and
231 the gap opening penalty lowered from 6 to 5. PCR duplicates were marked using the
232 *MarkDuplicatesWithMateCigar* module of Picard (http://broadinstitute.github.io/picard), and
233 then SAMtools (Li *et al.* 2009) was used to remove duplicates, secondary alignments, mappings
234 with a quality score less than ten, and reads with more than 80 bp clipped. SAMtools (Li *et al.*
235 2009) and Bedtools (Quinlan 2014) were used to calculate read and per bp coverage levels for
236 exons, introns, and intergenic regions. FreeBayes (Garrison and Marth 2012) was used to call
237 SNPs.

238 *Calculating Capture Efficiency*- EecSeq is unique amongst exome capture methods because the
239 probes are not designed directly, i.e. there is no set of *a priori* targets. Additionally, EecSeq is
240 designed to capture exons that are expressed in the samples used to create probes - not the entire
241 exome. To compare EecSeq to other capture methods, capture targets were defined as exons that
242 had more than 35X coverage in the RNAseq (probe) data and confidence intervals were generated
243 by defining capture targets as 20X RNAseq coverage and 50X RNAseq coverage. We also
244 calculated a conservative, near-target range of 150 bp on either side of the defined targets. This
245 range corresponds to the modal DNA fragment length used for the capture libraries with the
246 expectation that exon probes could capture reads that far from the original target.

# 247 Results

248 *RNA sequencing results*- RNA sequencing, filtering, and mapping statistics can be found in
249 supplemental Table 3. After filtering, a total of 21,990,025 RNA reads were mapped uniquely to
250 the eastern oyster genome. Of the total RNA reads, 78% mapped to genic regions of the genome,
251 and 58% mapped to annotated exon regions. Across all exonic bases in the genome, less than 5%
252 had more than 50X coverage; however, over 16% had at least 20X coverage and over 45% had at
253 least 5X coverage (Figure 2).

254 *Exome capture sequencing results*- Six replicate capture pools of the same eight individuals were
255 sequenced on half a lane of Illumina HiSeq (3 replicates from probes that had been enriched via
256 12 cycles of PCR and 3 replicates from probes that had been enriched via 24 cycles of PCR). A
257 summary of exome capture sequencing, filtering, and mapping statistics are shown in Table 2.
258 On average, there were 47,629,033 raw reads (forward and paired-end) per capture pool and an

259 average of 32,123,268 mapped reads per capture pool after filtration. Across the entire oyster
260 genome, RNA sequencing coverage and exome sequencing coverage was highly correlated
261 (Supplemental Figure 1), and across all exon regions total RNA coverage predicted 72.6% of the
262 variation in exome capture coverage (Figure 3; log-log transformation, $R^2 = 0.72619$, $p < 0.0001$).
263 Coverage across all exons and expressed exon targets was highly correlated ($0.984 < r < 0.996$)
264 across all replicate captures, and the average capture of pools with standard probes and the
265 average capture of pools with probes with extra PCR was virtually identical ($R^2 = 99.1$; $p <$
266 $0.0001$).

267

268 *Exome capture efficiency*- Capture sensitivity, or the percentage of targets covered by at least one
269 read (1X), was high across all replicate pools, regardless of target set (Table 3). Across all
270 known exons, sensitivity was on average 86.8% across replicate capture pools, and across all
271 defined target sets, sensitivity was over 99.4%. Increasing the sensitivity threshold from 1X to
272 10X lowers the sensitivity across all exons but has little effect on sensitivity across defined target
273 sets (Supplemental Table 4). Sensitivity can also be measured at the per bp level instead of per
274 exon. The percent of target bases captured is shown as a function of sensitivity threshold (read
275 depth of capture libraries) in Figure 4.

276 Capture specificity is the percentage of mapped reads that fall within target regions. Across all
277 exons, capture pools averaged 47.9% reads on target, 6.8% of reads near target (falling within
278 150 bp of an exon, one modal read length), and 45.3% of reads off-target (more than 150 bp away
279 from an exon). Across defined expressed exon targets (exons that sequenced to 35x read depth),

280 capture pools averaged 37.1% (C.I. 33.6% - 41.4%) reads on target, 3.55% (C.I. 3.0% - 4.4%) of
281 reads near target, and 59.38% (C.I. 54.2% - 63.4%) reads off target.

282 For all exons, between the 10th and 90th percentile of exon length (59bp - 517bp), the mean per
283 basepair coverage averaged 17.75X +/- 0.06X for each capture pool of 8 individuals. When
284 considering target exons (35X coverage in RNA-derived probes), the mean per basepair coverage
285 increased to 61.22X +/- 0.23X on average for each capture pool. This breaks down to
286 approximately 7.66 reads on average per individual per bp within expressed exome targets.
287 Within exons, mean per basepair coverage was evenly distributed across all base pairs with only
288 slightly lower coverage at the 5' or 3' edges of exons compared to the middle of exons (Figure 5;
289 Supplemental Figure 3).

290 Mean capture coverage also did not appear to relate to the GC content of the target exon (Figure
291 6), though it did appear to peak near the mean GC content of 43.57%. To test this, we calculated
292 the reciprocal of the absolute value of the difference between each exon GC content and the
293 average GC content, and then tested for a linear relationship to mean coverage. Though we found
294 this relationship to be significant ($p > 0.0008$), it explained only the 0.0033% of the variance in
295 coverage, confirming that exon GC content did not affect exon capture in a meaningful way.

296 Coverage did vary significantly between untranslated regions (UTR) within exons and coding
297 sequence (CDS) within exons (Welch's test $t = 40.063$; degrees of freedom = 135580; $p <$
298 $0.0001$) with a mean coverage for UTR equaling 11.59X +/- 0.0864 and a mean coverage for
299 CDS equaling 17.71X +/- 0.1261. This small but significant coverage difference was also
300 evident as the percent of target bases greater than a given read depth (Supplemental Figure 2).
301 This pattern was not surprising, however, because the same pattern was observed for the RNA

302 reads (CDS mean coverage = 13.65X +/- 0.2011; UTR mean coverage = 8.25 +/- 0.1275;
303 Welch's test $t$ = 22.677; degrees of freedom = 129300; $p < 0.0001$), indicating that the probes
304 also had lower coverage in UTR compared to CDS.

305 *Expressed exon capture*- To visualize the relationship between coverage and an expected
306 expressed target, we plotted coverage of the six capture pools along two heat shock proteins, Heat
307 Shock cognate 71 kDa (NCBI Reference Sequence: XM_022472393.1, Figure 7) and Heat Shock
308 70 kDa protein 12B-like (NCBI Reference Sequence: XM_022468697.1; Supplemental Figure 4).
309 As expected, exons in both genes show elevated coverage that corresponded to the coverage of
310 the mRNA-derived probes, especially along regions with corresponding CDS with few reads
311 mapping to intronic or intergenic regions.

312 *SNP Discovery*- A total of 1,011,107 raw SNPs were discovered with 909,792 SNPs having a
313 quality score higher than 20. A total of 99,169 high quality SNPs were found within known
314 exons. Of these, 31,579 exome SNPs had at least an average of 16X coverage, 15,760 exome
315 SNPs had at least an average of 32X coverage, 8,837 exome SNPs had at least an average of 48X
316 coverage, and 3,508 exome SNPs had at least an average of 80X coverage with an additional
317 2,443 80X-SNPs found outside of exon regions.

# 318 Discussion

319 Expressed exome capture sequencing (EecSeq) is a novel design for exome capture that uses *in-*
320 *situ* synthesized biotinylated cDNA probes to enrich for exon sequences, thereby removing the
321 requirement of *a priori* genomic resources, costly exon probe design, and synthesis. Here, we
322 showed that EecSeq target enrichment had high levels of sensitivity, with comparable if not

323 superior performance and specificity to traditional methods. EecSeq exon enrichment showed
324 even coverage levels with exons and across exons with differing levels of GC content. Lastly, we
325 showed that EecSeq can quickly and cheaply generate thousands of exon SNPs.

326 **Benefits of EecSeq**

327 *Diverse probes*- With EecSeq, cDNA exon probes are constructed *in-situ* from extracted mRNA,
328 and this allows for the design of a high-diversity probe pool. Traditional sequence capture probes
329 are typically designed from a single reference genome or individual, and this may limit capture
330 efficiency on individuals with different SNPs, insertions, or deletions than the reference. While
331 probes been successfully used to capture sequences in quite divergent species (less than 5%
332 sequence divergence, Jones & Good 2016), there is evidence that capture success declines as
333 sequences become less related to the reference. Portik *et al.* (2016) found that for each percent
334 increase of pairwise divergence, missing data increased 4.76%, sensitivity decreased 4.57%, and
335 specificity decreased 3.26%. Even with well-designed, commercially available capture kits for
336 human exon capture, Sulonen *et al.* (2011) found that allele balances for heterozygous variants
337 tended to have more reference bases than variant bases in the heterozygous variant position
338 across all methods for probe development. Insertions and deletions (InDels) are arguably an even
339 larger problem, since these would decrease hybridization with a probe due to a frameshift.

340 *Longer Probes*- Traditional exome capture relies on synthesized RNA or DNA baits. These baits
341 can be relatively small (60 bp; Bi *et al.* 2012) or range between 95 and 120 bp (Clark *et al.* 2011;
342 Sulonen *et al.* 2011; Nadeau *et al.* 2012; Chilamakuri *et al.* 2014). In contrast, EecSeq probes
343 have a modal length of 150 bp but also range up to over 400 bp (data not shown). The longer
344 length of EecSeq probes likely helps to buffer against divergence between probes and targets.

345 The longer probes may also be the reason why we observed relatively little GC bias in coverage
346 across exons, and may help explain the uniformity of coverage within exons in EecSeq data.

347 *Cost*- EecSeq provides significant cost and time savings over traditional exome capture and RNA
348 sequencing (RNAseq). No *a priori* genomic information is necessary for EecSeq, saving
349 substantial time and money for obtaining these data in non-model organisms. Likewise, the cost
350 of synthesizing the probes is significantly reduced because probes can be made in-house and do
351 not have to be designed by a company. On a per sample basis, EecSeq is also significantly
352 cheaper than RNAseq because (i) commercial DNA library preps are cheaper than those for
353 mRNA, and (ii) more individuals can be multiplexed on a single lane. For example, the cost of
354 RNA seq is $246 per sample (cost estimated using the same RNA kits used with EecSeq and ½
355 reactions) and assuming that 12 RNAseq libraries can be sequenced in a single lane of Illumina
356 HiSeq, the cost per sample is ($1,008; cost of the kit; Kapa Biosystems Stranded mRNA-Seq Kit
357 with 24 reactions or 48 half-reactions)*(1/48; the amount used per sample) + $2700/12 = $246
358 per sample). The equivalent cost per sample for EecSeq is $48.02 per sample (for 96 samples in
359 one lane of HiSeq; Supplemental Table 6) or $62.08 per sample if a more conservative
360 sequencing strategy is used (96 samples sequenced over 1.5 lanes of HiSeq; Supplemental Table
361 6).

362 *No dependency on restriction sites*- A recently published method, hyRAD-X, (Schmid *et al.*
363 2017) is similar to EecSeq in that it uses *in-situ* synthesized cDNA probes from expressed mRNA
364 to capture exome sequences. However, the protocol relies on a restriction digest to fragment
365 cDNA and ligate on probes. This may result in a reduced template of probes because not all
366 cDNA fragments will have restriction sites on both ends. To evaluate the possibility that the

367 hyRAD-X would produce a reduced template of probes, we performed crude calculations using
368 SimRAD in R (Lepais & Weir 2014) on the *C. virginica* exome. Of the 31,383 known mRNA
369 transcripts in the oyster genome (assuming 1 transcript variant), 29,555 contain at least 2 MseI
370 cut sites (TTAA). However, there is an SPRI cleanup on the digestion (2X), meaning that at best,
371 only fragments 100bp and larger are getting through to biotinylation
372 (http://www.keatslab.org/blog/pcrpurificationampureandsimple). SimRAD estimates 220,184 out
373 of a possible 440,881 fragments. Therefore, at the absolute best hyRAD-X is only sampling
374 (29,555/31,383)*(220,184/440,881) = 47% of the exome, though this number may increase
375 slightly due to transcript variations. Relying on restriction digests may also produce skewed size
376 distributions in probes which would be magnified in subsequent rounds of PCR. In Schmid *et al.*
377 (2017), hyRAD-X generated 524 exome SNPs at a minimum of 6X coverage across 27 samples
378 (compared to the 3,508 exome SNPs discovered at 80X coverage derived from only 8 effective
379 samples in 6 replicate capture using EecSeq), but they were also studying ancient DNA and so
380 whether the hyRAD-X protocol results in limited coverage across exons remains to be tested.

381 **Caveats of EecSeq**

382 Despite the demonstrated benefits of EecSeq, there are some potential caveats that should be
383 considered before employing the method. First, there is no ability to filter out probes that belong
384 to repetitive sequences, which are often present at high concentrations in large-genome organisms
385 such as amphibians (Keinath *et al.* 2015) or conifers (De La Torre et al. 2014). In one capture
386 study from designed probes, a small proportion of the probes (unknowingly at the time of probe
387 development) matched highly repetitive sequences (Syring *et al.* 2016). This resulted in an
388 inordinate number of reads to these few probe sequences (Syring *et al.* 2016). However, the

389 inclusion of known repetitive sequence blocker in hybridization, such as $c_0t$-1 that is used in the
390 EecSeq protocol, has been shown to nearly double capture efficiency (McCartney-Melstad *et al.*
391 2016). In general, repetitive elements, short repeats, and low complexity regions are problematic
392 for all types of probe design and capture.

393 Another caveat of using EecSeq is the need to obtain RNA from relevant samples, although
394 capture designs or gene expression studies based on transcriptomes face the same challenge.
395 Note, however, the advantage that EecSeq probes can be made from mRNA pooled from many
396 individuals, tissues, and conditions of interest. If genes of interest are expressed in tissues that are
397 difficult to dissect or are in small abundances (such as neurons), then the RNA-based methods
398 presented here would not be a feasible approach unless pooling multiple extractions.
399 Additionally, the probes are a limited resource - our results indicate, however, that additional
400 rounds of PCR on the probes have little effect on capture.

401 **Unique Aspects of EecSeq**

402 Our approach relies on expressed mRNA for probe synthesis and the abundance of particular
403 mRNAs will vary depending on gene expression. EecSeq includes a normalization step to
404 decrease the abundance of very common transcripts, but probe pools will still skew towards
405 highly expressed genes and therefore capture coverage will be higher for those exons. This
406 aspect of EecSeq can be customized for particular research questions. For projects focused on
407 total exome capture, pools from multiple individuals, tissue types, and environmental/laboratory
408 exposures can be constructed to generate a robust probe set. On the contrary, if an investigator is
409 focused on a subset of genes that are responding to a particular stressor, it is possible to make
410 probes from organisms exposed that specific condition and then use those probes to capture other

411 individuals. This reduced probe set may also allow for greater multiplexing, but this remains to
412 be specifically tested. While we have only used mRNA to create probes, there may be
413 possibilities to capture other types transcribed sequences such as long non-coding RNAs or
414 possibly even miRNA.

415 Previous work on exome capture probe design has focused on intron/exon boundaries. In
416 general, it is thought that capture probes that span exon boundaries will result in low coverage of
417 these regions (Jones & Good 2016) or that certain regions will not be covered at all (Neves *et al.*
418 2013). Inclusion of too many boundaries may also lower overall capture performance by
419 increasing off-target capture (Suren *et al.* 2016). EecSeq exome probes are derived from mature
420 RNA, so some of the probes will span inevitably span exon boundaries. Though exon/intron
421 boundaries cannot be eliminated in EecSeq, both input mRNA and genomic DNA were
422 fragmented down to a modal size of 150 base pairs, with the intention of making both smaller
423 than the average exon size of Eastern Oysters (note that this size is at the lower limit of what is
424 possible with Illumina sequencing). We found that coverage within exons was fairly uniform,
425 indicating a lack of "edge effects." We hypothesize that the relative long length of EecSeq probes
426 (compared to commercially synthesized probes), the near matching length of genomic DNA
427 fragments, and the length distribution relative to actual exon size helped to ensure uniform exon
428 coverage.

429 We compared our observed measures of sensitivity and specificity to other recently published
430 studies in non-model species where probes were designed from bioinformatic resources for the
431 same species. EecSeq capture efficiency performed as well as or outperformed almost all other
432 previously published exome capture studies in non-model species (excluding mice and humans;

433 Table 4) with the notable exception of black cottonwood (Zhou & Holliday 2012), a species with
434 exceptional genomic resources. Note, however, that we analyzed capture efficiency across pools
435 of 8 individuals, and there could be considerable variability at the individual level that remains to
436 be quantified.

437 **Conclusions and Future Directions**

438 Here, we have shown that EecSeq effectively targets expressed exons, delivers consistent and
439 efficient exome enrichment that is comparable to traditional methods of exome capture, and
440 generates thousands of exome-derived SNPS cost effectively. Additional tests are needed to
441 examine the efficiency of exome capture across individuals for different species, which should be
442 coupled with sequencing of EecSeq probes to investigate the effects of probe pool diversity and
443 sequence divergence between probes and targets on capture. Nonetheless, EecSeq holds
444 substantial promise as a universally applicable and cost-effective method of exome sequencing
445 for virtually any macroscopic organism.

# 446 Acknowledgements

447 The authors would like to thank Alan Downey-Wall and Sara Schaal for informative discussions
448 and input throughout the duration of this project. The authors also thank Nadir Alvarez for
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451

452

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548 individually barcoded samples for population and evolutionary genomics in nonmodel species.
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551 related conifers. *Science*, **353**, 1431–1433.
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553 space using sequence capture. *BMC genomics*, **13**, 703.

554

# 555 Data Accessibility

556 Raw, demutliplexed sequences are archived at the NCBI Short Read Archive (BioProject:
557 PRJNA423022). A complete and updated EecSeq protocol can be found at
558 (https://github.com/jpuritz/EecSeq) along with bioinformatic code to repeat all analyses described
559 in this paper.

# 560 Author Contributions

561 JP conceived the original concept of this work and performed all laboratory and data analysis.
562 KL contributed all reagents and experimental materials. JP and KL designed the research,
563 experiments, and data analysis, and wrote the manuscript.

564

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566

567

576 # Tables

577

| Oligo Name | Sequence |
|---|---|
| Universal_SAI1_Adapter | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGTCGACT*T |
| Indexed_Adapter_SAI1_I5 | P*AGTCGACAGATCGGAAGAGCACACGTCTGAACTCCAGTCACACAGTGATCTCGTATGCCGTCTTCTGCTTG |
| Indexed_Adapter_SAI1_I8 | P*AGTCGACAGATCGGAAGAGCACACGTCTGAACTCCAGTCACACTTGAATCTCGTATGCCGTCTTCTGCTTG |
| Indexed_Adapter_SAI1_I9 | P*AGTCGACAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGATCAGATCTCGTATGCCGTCTTCTGCTTG |
| Indexed_Adapter_SAI1_I11 | P*AGTCGACAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGGCTACATCTCGTATGCCGTCTTCTGCTTG |

578 **Table 1. Corrected adapter sequences for mRNA library preparation.**
579 Oligos are listed in a 5' to 3' orientation with "P" indicates a phosphorylation modification to enable
580 ligation.
581

582

| Replicate Capture Pool | Raw Reads | Filtered Reads | Mapped Reads | % Duplicates | Filtered Mapped Reads | % mapping to mitochondrial genome |
|---|---|---|---|---|---|---|
| EC_2 | 53,493,950 | 53,118,952 | 42,403,525 | 5.8 | 35,955,539 | 1.7% |
| EC_4 | 44,935,340 | 44,651,228 | 35,275,663 | 6.1 | 29,519,347 | 1.6% |
| EC_7 | 43,745,614 | 43,448,296 | 35,007,184 | 5.6 | 29,723,437 | 2.2% |
| EC_1 | 41,402,996 | 41,145,724 | 32,668,750 | 4.5 | 27,940,717 | 1.8% |
| EC_3 | 56,127,536 | 55,753,268 | 44,605,960 | 5.0 | 38,103,268 | 1.9% |
| EC_12 | 46,068,760 | 45,750,394 | 37,227,067 | 6.2 | 31,497,298 | 2.2% |

583 **Table 2. Exome capture sequencing, filtering, and mapping statistics.**
584 EC_2, EC_4, and EC_7 are the three replicate captures with the original probe pool, and EC_1, EC_3, and
585 EC_12 are the replicate captures with the probe pool exposed to 12 extra rounds of PCR.
586

587

| | Capture Pool | | | | | |
|---|---|---|---|---|---|---|
| Targets | **EC_2** | **EC_4** | **EC_7** | **EC_1** | **EC_3** | **EC_12** |
| **All Exons** | 88.0% | 86.0% | 85.8% | 86.5% | 87.9% | 86.4% |
| **20XR Exons** | 99.5% | 99.4% | 99.4% | 99.4% | 99.5% | 99.4% |
| **35XR Exons** | 99.6% | 99.6% | 99.6% | 99.6% | 99.6% | 99.6% |
| **50XR Exons** | 99.7% | 99.7% | 99.7% | 99.7% | 99.7% | 99.7% |

588 **Table 3. Exome capture sensitivity with a 1x threshold.**

589 Sensitivity is the percentage of target bp with at least one read mapping successfully. Here, targets are
590 broken up into subsets: All annotated exons, exons with at least 20X coverage from the RNA library,
591 exons with at least 35X coverage from the RNA library, and exons with at least 50X coverage from the
592 RNA library. EC_2, EC_4, and EC_7 are the three replicate captures with the original probe pool, and
593 EC_1, EC_3, and EC_12 are the replicate captures with the probe pool exposed to 12 extra rounds of PCR.
594

| Reference and species | Num. target genes or exons | Sensitivity % of targeted regions > 10x depth | Specificity % of reads mapping to targeted bases | % of reads mapping near target | % of reads mapping off target | Notes |
|---|---|---|---|---|---|---|
| EecSeq (this study) eastern oyster *Crassostrea virginica* | 71,105 (51,096-110,020) | All exons: 54.7% Expressed Exons: 98.8% ( 97.4% - 99.1%) | All exons: 47.8% Expressed Exons: 37.0% ( 33.6% - 41.4%) | All exons: 28.4% Expressed Exons: 23.6% (22.3% - 25.2%) | All exons: 23.7% Expressed Exons: 39.3% (33.3% - 44.1%) | |
| (Suren *et al.* 2016) pine and spruce *Picea glauca x engelmanii* and *Pinus contorta* | 26824 genes (pine) 28649 genes(spruce) | 51% (spruce) and 59% (pine) (all samples, also metrics for 75% of samples) | 18.5% (spruce) and 21 % (pine) | 37% (spruce) 38% (pine) | 44% (spruce) and 41% (pine) | Non-model species, large genomes, near target defined as 500 bp |
| (Zhou & Holliday 2012) black cottonwood *Populus trichocarpa* | 20.76Mb (5%) of exons, regulatory regions | 86.8 % (at 100X coverage about 0-8%) | ~93% | On average, approximately 80 base pairs nearest the bait were sequenced at a depth of > 10X | NR | Model species with good genome. Off target defined as > 250bp away. |
| (Hebert et al. 2013) lake whitefish *Coregonus clupeaformis* | 11,975 nuclear exons, and other genomic markers using 62,438 probes | NR | 11.8% | NR | NR | 98% of targeted genes (2728) were successfully captured a mean read depth of 31X |
| (Bi *et al.* 2012) chipmunk *Tamias alpinus* | 11,975 exons | 40.3% | 25% | NR | NR | % of exons that were covered by at least one read, > 99% |
| (Christmas *et al.* 2017) narrow-leaf hopbush *Dodonaea viscosa* ssp. *angustissima* | 700 genes | NR | 15.7% | NR | NR | Did not account for intron sites |
| (Syring *et al.* 2016) whitebark pine *Pinus albicaulis* | 7,849 distinct transcripts | NR | 13% | NR | NR | |
| (Müller *et al.* 2014) douglas-fir *Pseudotsuga menziesii* | 57,110 exons | 90% | 32-52% per individual | NR | NR | |
| (Nadeau *et al.* 2012) butterflies | BAC loci (3.5 MB; 57,610 baits) | 75.6% | 33.5% | NR | NR | |

595 **Table 4. Comparing specificity and sensitivity across capture methods.**

596 A summary of sensitivity and specificity of recent exome-capture studies in which probes were
597 designed from the same species. NR: not reported.
598

599 # Figure Legends

600 **Figure 1. Conceptual Diagram of Expressed Exome Capture Sequencing.**

601 Upper left panel: The shotgun genomic DNA library that will be captured with probes. Middle left panel: EecSeq
602 relies on custom RNA adapters that contains a SAlI restriction site. Middle upper panel: The adapters are
603 incorporated into a mRNA library preparation that is normalized with duplex-specific nuclease. Adapters are then
604 removed with a SA1I restriction digest, cDNA probes are subsequently blunted with mung bean nuclease, and
605 biotinylated via a PCR reaction. Upper right panel: The probes are then hybridized to the shotgun genomic library
606 with TruSeq style adapters. Exon loci bind to the cDNA probes. Lower panel: Hybridized exon loci and probes are
607 then captured with magnetic Streptavidin beads. The captured exome fragments are washed several times, eluted,
608 enriched with PCR, and then sequenced.

609 **Figure 2. Distribution of RNA reads across regions of the oyster genome.**

610 Percentage of bases within exons- both coding sequences (CDS) and untranslated exon regions (UTR), intergenic,
611 and intron regions at various coverage levels.

612 **Figure 3. Total DNA and RNA coverage across all exons.**

613 Depth was calculated as the total number of reads overlapping with an exon region. For exome capture depth (y-
614 axis), reads were summed across all 6 replicate captures. For RNA read depth, reads were summed across all four
615 libraries. The shape and color of each point was determined by the percentile size of the respective exon (lower 10%
616 < 59 bp, upper 10% > 517 bp, and the middle 80% was between 57 bp and 517bp). Note that the DNA reads were
617 sequenced to greater depth than the RNA-derived probes.

618 **Figure 4. Per base pair EecSeq capture sensitivity.**

619 To measure EecSeq capture (DNA) sensitivity, capture targets were defined as exons that had more than 35X
620 coverage in the RNAseq (probe) data. Confidence intervals were generated by defining capture targets between 20X
621 RNAseq coverage and 50X RNAseq coverage. Near-target mapping were 150 bp on either side of the defined
622 targets. This range corresponds to the modal DNA fragment length used for the capture libraries with the expectation
623 that exon probes could capture reads that far from the original target. EC_2, EC_4, and EC_7 are the three replicate
624 captures with the original probe pool, and EC_1, EC_3, and EC_12 are the replicate captures with the probe pool
625 exposed to 12 extra rounds of PCR. Depth in this figure is the depth of DNA reads from EecSeq captures.

626 **Figure 5. Boxplots of mean per basepair coverage levels plotted across exons size windows.**

627 All annotated exons were broken into 10bp - 30 bp windows depending on overall size and the mean per basepair
628 coverage per capture was calculated for each window size. The line each box represents the median of mean
629 coverage values and the box surrounds the $25^{th}$ and $75^{th}$ percentiles. The mean of each bin class is plotted as a black
630 diamond with standard error bars around it. Outlier points were not plotted. Note that the data for this graph is for
631 all annotated exons, regardless of expected capture. See Supplemental Figure 3 for a similar plot focused on an
632 expressed target set.

633 **Figure 6. Mean capture depth plotted against exon GC content.**

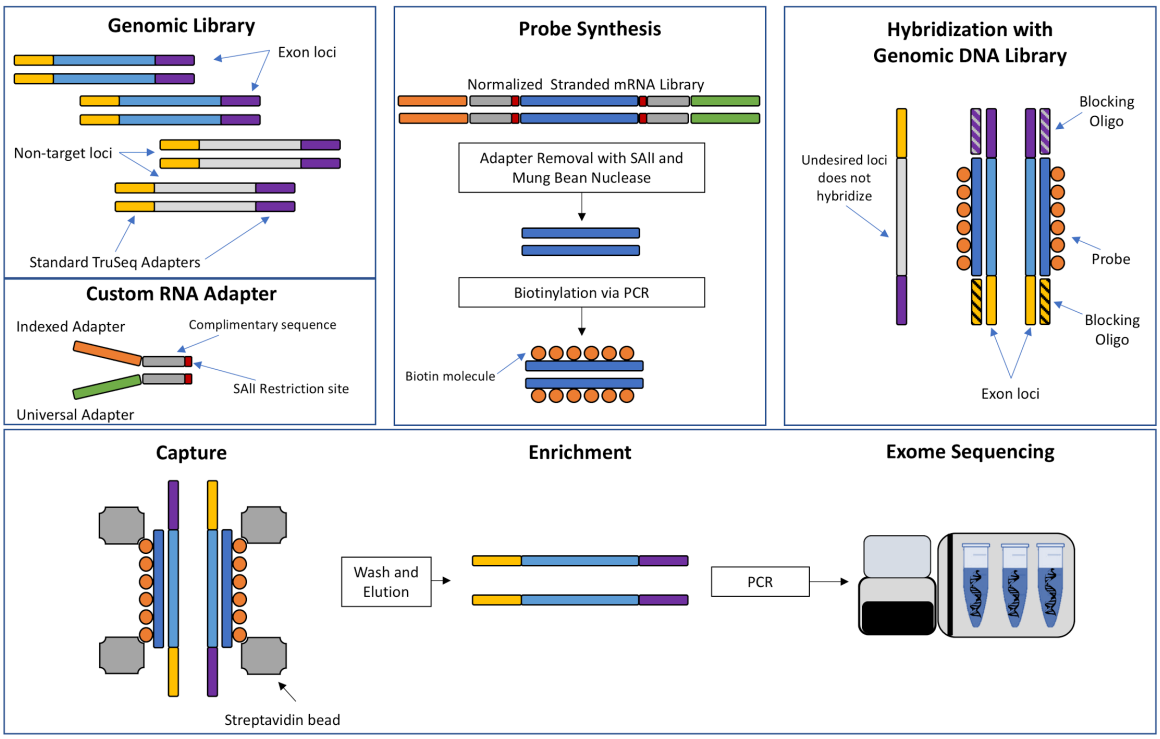
634 Exons were broken up into three size windows: (1) Lower 10%- exons less than 57 bp, (2) Middle 80%- exons
635 greater than 56 bp and less than 518, (3) Upper 10%- exons greater than 517 bp.

636 **Figure 7. EecSeq capture and probe coverage across Heat Shock cognate 71 kDa.**

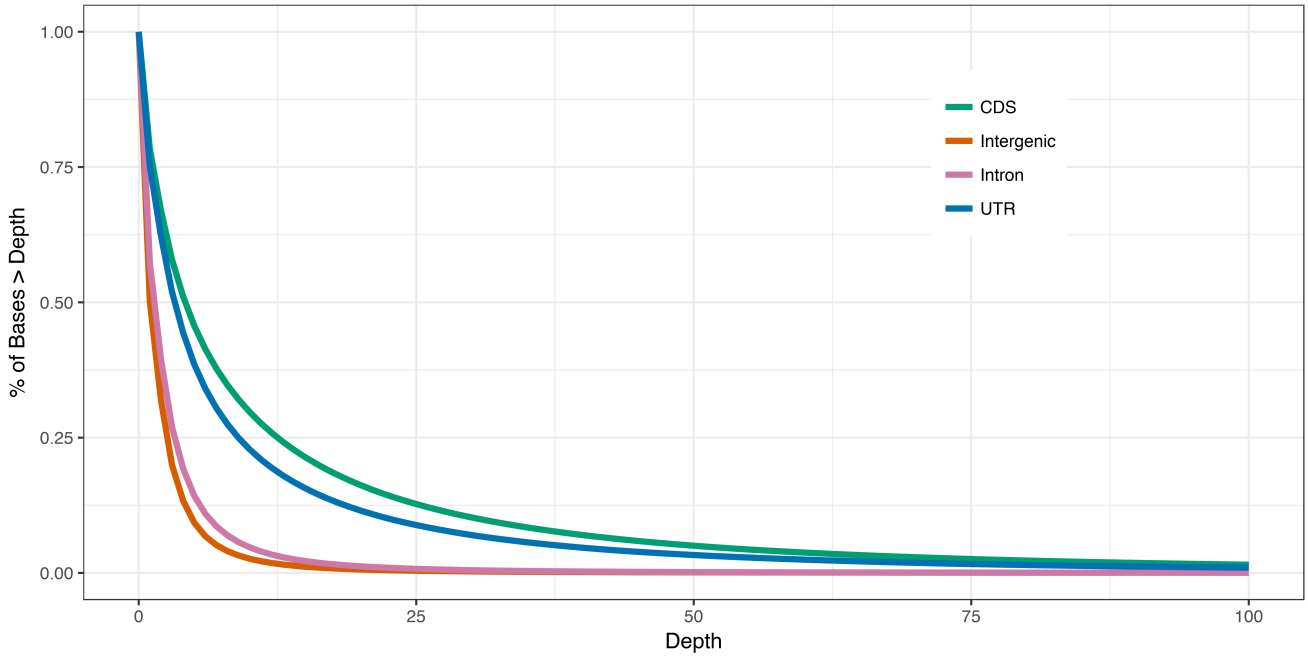
637 Coverage for each replicate capture pools is plotted along base pairs 32,740,000 to 32,755,000 of reference
638 Chromosome NC_035780.1 containing the full gene region of Heat Shock cognate 71 kDa (NCBI Reference
639 Sequence: XM_022472393.1), predicted glucose-induced degradation protein 8 homolog (NCBI Reference
640 Sequence: XM_022486802), and a partial gene region for rho GTPase-activating protein 39-like (NCBI Reference
641 Sequence: XM_022486743.1). Each exome capture pool coverage is plotted in light blue with dashed grey border,
642 and a rolling 100 bp window average across all pools is plotted in dark blue. Each RNAseq (probe) sample coverage
643 is plotted in light red with dashed grey border and a rolling 100 bp window average across all pools is plotted in dark
644 red. Gene regions are marked in purple with exons color coded by gene. Coding sequence (CDS) is marked by a
645 white bar within exon markers.

# Figures

**Figure 1. Conceptual Diagram of Expressed Exome Capture Sequencing.**



651 **Figure 2. Distribution of RNA reads across regions of the oyster genome.**

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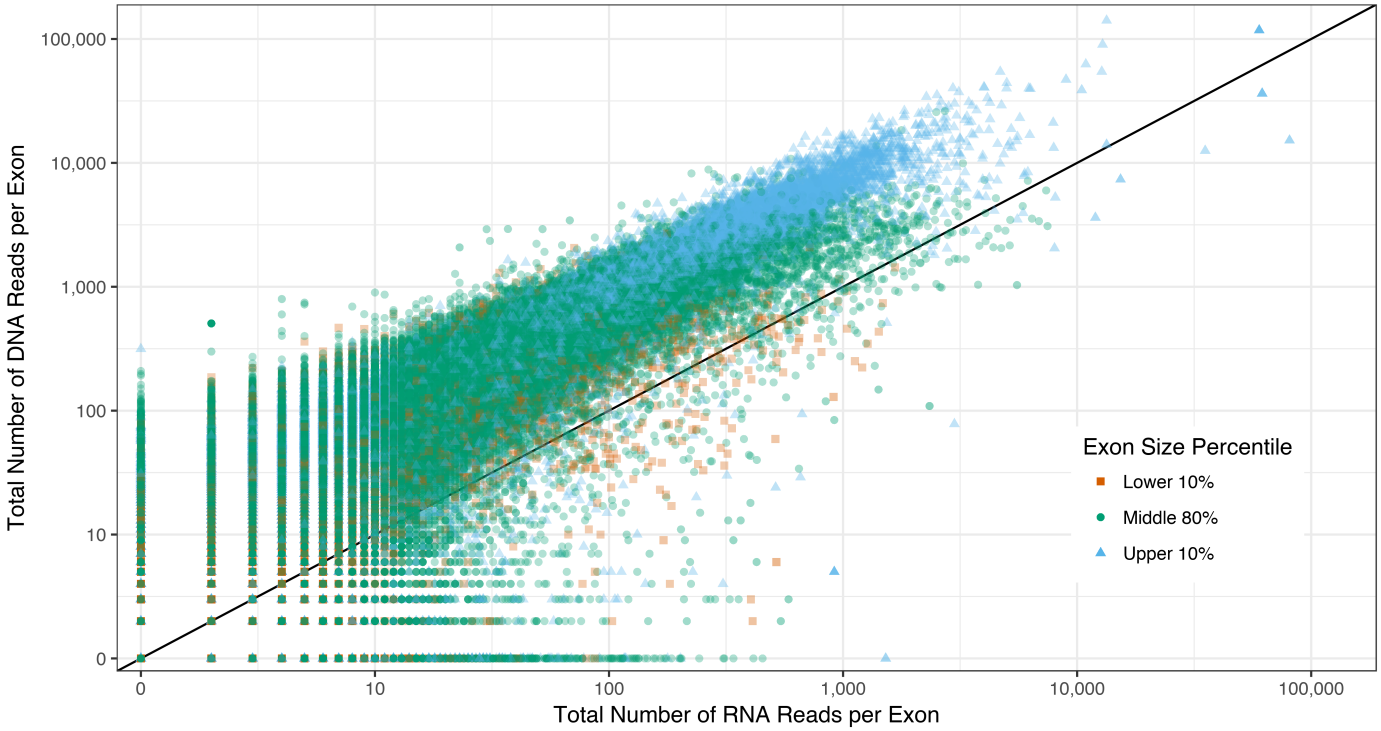
655 **Figure 3. Total DNA and RNA coverage across all exons.**

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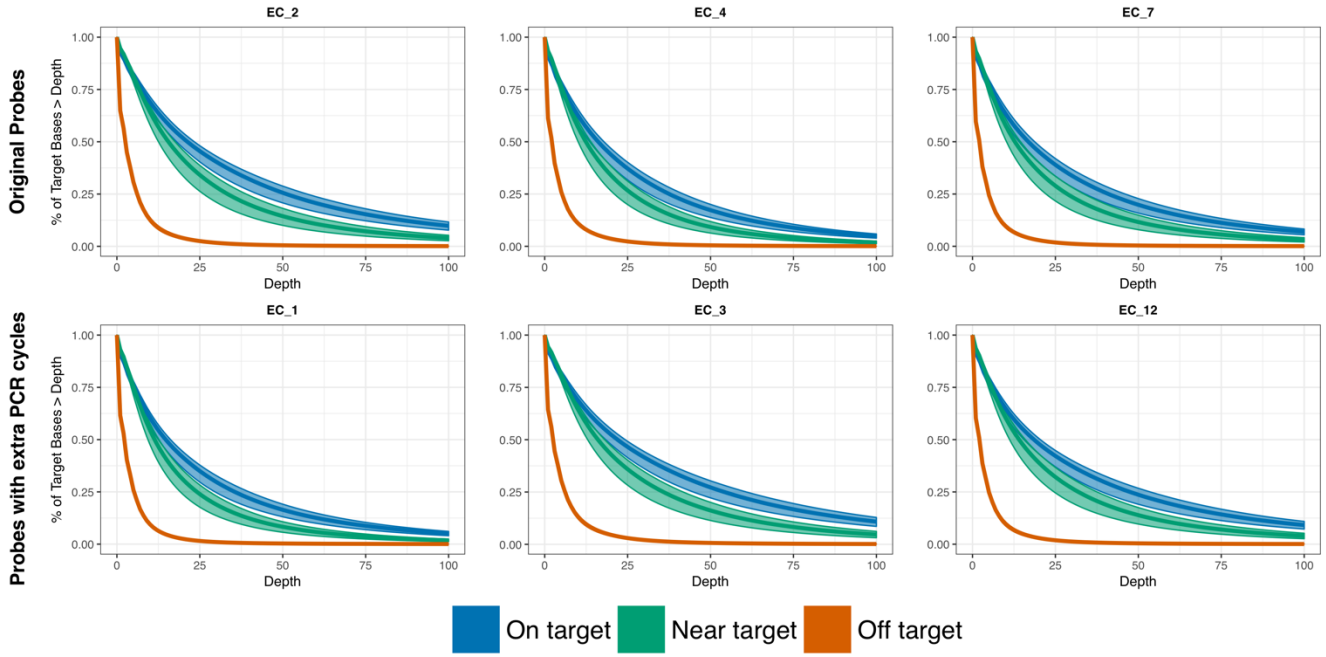



659 **Figure 4. Per base pair EecSeq capture sensitivity.**

660

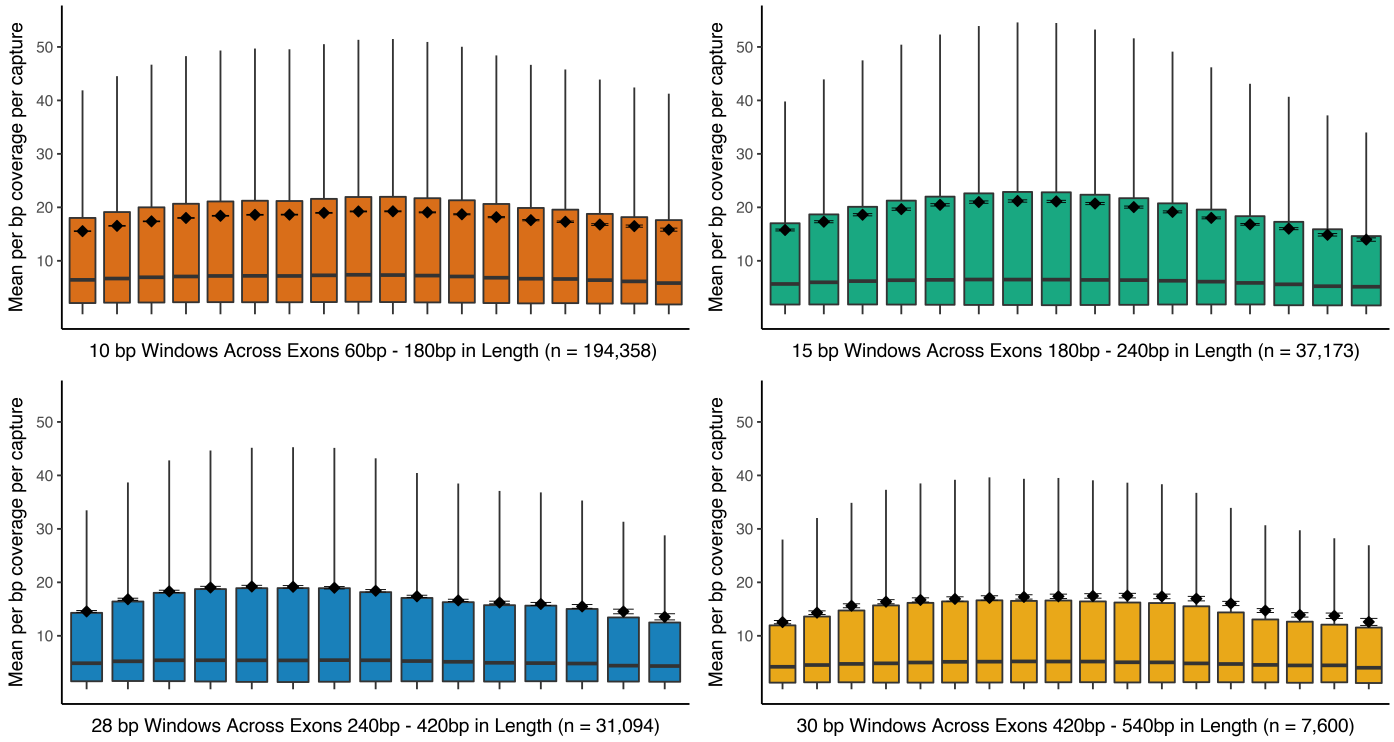
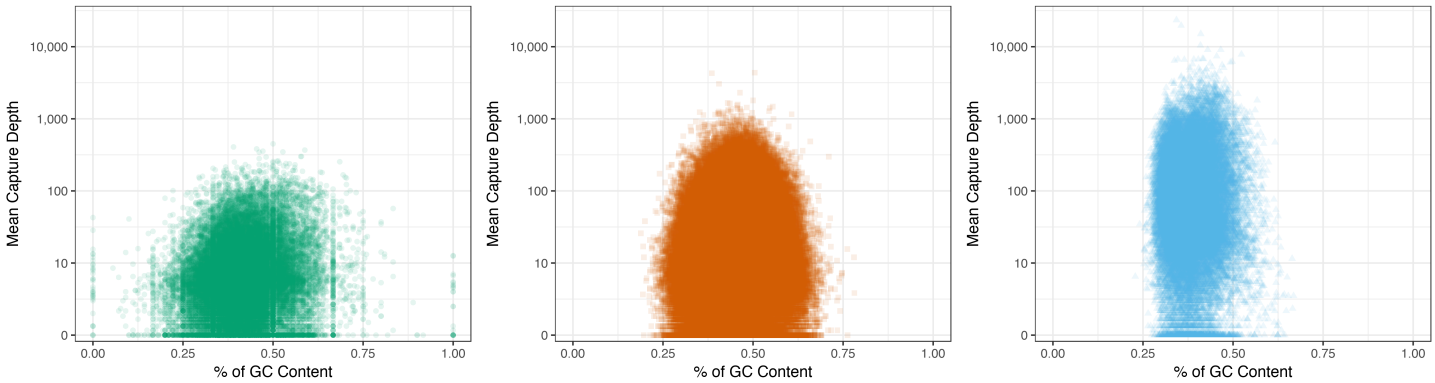



**Figure 5. Boxplots of mean per basepair coverage levels plotted across exons size windows.**

665 **Figure 6. Mean capture depth plotted against exon GC content.**

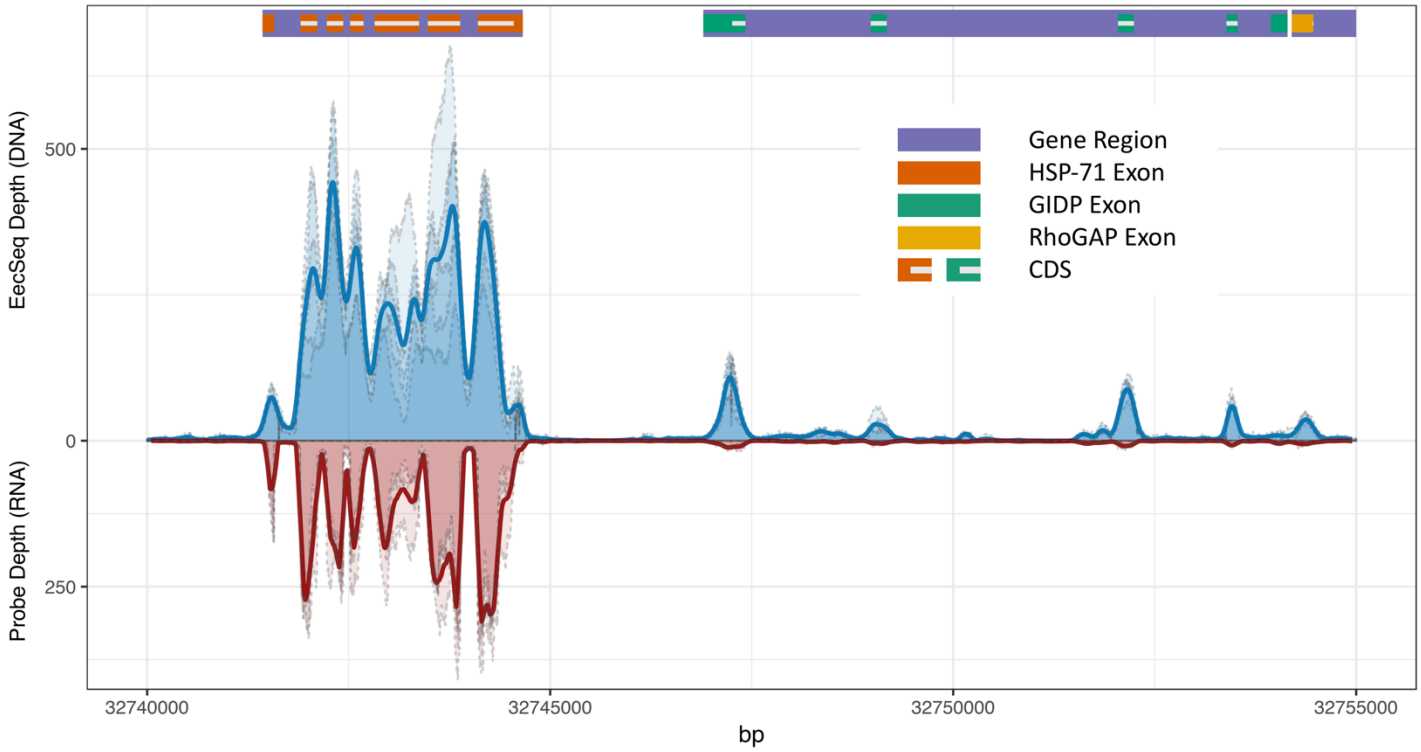
666



667 **Figure 7. EecSeq capture and probe coverage across Heat Shock cognate 71 kDa.**

668

669

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# Expressed Exome Capture Sequencing (EecSeq): a method for cost-effective exome sequencing for all organisms with or without genomic resources

## Supplemental Material

Jonathan B. Puritz and Katie E Lotterhos

# Tables

## Supplemental Table 1: Original RNA Adapters

| Oligo Name | Sequence |
|---|---|
| Universal_SAI1_Adapter | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCGTCGACT*T |
| Indexed_Adapter_SAI1_I5 | P*AGTCGACGATCGGAAGAGCACACGTCTGAACTCCAGTCACACAGTGATCTCGTATGCCGTCTTCTGCTTG |
| Indexed_Adapter_SAI1_I8 | P*AGTCGACGATCGGAAGAGCACACGTCTGAACTCCAGTCACACTTGAATCTCGTATGCCGTCTTCTGCTTG |
| Indexed_Adapter_SAI1_I9 | P*AGTCGACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGATCAGATCTCGTATGCCGTCTTCTGCTTG |
| Indexed_Adapter_SAI1_I11 | P*AGTCGACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGGCTACATCTCGTATGCCGTCTTCTGCTTG |

**Supplemental Table 2: Original DNA Adapters.** Oligos should be paired for adapter formation following 1.1.X pairs with 1.2.X.

| | |
|---|---|
| DNA_P1.1.1 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCATGG*T |
| DNA_P1.1.2 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACCAG*T |
| DNA_P1.1.3 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATCG*T |
| DNA_P1.1.4 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGATG*T |
| DNA_P1.1.5 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGCATG*T |
| DNA_P1.1.6 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAACCG*T |
| DNA_P1.1.7 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTTGG*T |
| DNA_P1.1.8 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAGGAG*T |
| DNA_P1.2.1 | PC*CGTACAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT |
| DNA_P1.2.2 | PC*TTGGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT |
| DNA_P1.2.3 | PC*GCTAGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT |
| DNA_P1.2.4 | PC*AGCTAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT |
| DNA_P1.2.5 | PC*ACGTAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT |
| DNA_P1.2.6 | PC*GTTGGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT |
| DNA_P1.2.7 | PC*CCAACAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT |
| DNA_P1.2.8 | PC*TTCCTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT |
| DNA_P2.1 | P*GATCGGAAGAGCGAGAACAA |
| DNA_P2.2 | GTGACTGGAGTTCACACGTGTGCTCTTCCGATC*T |

**Supplemental Table 3: Correct DNA Adapters.** Oligos should be paired for adapter formation following 1.1.X pairs with 1.2.X.

| | |
|---|---|
| DNA_P1.1.1 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCATGG*T |
| DNA_P1.1.2 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACCAG*T |
| DNA_P1.1.3 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATCG*T |
| DNA_P1.1.4 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGATG*T |
| DNA_P1.1.5 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGCATG*T |
| DNA_P1.1.6 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAACCG*T |
| DNA_P1.1.7 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTTGG*T |
| DNA_P1.1.8 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAGGAG*T |
| DNA_P1.2.1 | PC*CATGCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT |
| DNA_P1.2.2 | PC*TGGTTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT |
| DNA_P1.2.3 | PC*GATCGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT |
| DNA_P1.2.4 | PC*ATCGAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT |
| DNA_P1.2.5 | PC*ATGCAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT |
| DNA_P1.2.6 | PC*GGTTGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT |
| DNA_P1.2.7 | PC*CAACCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT |
| DNA_P1.2.8 | PC*TCCTTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT |
| DNA_P2.1 | P*GATCGGAAGAGCGAGAACAA |
| DNA_P2.2 | GTGACTGGAGTTCACACGTGTGCTCTTCCGATC*T |

**Supplemental Table 4. RNA sequencing statistics.** Samples 3E and 4E were heat-shocked individuals and Samples 1C and 3C were control individuals.

| Sample | Raw Reads | Filtered Reads | Mapped Reads | Filtered Mapped Reads |
|---|---|---|---|---|
| **3E** | 25259714 | 23736708 | 12499756 | 5682642 |
| **4E** | 24794376 | 23304626 | 12619770 | 5843708 |
| **1C** | 22749260 | 21494102 | 12499756 | 4717835 |
| **3C** | 25485364 | 23900618 | 12594813 | 5745840 |

**Supplemental Table 5. Exome capture specificity with a 10X threshold.**

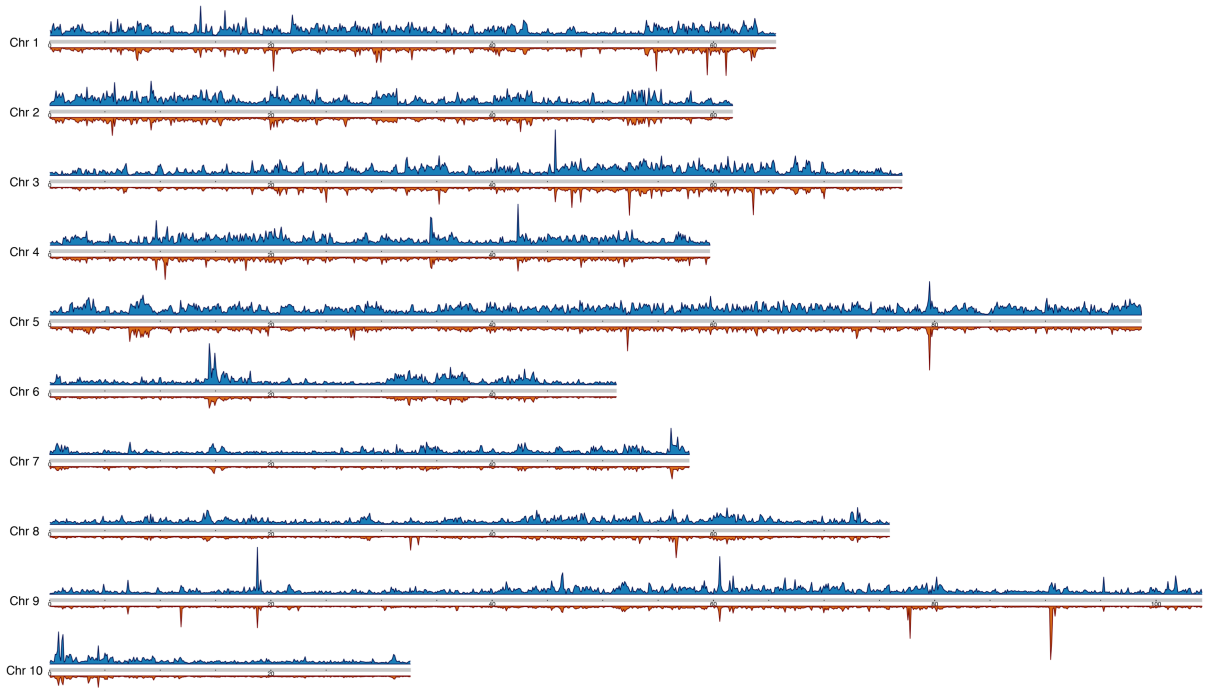
Sensitivity measured here is the percentage of targets with at least 10 reads mapping successfully. Here, targets are broken up into subsets: All annotated exons, exons with at least 20X coverage from the RNA library, exons with at least 35X coverage from the RNA library, and exons with at least 50X coverage from the RNA library. EC_2, EC_4, and EC_7 are the three replicate captures with the original probe pool, and EC_1, EC_3, and EC_12 are the replicate captures with the probe pool exposed to 12 extra rounds of PCR.

| 10X | **Capture Pool** | | | | | |
|---|---|---|---|---|---|---|
| Targets | **EC_2** | **EC_4** | **EC_7** | **EC_1** | **EC_3** | **EC_12** |
| **All Exons** | 58.8% | 51.9% | 52.2% | 52.3% | 58.7% | 54.2% |
| **20XR Exons** | 98.2% | 96.7% | 97.1% | 96.6% | 98.2% | 97.6% |
| **35XR Exons** | 99.1% | 98.6% | 98.8% | 98.6% | 99.0% | 98.9% |
| **50XR Exons** | 99.3% | 99.0% | 99.1% | 99.0% | 99.2% | 99.2% |

**Supplemental Table 6. Per sample cost calculations for EecSeq.** The calculations below assume 8 mRNA libraries are used to create probes to capture 96 samples in 8 capture reactions (12 samples per capture). Costs assumes the captured DNA is sequenced in one to one and a half lane(s) of Illumina High Seq 4000. This assumes that coverage levels for 96 samples in one lane would be equivalent to coverage levels seen in six pools of eight samples in half a lane. Cost does not include DNA or RNA extraction. See the github respository for more information on library preps and capture. Based on results in the main paper, this multiplexing strategy would give ~7.66x coverage per individual at exons represented by 35X sequencing depth at RNA-derived probes and would give ~3,508 exome SNPs at 10X coverage. Whether 96 individuals can be sequenced to enough depth in a single lane will depend on the number of megabases represented by the probes, the desired read depth, and the sensitivity and specificity of capture in the focal species. We have included the cost if 96 samples were sequenced over one and half lanes to include this potential variance.

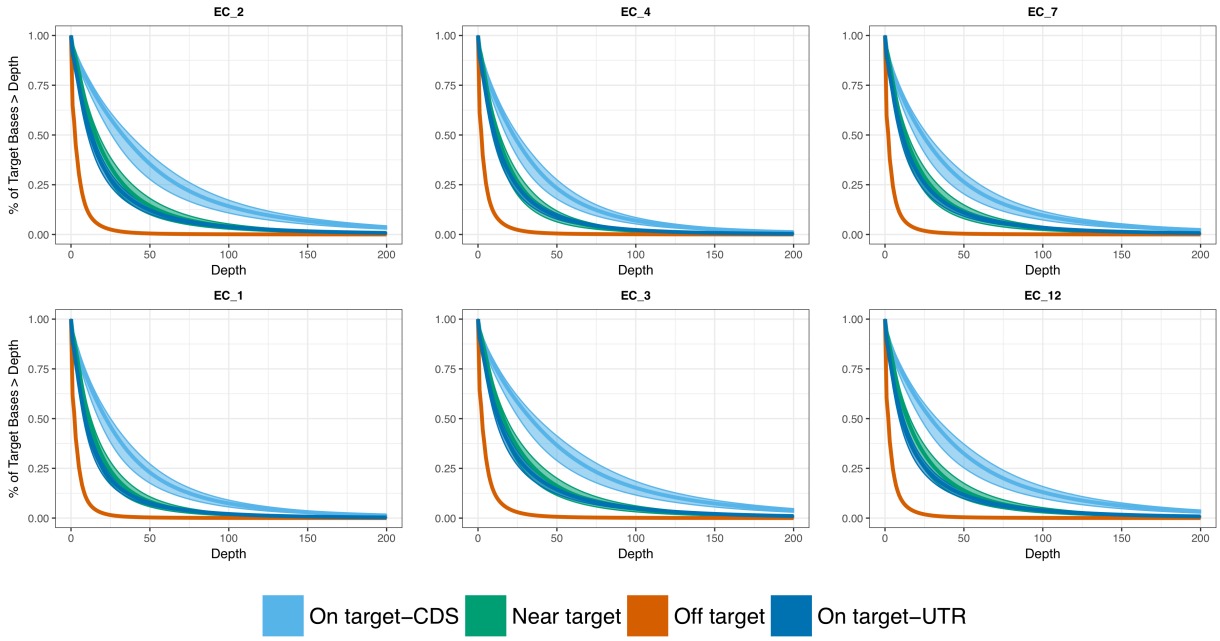
| **Reagent** | **Vendor** | **Price** | **Total Units** | **Units Used** | **Project Cost** |
|---|---|---|---|---|---|
| Genomic DNA Shearing | Genomic Core Lab | 1.50 | 1 | 96 | $144.00 |
| Kapa-Stranded mRNA-Seq 24 rxn kit - Illumina | KapaBiosystems KK8420 | $1,008.00 | 24 rxn | 4 (1/2 rxns are used) | $168.00 |
| Hyper Prep gDNA kit with KAPA Library Amplification Primer Mix (10X) 96 rxn kit | KapaBiosystems KK8504 | $2,496.00 | 96 rxn | 48 (1/2 rxns are used) | $1,248.00 |
| KAPA Pure Beads (5 mL) | KapaBiosystems KK8000 | $150.00 | 100 | 100 | $150.00 |
| Oligos | IDT | $2,391.85 | 1000 | 2 | $4.78 |
| DSN | Evrogen EA001 | $350 | 50 | 20 | $140.00 |
| Library Amplification Polymerase | KapaBiosystems KK2611 | $126.00 | 50 | 5 | $12.60 |
| SAII-HF Enzyme | NEB R3138S | $61.00 | 2000 | 100 | $3.05 |
| Mung Bean Nuclease | NEB M0250S | $63.00 | 1500 | 50 | $2.10 |
| DecaLabel™ Biotin DNA Labeling Kit | ThermoFisher K0651 | $158.00 | 10 | 1 | $15.80 |
| Denhardt's Solution (50X) | ThermoFisher 750018 | $149.00 | 100 | 0.0128 | $0.02 |
| Dynabeads™ M-280 Streptavidin | ThermoFisher 11205D | $496.00 | 2 mL | 0.08 | $19.84 |
| Human Cot-1 DNA (1 mg/ml) | ThermoFisher 15279011 | $230.00 | 500 µg | 4 | $1.84 |
| **Total Prep Cost** | | **$7,680.35** | | | **$1,910.03** |
| | | | | **Per Sample** | **$19.90** |
| Sequencing on Illumina Hi-Seq 4000 | Genomic Core Lab | $2,700 | 1 | 1-1.5 | $2,700 |
| | | | | **Per Sample** | **$28.13-$42.18** |
| | | | **Total Per Sample** | | **$48.02-$62.08** |

# Supplemental Figures



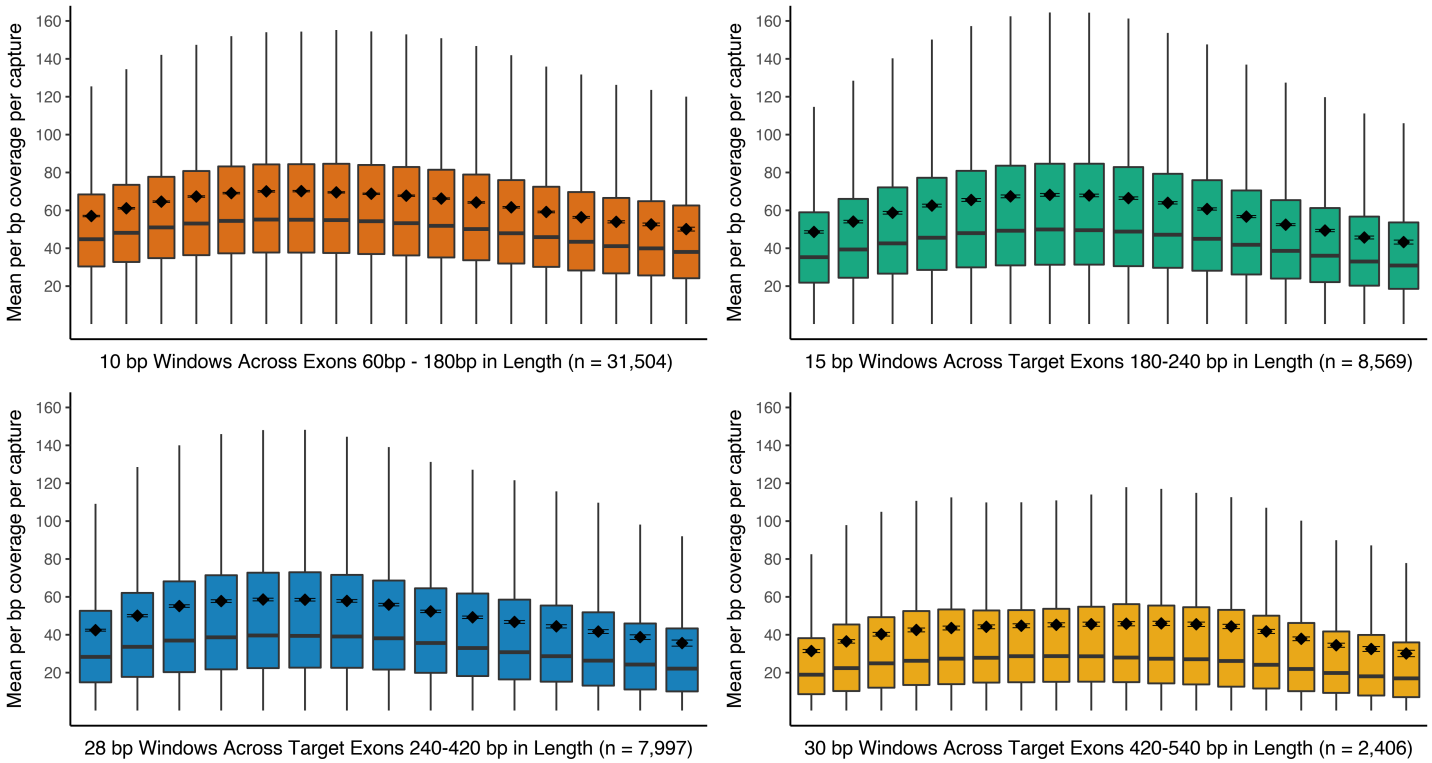

**Supplemental Figure 1. Expressed exome capture reads and RNAseq reads from probes plotted across the eastern oyster genome.**

Read coverage density was plotted in 10,000 bp sliding windows for both total RNA reads (red; below chromosome) and total EecSeq reads (blue; above chromosome) using the karyoploteR package (https://bioconductor.org/packages/release/bioc/html/karyoploteR.html).



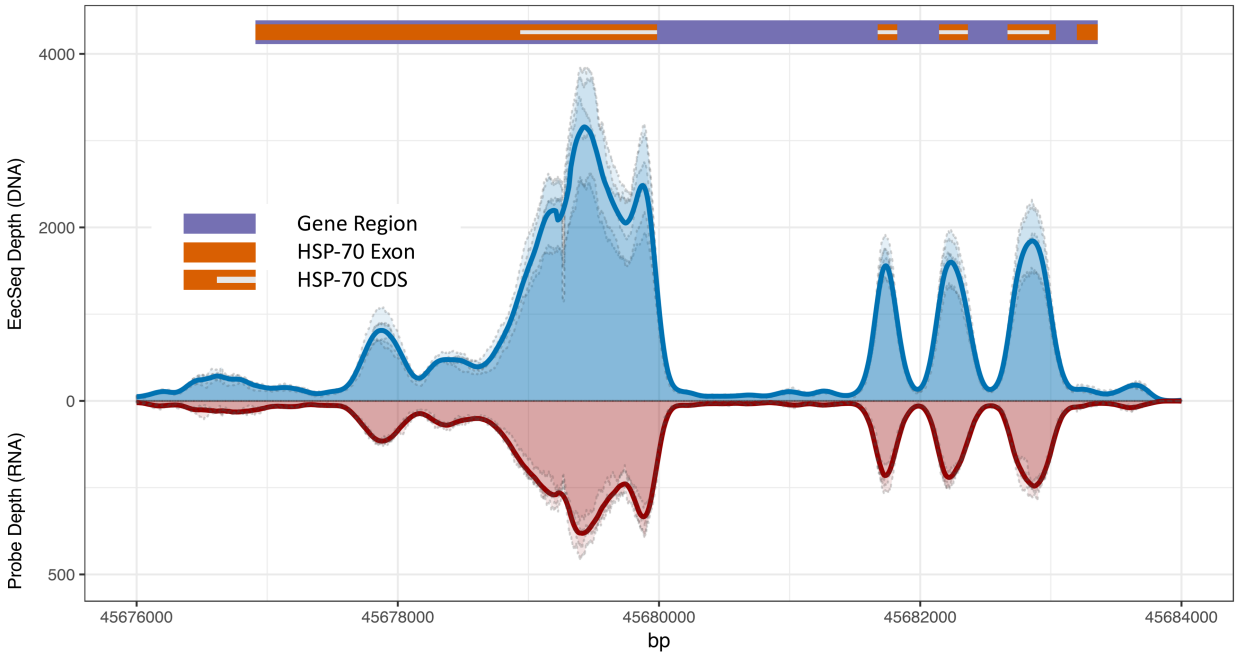

**Supplemental Figure 2. Per base pair sensitivity plot of EecSeq captures including CDS and UTR.**

To compare EecSeq to other capture methods, capture targets were defined as exons that had more than 35X coverage in the RNAseq (probe) data and confidence intervals were generated by defining capture targets between 20X RNAseq coverage and 50X RNAseq coverage. Near-target mapping were 150 bp on either side of the defined targets. For this figure, target exons were broken into coding sequence (CDS) and untranslated regions (UTR) for comparisons. This range corresponds to the modal DNA fragment length used for the capture libraries with the expectation that exon probes could capture reads that far from the original target. EC_2, EC_4, and EC_7 are the three replicate captures with the original probe pool, and EC_1, EC_3, and EC_12 are the replicate captures with the probe pool exposed to 12 extra rounds of PCR.




**Supplemental Figure 3. Boxplots of mean per basepair coverage levels plotted across target exons size windows.**

Target exons (those with at least 35X coverage in the RNA data) were broken into 10bp - 30 bp windows depending on overall size and the mean per basepair coverage per capture was calculated for each window size. The line each box represents the median of mean coverage values and the box surrounds the 25$^{th}$ and 75$^{th}$ percentiles. The mean of each bin class is plotted as a black diamond with standard error bars around it.




**Supplemental Figure 4. EecSeq capture and probe coverage across Heat Shock cognate 70 kDa.**

Coverage for each replicate capture pools is plotted along basepairs 45,760,000 to 45,684,000 of reference Chromosome NC_035782.1 containing the full gene region of 70 kDa protein 12B-like (NCBI Reference Sequence: XM_022468697.1). Each exome capture pool coverage is plotted in light blue with dashed grey border and a rolling 100 bp window average across all pools is plotted in dark blue. Each RNAseq (probe) sample coverage is plotted in light red with dashed grey border and a rolling 100 bp window average across all pools is plotted in dark red. Gene regions are marked in purple with exons color coded by gene. Coding sequence (CDS) is marked by a white bar within exon markers.