Chapter 11

Multiplex PCR Design for Scalable Resequencing

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Abstract

While conventional PCR applications typically focus on a single PCR assay per reaction, multiplex PCR applications are a convenient and scalable solution becoming more routine. Multiplex methods can be applied to virtually any DNA template source (e.g., plant or human DNA, FFPE DNA isolated from clinical samples, bisulfite-converted DNA for DNA methylation analysis), and offers a cheap, convenient, and scalable solution for experiments that require characterization and analysis of multiple genomic regions.

This method will detail the procedures to successfully design, screen, and prepare multiplex amplicon libraries; as well as supporting instructions on how to prepare these libraries for sequencing on Illumina, Ion Torrent, and Oxford Nanopore platforms. The flexibility of assay design allows means that custom multiplex panels can range in size from two assays up to a few hundred amplicons or more. Notably, the method described here is also amenable to whatever PCR buffer system the user prefers to use, making the system globally adaptable to the needs and preferences of the end user.

Key words PCR, Multiplex PCR, Sequencing, Resequencing, NGS, DNA methylation, SNP screening, Bisulfite DNA

1 Introduction

The polymerase chain reaction was first described in 1985 [1], and detailed a method wherein a region of DNA could be exponentially amplified 220,000 fold in a day. While the technique was rapidly adopted by molecular biology labs as a convenient and cheap way to characterize DNA, the initial application of the technique focused exclusively on the use of a single pair of DNA primers to amplify a single region of interest; if multiple different targets were under investigation, they would be run individually in separate reactions. This focus on limiting the methodology to single amplicon reactions was initially driven by a variety of practical limiting factors; for example, the relative cost of oligonucleotide synthesis for primer manufacture could be cost-prohibitive for many labs, and effective solutions for PCR-specific problems such as the production of primer dimers and off-target amplification effects were not well

Chhandak Basu (ed.), *PCR Primer Design*, Methods in Molecular Biology, vol. 2392, https://doi.org/10.1007/978-1-0716-1799-1_11,

established, both of which could render expensive DNA oligonucleotides unusable.

Since that time, advances in primer design, PCR formulations, and the assembly of the whole genomes have all worked to increase the fidelity of PCR applications and reduce its overall cost. This price reduction now allows multiplex panels of dozens [2], hundreds [3], and even tens-of-thousands of amplicons [4] to be concurrently amplified in a single reaction, thereby offering a convenient, flexible, and scalable method for profiling DNA from any source. These multiplex reactions have now been utilized for screening DNA variants [5], analysis of bisulfite-converted DNA for DNA methylation studies [6], gene expression profiling [4], and quantitative PCR applications such as qPCR [7] and ddPCR [8].

These advances are reflected in the current scientific marketplace, as many molecular biology vendors now offer multiplex amplification solutions. At the time of this manuscript, the Ampliseq methodology developed by Thermofisher has achieved some of the densest reactions, with over 20,000 different primer pairs in a single pool. However, other molecular biology vendors have also developed similar methods to amplify large numbers of primers within a single tube. One shared feature of these different commercial solutions is the fact they each employ slightly different methods to maintain the specificity and remove unwanted amplification artifacts, typically based on proprietary buffer and enzyme systems. While these unique formulations are effective, they can also rapidly inflate the per-reaction price to a point where it is not cost-effective, particularly for projects with small numbers of regions but a large number of samples to screen.

This method will therefore take users through the steps to design and implement their own cheap, effective multiplex PCR panels, using a methodology that has been routinely used for multiple different applications and samples [2, 9–12]. The standard process for designing and implementing multiplex assays involves an initial assessment of the dimer-score at which primer dimers occur using PrimerROC [12] (http://www.primer-dimer.com/roc/). After this is determined, individual PCR assays are designed using PrimerSuite [10] (http://www.primer-suite.com/) and ordered as individual oligonucleotides, followed by subsequent QC of the individual assays and multiplex pool. Once QC is finished, the final library pools are prepared using ligation-mediated PCR (Fig. 1), which is a second PCR step that employs a universal fusion sequence at the 5′ end of every primer to add platform-specific sequencing adaptors and sample barcodes, resulting in the construction of a sequencing-ready pool of samples (*see* **Note 10** for a more detailed description about ligation and barcoding). The methodology described has been routinely used for multiple applications [2–5] and can be applied to small assay pools (for example, two to four primer pairs for multiplexing qPCR or droplet-digital

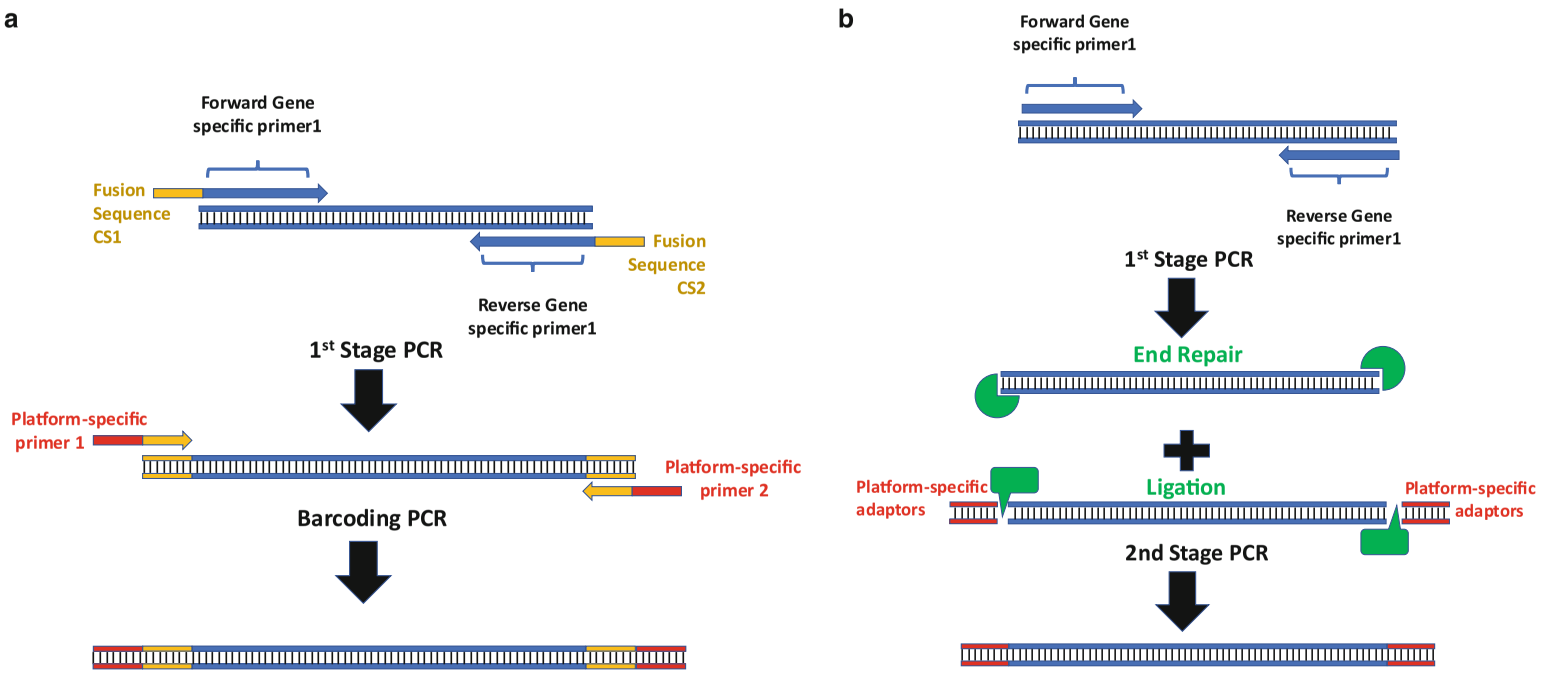

Fig. 1 Conceptual illustration of the difference between fusion primers (**a**) and the conventional PCR primer workflow (**b**). Fusion primers have an additional sequence of DNA appended to their 5′, which is shared by all primers in the reaction. The incorporation of the fusion sequence (which is referred to as the CS1 and CS2 sequences within this method) allows the sequencing adaptors and library barcodes to be incorporated using ligation-mediated PCR. This is in contrast to the standard PCR workflow (**b**), which will require a separate, enzymatic end-repair and ligation treatment steps to prepare the sample for sequencing

PCR assays [7, 8]), but scales up easily, and we have successfully validated and run larger pool pools of 50–100 primer pairs in a single reaction for multiple different applications. By running multiple large pools at the same time, users can easily scale up to screen hundreds of amplicons simultaneously and adapt the protocol to suit their sequencing platform of choice.

2 Materials

The method described how to construct libraries using PCR primers with universal consensus sequences appended to their 5′ end, followed by a ligation-mediated PCR step to add sequencing adaptors. However, an alternate method for multiplex resequencing can also be performed using non-fusion primers and enzymatic ligation; *see* **Note 1** and Fig. 1 for a more detailed description of the key differences between the two.

2.1 PCR Primer Ordering

1. PCR primers can be ordered from whichever supplier the user prefers. Our lab has had success with *Integrated DNA Technologies* (IDT), but any vendor will suffice.
2. This method employs fusion primers and requires that the following sequences are appended to the 5′ ends of all forward and reverse primers. *See* **Note 2** for more background information.

   Critical note: All forward primers must have the CS1 sequence appended to their 5′ end, and all reverse primers must have the CS2 sequence appended to their 5′ end.

   CS1-14 bp forward fusion sequence GACATGGTTC TACA.

   CS2-14 bp reverse fusion sequence CAGA GACTTGGTCT.

   For example, assuming two gene-specific primers where the forward primer was **AAACC** and the reverse primer was **GGGTT**, the oligos to order would be:

   Forward: 5′-GACATGGTTCTACA**AAACC**-3′.

   Reverse: 5′-CAGAGACTTGGTCT**GGGTT**-3′.
3. For most projects, ordering the smallest synthesis scale (25 nmole) provides more than enough primer, and it is recommended users begin with the smallest scale for screening and optimization purposes.
4. If less than eight primer pairs are being ordered, then ordering in tube format is appropriate. However, if more than eight primer pairs are being ordered, it is convenient (and frequently cheaper) to have them delivered in plate format, as this expedites the resuspension and initial primer screen. *See* **Note 3** if ordering in plate format.

2.2 PCR Reagents

While users can employ whichever PCR buffer system they are comfortable with, a hot-start polymerase is critical for successful multiplex amplification. Given this, the following two PCR buffer systems have been previously validated for this method.

1. High fidelity polymerase: Phusion Green Hot Start II High-Fidelity DNA Polymerase (2 U/μL), F537L, Thermofisher Scientific, USA. Suitable for standard DNA and variant/SNP screening applications.
2. Standard Taq Polymerase: Promega Gotaq Flexi Hotstart, M5005, Promega, USA. Suitable for bisulfite DNA methylation analysis.
3. 10 mM dNTP solution mix: New England Biolads (NEB), USA. N0447L.
4. Exonuclease I: New England Biolads (NEB), USA, cat # M0293L.
5. Library adaptors compatible with the CS1 and CS1 fusion sequences: Access Array Barcode Library for Illumina Sequencers—384, Single Direction, Fluidigm, USA: Product # 100-4876. If users wish to use Ion Torrent or Oxford Nanopore systems, *see* **Notes 1** and **10.**
6. Low Tris-EDTA (LTE) buffer: 10 mM Tris–HCl (pH 8.0), 0.1 mM EDTA.
7. AMPure XP beads: Beckman Coulter, USA: Cat# A63880. *See* **Note 4**.
8. Qubit HS Flourmetric DNA measurement kit: Q32851, Thermofisher Scientific, USA.

3 Methods

3.1 Primer Design and Resuspension

The method described details on how to construct libraries using PCR primers with universal consensus sequences appended to their 5′ end, followed by a ligation-mediated PCR step to add sequencing adaptors. However, an alternate method for multiplex resequencing can also be performed using non-fusion primers and enzymatic ligation; *see* **Note 1** and Fig. 1 for a more detailed description of the key differences between the two (Fig. 2).

1. Prior to primer design, users should employ PrimerROC [6] (http://www.primer-dimer.com/roc/) to determine the dimer score, which will assist in determining the threshold at which primer dimer artifacts occur within their PCR buffer system.
2. Go to PrimerSuite (http://www.primer-suite.com/) [5] and paste or upload a FASTA file containing the regions of interest. PrimerSuite accommodates assay design for standard genomic

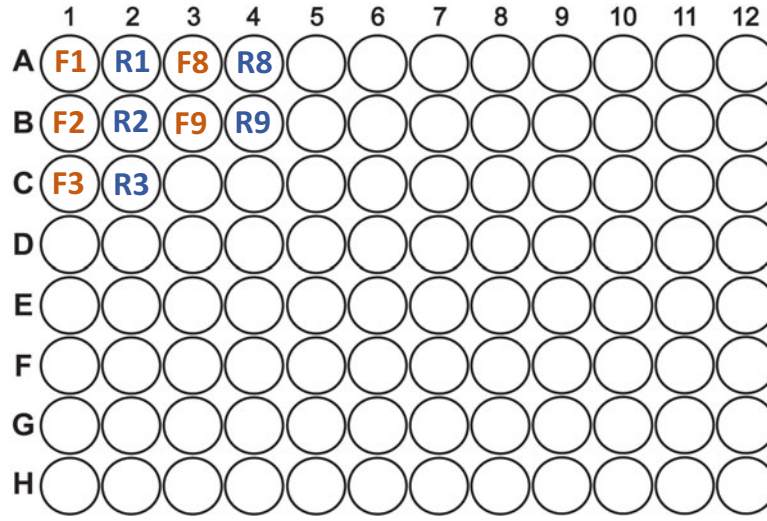

Fig. 2 Suggested layout for primer pairs when ordering in a plate format. For ease of use it is recommended that users order oligos arrayed so that forward and reverse primers are in alternating columns. This simplifies the process of combing the forward and reverse pairs when performing screening and pooling

DNA and bisulfite DNA methylation assays, and contains a variety of parameters thatthe user can tune to their design specifications. The default options PrimerSuite uses are generally a good starting point for most applications; however, if using a PCR buffer system different from what is recommended in this method, the *Minimum Dimer Score Cutoff* should be changed to that determined using PrimerROC in **step 1**.

The current version of PrimerSuite is restricted to 15 DNA regions. If attempting to design more than 15 regions, then more than multiple different multiplex pools will need to be designed.

3. Once the primers have arrived, resuspend all oligos to the same concentration using low Tris-EDTA buffer (LTE). A standard concentration to resuspend to is 100 μM. The calculation for determining the total volume of LTE to add to achieve a final concentration of 100 nM is

   total nmole of oligonucleotide × 10 = volume of LTE to add for 100 μM final concentration.

   Example: 9.7 nmole of oligo × 10 = 97 μl of LTE for a final concentration of 100 μM.

   After adding LTE, it is recommended that the tubes be vortexed and then quickly heated for 10 min at 37 °C to ensure they are fully dissolved, as the multiplex reaction can be sensitive to differences in primer concentration.

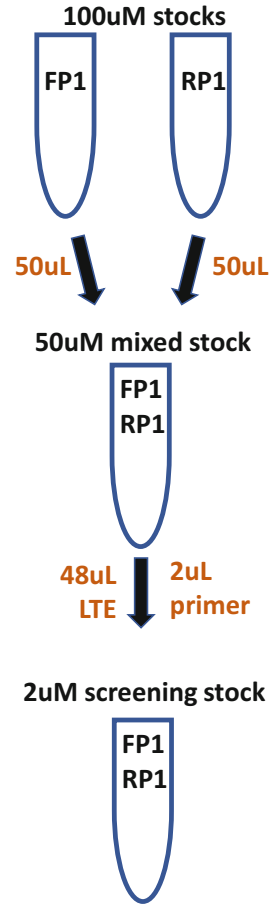

Fig. 3 Primer dilution schema for preparing the 50 μM and 2 μM working solutions

4. Make a 50 μM primer-pair stock by combing equal volumes of the 100 μM forward and reverse primers into a single tube. For example, combining 50 μl of forward-primer1 with 50 μl of reverse-primer1 into a single tube gives a total of 100 μl, with each primer at 50 μM individually (Fig. 3).
5. Perform an additional dilution of the 50 μM primer-pair stock to 2 μM, which is required to perform the initial singleplex screen. 2 μl of the 50 μM primer-pair stock added to 48 μl LTE is recommended (Fig. 3).

3.2 Singleplex Screening and Quality Control

After the primer pairs have been resuspended and working stocks have been made, the assays are first run individually in singleplex reactions for preliminary QC, as outlined in the following section.

1. Using the 2 μM primer pair stocks, perform a screening reaction using the PCR conditions below to check the amplification

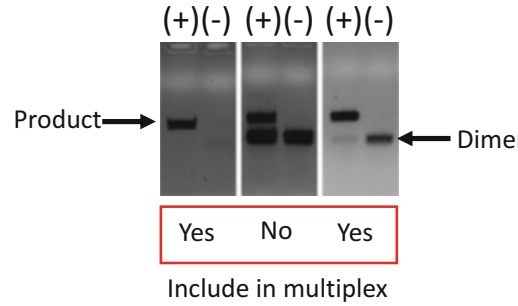

Fig. 4 Representative gel image showing expected PCR products for the singleplex screen. (+) PCR with template; (−) no template control. The primer pairs on the left and right can both be included in the final multiplex pool. The primer pair in the middle gives a strong dimer band in both the template and no-template controls, and should be excluded from the multiplex pool

fidelity of the primer pair, and to assess whether individual assays form primer dimers. Ensure that no template controls are run for all assays, as the no-template controls assist in determining the tendency of the assay to form primer dimers (Fig. 4).

Note: the following reaction conditions will work for both Phusion Green (Thermofisher) and GoTaq Flexi Green (Promega). Alternately, the PCR assays can be screened using your buffer system of choice.

Component	Volume	Final concentration	Notes
Water	Add to 25 µl		
5× green	5 µl	1×	Both Phusion and GoTaq are supplied at 5× concentration
10 mM dNTPS	0.5 µl	200 µM each	
2 µM forward/ reverse primer pair mix	2.5 µl	200 nM each	
Template DNA	× µl		5–50 ng input recommended. *See* **Note 5**
Hot start DNA polymerase	0.25 µl	0.02 U/µl	
25 mM $MgCl_2$	6 µl	6 µM	*See* **Note 6**

PCR cycling conditions

$$\begin{array}{l} 95^{\circ}\mathrm{C}\ 7\ \mathrm{min} \\ \left.\begin{array}{l} 95^{\circ}\mathrm{C}\ 20\,\mathrm{s} \\ 56^{\circ}\mathrm{C}\ 30\,\mathrm{s} \\ 62^{\circ}\mathrm{C}\ 30\,\mathrm{s} \\ 72^{\circ}\mathrm{C}\ 90\,\mathrm{s} \end{array}\right\} 35\ \text{cycles} \end{array}$$

The above cycling conditions are sufficient for the majority of multiplex reactions (i.e., amplicons less than 200 bp in size, and less than 20 amplicons in a single pool). For denser amplicons and/or amplicons which are larger in size, it may be beneficial to increase the extension time, to ensure equal amplification of all products.

Perform QC by running the PCR products out using DNA gel electrophoresis. Assays which give strong dimers with relatively little amplicon of the expended size should be excluded from future use. However, PCR assays which amplify well but produce a small amount of dimer can still be utilized in the multiplex reaction (Fig. 4).

3.3 Multiplex Screening and QC

1. Combine equal volumes of all primer pair assays which passed the singleplex QC to make the final multiplex pool.
2. Based on the pooling, work out the concentration of each *individual primer* within the reaction.

   For example, if 20 μl from 14 different primer pairs were combined to create a multiplex pool, and the starting concentration of each primer pair stock was 50 μM, the final concentration of each individual primer would be 3.57 μM. This can be calculated in the following way:

   14 primer pairs × 20 μl volume each pair at 50 μM = 280 μl total volume.

   Using the standard dilution factor formula C1V2 = C2V2.
   (50 μM)(20 μl) = C2 (280 μl).
   C2 = 3.57 μM.
3. Perform a PCR screen using the same conditions as outlined in the singleplex screening reaction above, with the following changes.
   (a) The total volume of primers put into the PCR reaction needs to be adjusted so that the final volume of each *individual primer* within the reaction is 200 nM. Using the example calculation detailed in **step 2** above, this would be:

   C1V2 = C2V2.

   (0.2 μM final primer concentration) × (25 μl final PCR reaction)

   =

   (3.57 μM initial primer concentration) × V2.

Table 1
Recommended cycles for multiplex amplification

# of PCR assays in multiplex	# of PCR cycles to perform for QC	# of PCR cycles to perform for library preparation
2	34	31
4	33	30
8	32	29
16	31	28
32	30	27
64	29	26

V2 = 1.4 μl of multiplex primer pool to add to the 25 μl PCR reaction.

(b) The total number of PCR cycles needs to be reduced, since product formation is now occurring at a faster rate proportional to the number of assays in the pool. To estimate the number of cycles, the PCR should be reduced by, refer to Table 1.

4. Assess multiplex quality by running the PCR products out using DNA gel electrophoresis. A single strong band should be observed of the expected size, with minimal-to-no primer dimer observed, similar to the first panel in Fig. 4.

3.4 Multiplex Amplification of Target Samples and Library Preparation

1. Once the multiplex pool has been confirmed to work, proper samples can now be amplified. PCR conditions remain identical, except that the total number of PCR cycles should be further reduced as outlined in Table 1. *See* **Note 7**.
2. Add 2 μl of Exonuclease I to each multiplex pool reaction to remove unincorporated primers. Incubate at 37 °C for 15 min.
3. Clean up the PCR reaction using AMPure XP beads, or a similar SPRI bead system, following the vendor's recommended protocol (*see* **Note 4**). Based on the size of the amplicons produced, the following ratio of beads to PCR reaction is recommended.

   Amplicons equal to or smaller than 150 bp in size: Use 1.5× volume of beads.

   Amplicons larger than 150 bp in size: Use 1.2× volume of beads.
4. Resuspend the sample in 20 μl of LTE and incubate at 37 °C for 5 min to fully elute the DNA from the beads.
5. Prepare the barcoding PCR reaction according to the following recipe. Note: Only the Phusion polymerase is used for this step to ensure high-quality sequencing data.

Component	Volume	Final concentration
Water	Add to 30 µl	
5× Phusion Green	5 µl	1x
10 mM dNTPS	0.5 µl	200 µM each
2 µM forward/reverse access array barcoding primer	5 µl	333 nM each *See* **Notes 1** and **10** for sequencing on other platforms
Purified amplicons from **step 4**	10 µl	
Phusion hot start DNA polymerase	0.25 µl	0.02 U/µl
25 mM $MgCl_2$	7.2 µl	6 µM

6. Perform the barcoding PCR using the following cycling conditions

Barcoding PCR
94°C 5 min
94°C 20 s
45°C 30 s
72°C 120 s
} 9 cycles

7. Perform QC by running the PCR products out using DNA gel electrophoresis. The expected size of the final library is roughly 150 basepairs larger than the original amplicon size, and if the process has been successful, a single strong band should be observed, although frequently a smaller dimer band around 150 bp can be seen. If too much or too little product is observed, the total PCR cycles may have to be adjusted up or down. If the predominant product is less than 200 bp in size, then primer-dimer artifacts have dominated the barcoding PCR reaction. In this case, refer to **Note 8** for troubleshooting.
8. Once the final libraries are confirmed to have worked, the individual samples can be pooled together for sequencing. If the total amount of product as visualized by gel electrophoresis is roughly equivalent between all samples, then equal volumes of every sample can be combined together. If the samples vary in their total yield, *see* **Note 9** for pooling recommendations.

Once the libraries are pooled in this way, sequencing on the platform of choice can be performed. In determining the best sequencing configuration, users should consult with their local genomic service provider.

4 Notes

1. The choice of whether to use primers with 5′ fusion sequences verses non-fusion primers has several implications. The following consideration may assist users in determining which is the best to use.
   (a) For projects that have a large number of samples (i.e., 100 samples or more) with a small number of regions for analysis (i.e., less than 20 regions being interrogated), using fusion primers is recommended as this allows up to 384 samples to be batched together on a single sequencing run, which is the most cost-effective option.
   (b) For projects with a large number of regions which require relatively dense multiple reactions per pool (i.e., more than 50 primer pairs in a single reaction), non-fusion primers may be preferable, as the inclusion of the fusion sequence increases the likelihood that primer-dimers artifacts will occur [6].

   If users wish to use a ligation method for their library construction, some additional components are required. A separate set of index adaptors that suit the sequencing platform of choice will need to be ordered directly from the platform vendor, e.g., Illumina, Ion Torrent, Oxford Nanopore, or others. Next, molecular biology reagents for end repair and ligation must also be purchased to perform ligation. NEB's Ultra II End Prep and Ultra II Ligation Module is one system users can refer to.
2. The fusion sequences employed were originally developed by Fluidigm for their Access Array platform, and were originally 23 bases long. However, the sequences provided for the method described in this chapter are 14 bp in length. Both sequences are illustrated below.

Fluidigm's original CS1	ACACTGACGACATGGTTCTACA
Truncated 14 bp CS1	GACATGGTTCTACA
Fluidigm's original CS2	TACGGTAGCAGAGACTTGGTCT
Truncated 14 bp CS2	CAGAGACTTGGTCT

The 14 bp truncated version is preferred since shorter fusion sequences generally result in less dimer formation during PCR [6]. As well, 14 bp was empirically determined to be the smallest length that could be used which still functions well using ligation-mediated PCR to incorporate the sequencing adaptors into the amplicon library.

3. When more than eight primer pairs are being ordered, it is cheaper and easier to order them in a 96-well plate format.

   If ordering in a plate format, users should attempt to order in such a way as that the Forward and Reverse primer pairs are laid out in alternating columns, as illustrated below in Fig. 2. Doing so allows the user to rapidly combine the primer stocks into forward + reverse primer pair aliquots using an 8-well multichannel pipette.

   When ordering in plate format, users should also choose the synthesis option wherein the total nmole in every well is standardized to the same quantity, as this greatly simplifies oligo resuspension and pooling.

4. Although Agencourt Ampure XP beads are listed in this method, any type of Solution Phase Reversable Immobolization (SPRI) bead-based DNA purification can be used. If using a different SPRI system than AMPure XP, some minor optimization regarding the ratio of beads:PCR may be required.

5. The method is flexible in the amount of input template to use, with the method having previously worked well with as little as 5 ng of input DNA. However, putting more templates into the reaction up to 50 ng can be beneficial and can help to reduce primer-dimer formation. If possible, users can screen a variety of template input amounts to determine the best quantity to use.

6. This recommended workflow uses a 6 mM final concentration of Mg^{2+} in the PCR conditions. In general, we have found the increased Mg^{2+} concentration does not increase off-target amplification, but does greatly enhance product formation. If users are having difficulty with off-target amplification, then a lower concentration of Mg^{2+} can be used.

7. The total number of PCR cycles is further reduced when amplifying actual target samples for two reasons. The first is to ensure that the reaction does not enter into a non-exponential product formation phase, which can be problematic and occurs at higher cycles. Second, differences in efficiency between high- and low-performing assays are magnified if too many PCR cycles occur. The functional outcome of this is that read coverage in the final sequencing reaction can be dominated by a just a few highly performing primer pairs, and the easiest way to control for this is to reduce the total number of PCR cycles.

8. The final product produced in the final barcoding PCR reaction should be approximately 150 bp larger than the original amplicon size, and should be readily visible when run on a gel. However, if the dominant product is 200bp or less, then the exonuclease I treatment and SPRI bead cleanup were not

effective in completely removing the residual unincorporated multiplex PCR primers. If this is the case, additional exonuclease I can be put into the reaction and the digest allowed to proceed for longer; and/or an additional SPRI bead clean may have to be performed.

9. Frequently some samples will produce more library than others due to variation in sample input and/or quality. In such cases, it is recommended to pool samples together based on their relative product yield first, then combine equal masses of each library into a final pool.

   For example, pool equal volumes of the high-yield samples into one tube, and the low-yield samples into a second tube. After pooling in this manner, each pool is then cleaned with SPRI beads and resuspended in 30ul LTE. Next, measure the concentration of each pooled sample using a fluorometric method such as Qubit HS (High Sensitivity). Finally, based on the concentration of the samples, combine the two pools in such a way so that there is an equal mass of each library in the final pool.

   Performing the pooling this way ensures that low-yield samples are not titrated out in the final sequencing reaction by the high-yield samples, and helps to normalize read coverage.

10. The process of preparing amplicon pools for sequencing requires an additional step which adds the platform-specific DNA sequences that enable sequencing, as detailed in Fig. 1. For example, Illumina utilizes DNA sequences referred to as P5 and P7 which are specific to their platform; Ion Torrent utilizes the A and P1 sequences. Sequencing adaptors are therefore specific and unique to each platform.

    The sequencing adaptors also contain an extra segment of DNA referred to as the barcode. The barcode is a small string of DNA bases typically 8–10 bp in length which are unique for every sample; in this way multiple samples can be combined together on a single sequencing run, and after the barcode is sequenced, the amplicons that relate to the original starting sample can be determined.

    Currently, Fluidigm only directly sells barcode adaptors to be used with the CS fusion sequences and Illumina sequencing platforms. However, the multiplex method can easily be adapted to other platforms with relative ease.

    If the user wishes to use Oxford Nanopore to sequence, the CS1 and CS2 sequences should be replaced with the following sequences instead:

    Forward primer

    5′- TTTCTGTTGGTGCTGATATTGC —your primer sequence-3′

Reverse primer

5′- ACTTGCCTGTCGCTCTATCTTC —your primer sequence-3′

Users are also referred to Oxford Nanopores *Four-primer PCR* workflow (Documents SQK-PSK004 or SQK-PBK004).

If the user wishes to use Ion Torrent platforms to sequence, the CS1 and CS2 sequences can still be used, but additional HPLC purified PCR barcode primers must be ordered in, since (at the time of this protocol) no commercial set can be purchased.

Note: Substitute in the DNA barcode of choice for the A-barcode X-CS2 primer, and order sufficient barcode primers to ensure every sample of interest can be uniquely barcoded.

Order the following at 100nmol synthesis scale, HPLC purified:

A-barcode X-CS1

CCATCTCATCCCTGCGTGTCTCCGACTCAG [barcode]GATACACTGACGACATGGTTCTACA

Order the following at 100 nmol synthesis scale, HPLC purified

P1-CS2 CCTCTCTATGGGCAGTCGGTGATTACGG TAGCAGAGACTTGGTCT

For example, if the IonExpress 001 and 002 barcodes were selected, the following sequences would be used (barcode sequence is **bolded and underlined**):

A-IonXpress001-CS1

5′ CCATCTCATCCCTGCGTGTCTCCGACTCAG **CTAAGGTAAC**GATACACTGACGACATGGTTCTACA

A-IonXpress002-CS1

5′ CCATCTCATCCCTGCGTGTCTCCGACTCAG **TAAGGAGAAC**GATACACTGACGACATGGTTCTACA

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