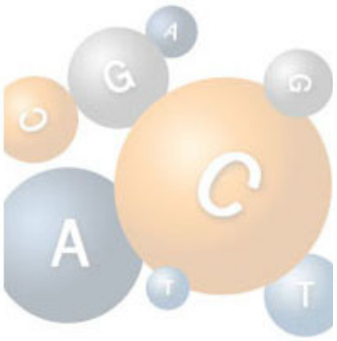
MASSACHUSETTS GENERAL HOSPITAL

Simches Research Center • 185 Cambridge St., Boston, MA 02114 • nextgen@research.mgh.harvard.edu



next generation @MGH Sequencing Core

RESOURCES

Custom Primer Design: Things to Consider

Topics in this section:

- Uses of custom sequencing primers
- Review of Illumina technology and chemistry
- Designing a custom sequencing primer
- Validating your custom sequencing primer
- Ordering sequencing using a custom primer

Uses of custom sequencing primers

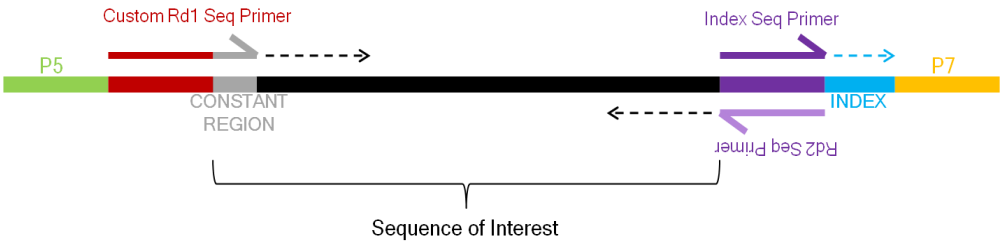
Custom sequencing primers are not recommended nor supported by Illumina. However, there are instances when an experiment can greatly benefit from the use of a custom primer. If designed correctly to be compatible with Illumina's chemistry, it can be used to overcome certain obstacles. One use is for unique library construction protocols which alter the sequence where an Illumina primer would anneal.

Another more common example of custom primer use is for sequencing libraries with an initial constant region. This region can be biological in nature, a byproduct of the method, a linker, etc. Illumina's platform uses an algorithm which defines unique clusters based on the first FOUR cycles of sequencing. Initial constant regions can obscure this process. In the past, the Genome Analyzer (GA) platform could be programmed to define clusters at slightly later cycles to accomodate this, but the new HiSEQ platform does NOT have this capability.

Below is the structure of a typical Illumina library:

The role of a custom primer in sequencing these samples is shown below. It allows sequencing to begin AFTER the constant region. This both makes the sample compatible with the HiSEQ's algorithm and gives the customer more usable data.

Example of the use of a custom seq. primer:



Review of Illumina technology and chemistry

In order to successfully design a custom sequencing primer, it is important to understand the basic chemistry behind Illumina's technology.

Please refer to our Overview of Illumina Chemistry page for illustrated descriptions of the next generation sequencing process. It is important that you realize the order in which things happen, as well as the structure of the library, to ensure that your custom primer will anneal efficiently and at the correct location. Our core is currently shifting between the GA and our new HiSEQ, and will soon be taking our GA out of service. As mentioned above, the HiSEQ CANNOT use any other cycle combination besides 1-4 for cluster identification. After clicking the link above, please continue reading.

How this relates to custom primer design:

- You can no longer "skip cycles" to adjust for constant regions.
- In Illumina's design, the P5 end is sequenced first, then the P7 region (if a paired-end read). You cannot change this.
- Sequencing for each read is unidirectional. Remember that extension can only occur in the 5'-->3' direction! At the beginning of each read, each library fragment is attached to the flowcell by the 5' end, with a free 3' end. Thus, sequencing must always occur "from the top, down."
- The sequencing steps are the same for read 1 of both PE and SE runs.
- Read 1 clustering is done on an instrument called the cBot, which allows the user to apply a different sequencing primer to each lane. Read 2 clustering is done on the HiSEQ (or GA), which applies the same primer to all lanes. Thus, you can ONLY use a custom primer for read 1.

Designing a custom sequencing primer

The design requirements listed below are meant to clarify the information on this page. There may be additional design requirements depending on the nature of your project.

The following sequences have been released by Illumina and may be helpful in the design of your custom primer:

P5: 5' AAT GAT ACG GCG ACC ACC GA 3'
P7: 5' CAA GCA GAA GAC GGC ATA CGA 3'

Remeber that these sequences make up only a piece of a typical Illumina adapter construct. Full Illumina adapter sequences may be available to you. Please email Ulandt Kim to inquire.

Custom primers must:

- Anneal to the P5 end of the library (refer to sequences above).
- Be used for read 1 ONLY.
- Span any initial constant regions.
- Be positioned so that 5'-->3' extension will occur using the sequence of interest as the template.
- Have properties which match those of Illumina's primer as closely as possible:
  - Tm = 66°C
  - 33bp
  - 52% GC
- Not have a risk of forming any significant 2° structures (i.e. won't stick to itself, form loops, etc.)

Custom primers submitted to the core must:

- Be HPLC purified.
- Be 100 uM.
- Have a volume of at least 10ul.
- Be reconstituted in EB, DI water, etc. (we recommend a standard SSC buffer with TWEEN).
- Be submitted in a low-bind tube.

We require that primers be HPLC purified because HPLC removes incomplete sequences generated during oligo synthesis, while standard desalting does not.

Validating your custom sequencing primer

Before submitting your samples and custom primer, we recommend that you QC the primer. Sanger sequencing can be used to judge the primer's annealing capabilities and rule out any adverse 2° structures. However, it is important to note that a primer and its corresponding sample may be successfully Sanger sequenced but still fail next generation sequencing. This is because of Illumina's unidirectional method.

Therefore, we recommend setting up a PCR reaction using your custom sequencing primer as the forward primer, and the P7 sequence as the reverse primer. You will get product if you primer both anneals effectively AND anneals to the correct end of the library fragment.

Ordering sequencing using a custom primer

At this time, there is no extra charge for sequencing using a custom primer, and samples are submitted in the same fashion (Galaxy for MGH users, an emailed form for external users).

When submitting to Galaxy, note the plan to use a custom primer in the "Library Construction Method" field, including where the primer anneals and its sequence. For external users, there is a space on the submission form where you can enter the sequence of the custom primer, and you can add additional information, such as where it anneals, in the notes section.

It is also requested that you notify us via email when submitting a sample which uses a custom sequencing primer.