



Request for Proposals: Pilot of Global Hepatitis Outbreak and Surveillance Technology (GHOST) for Molecular Surveillance of HCV Infection

Application Due date: September 9, 2016

Submit to: Anne Gaynor, Manager of HIV, viral Hepatitis STD and TB
(Anne.Gaynor@aphl.org)

Summary

The Association of Public Health Laboratories (APHL), in cooperation with the US Centers for Disease Control and Prevention's (CDC) Division of Viral Hepatitis (DVH) is seeking to identify up to four state or local public health laboratories that will perform next generation sequencing (NGS) for hepatitis C virus (HCV) and pilot the utilization of GHOST for tracking HCV transmission during outbreak investigation and molecular surveillance in different epidemiological settings. Selected laboratories will regularly report their experiences using GHOST to identify areas where corrections, improvements or enhancements might be made.

Background

Worldwide approximately 390 million persons are living with HBV or HCV infection with 1.3 million persons dying each year from these diseases. In the US, approximately 5.7 million persons are living with HBV or HCV infection, which are major causes of chronic liver disease and liver cancer. In 2013, the number of HCV-associated deaths exceeded the number of deaths from all other nationally notifiable diseases combined. At least 50% of persons born 1945-1965 do not know they are living with HCV infection. From 2006-2010 the number of acute HCV cases was between 800-1,000 cases per year. Since that time the number of acute HCV cases continues to rise each year, with 2,138 cases having been reported recently. The number of new HCV infections increased >150% from 2010-2013, mostly among adolescents and young adults. These numbers are expected to rise as the main route of transmission is through injection drug use, which is also on the rise in the US. Continued transmission of HBV and HCV

through unsafe injection practices challenges the U.S. efforts to prevent new infections and reduce viral hepatitis-related morbidity and mortality.

Efficient molecular surveillance for viral hepatitis is needed to track infections and devise effective public health strategies for timely interventions to interrupt viral transmissions. Although NGS provides novel opportunities for efficient detection and tracking of HCV transmission, molecular surveillance remains complex and costly. Global Hepatitis Outbreak and Surveillance Technology (GHOST), recently developed by DVH, is an online portal that enables sites to upload raw sequence files and hosts a set of computational tools for automated sequence analysis. The user can upload raw sequence files to GHOST which can then be run through the automated algorithms to clean the data and return visual representations of potential transmission links.

To reduce potential laboratory errors, to achieve cost-reductions and to develop seamless transition to the GHOST-based surveillance, we are looking for laboratories to pilot GHOST. Pilot sites will follow a standard protocol (explained in Appendix D) and each site is expected to perform 7-8 sequence runs over the course of the project which is the equivalent of 48-120 samples in an approximately 9 month time frame (October 1, 2016-June 30, 2017). Sequences must be uploaded to GHOST within 1 week of completion of the sequencing run.

This pilot will be used to guide improvements to GHOST functionalities. We will look at adaptation to user needs and improved integration of HCV GHOST into enhanced viral hepatitis surveillance activities supported by CDC. Additional outcomes could include establishing regional, independent and sustainable HCV GHOST-based molecular surveillance programs in support of public health interventions such as outbreak response, investigations of endemic HCV transmission among high-risk populations, "cure-as-prevention" and other strategies to stop transmission.

Eligibility

Eligible laboratories include all member public health laboratories with the following capabilities and facilities in place. Specific expectations regarding methodologies to be used by the awardees are outlined in [Appendix A](#). Training in HCV NGS will be provided upon request. Technical assistance and troubleshooting will be provided by regular teleconference. All applicants are required to agree to the following minimum requirements (as also outlined in [Appendix B](#)):

- Established capacity for isolating high quality viral RNA;
- Established and demonstrated capacity for sequencing using Illumina MiSeq; and
- Sufficient equipment, laboratory space and workforce capacity for the proposed workload

Anticipated RFP Schedule

APHL anticipates the following schedule:

- | | | |
|----------------|---|---|
| July 27, 2016 | – | RFP Issued |
| August 8, 2016 | – | Letter of Intent Due to APH (see below) |

| | | |
|--------------------------|---|---|
| September 9, 2016 | – | RFP Responses Due |
| September 19, 2016 | – | Proposal review completed |
| September 20-21, 2016 | – | If needed, follow-up interviews and updated proposals due |
| September 22, 2016 | – | Final review completed and awardees selected |
| September 30, 2016 | – | Draft contracts submitted to APHL Legal Dept. for final internal review |

Any modification to this anticipated schedule will be communicated on APHL's procurement website (www.aphl.org/rfp) and via an email blast to the public health laboratories (PHLs).

Response Submittal

Confirmation of Intent to Respond

APHL requests that prospective applicants submit a brief email statement indicating an intent to submit a proposal. This must be received by **5:00pm EST on August 8, 2016**.

Final Response

APHL must receive complete responses by **5:00 pm EST, on September 9, 2016**. Please see page 6, section "Proposal – Required Submissions" for items that must be included in the completed proposal. Applicants may send proposals by the following methods:

Via email to Anne.Gaynor@aphl.org or

Via Mail (USPS, FedEx, UPS) addressed to:

Association of Public Health Laboratories

Attn: ANNE GAYNOR

8515 Georgia Avenue, Suite 700

Silver Spring, MD 20910

APHL will send an email acknowledging the receipt of your application; if you do not receive an acknowledgement within 48 hours, please email the RFP points of contact above to confirm receipt.

Award

Up to four laboratories, depending on the strength of applications, will be selected. Each selected site will be eligible for an award of up to \$25,000 which will be distributed via a contract administered with APHL.

Term of Project

The project term will be from date of contract signing (approximately October 1, 2016) through June 30, 2017. APHL anticipates the potential for annual renewals (with each additional funding year running from July 1 to June 30) for a maximum of three additional years. Each of the potential

renewals may involve some adjustment to the scope of work in order to address any change in the funding received by APHL and to accommodate CDC programmatic needs in that funding year. Each awardee would be notified in advance of any modification to the anticipated scope of work in a future funding year.

Evaluation Team

APHL staff, led by the HHST Program Manager, will conduct an initial review of all proposals for completeness. Any incomplete application on the proposal due date specified in [Anticipated RFP Schedule](#) Section above will not be considered and will not receive a formal evaluation.

Complete proposals will be reviewed by a team of three subject matter experts (SMEs) from CDC DVH and a panel of three APHL members selected from non-applicant public health laboratories. SMEs from CDC will be identified and selected by the Branch Chief of the DVH Laboratory Branch based on their familiarity with laboratory techniques and project requirements. APHL member experts will be identified from among the non-applicant PHLs by the APHL HHST Program Manager and will have expertise in the laboratory testing methods described in this RFP and familiarity with APHL reference center structure.

APHL will ask potential reviewers to disclose any real or perceived conflict of interest prior to the start of the evaluation process or to affirm that they have no conflict of interest that would preclude an unbiased and objective review of the proposals received. APHL will not select reviewers with a perceived conflict of interest.

Once potential reviewers have been identified, APHL's Director of Infectious Disease Programs will have final approval over the review team's composition.

Evaluation Criteria

Proposals will be evaluated based on the responses to the questions above and will receive a numeric score of up to 100 maximum points based on the scorecard template in [Appendix C](#).

Laboratories with prior experience with the protocol and utilization of GHOST will be considered as an enhancing factor for this RFP. Laboratories will also be given preference based on more extensive experience with the test methods, ability to handle increased test volume for HCV sequencing, existing in-house subject matter expertise, ability to comply with expectations laid out in [Appendix A](#) and ability to meet the minimum expectations outlined in [Appendix B](#).

Evaluation Process

The entire review will be conducted via a combination of email communication between APHL's HHST Program Manager and the members of the evaluation team or among the evaluation team members and teleconference and/or webinar evaluation sessions. APHL's HHST Program Manager will coordinate the review process and the evaluation sessions.

The reviewers may request follow-up interviews with all or some of the applicant laboratories and, following these interviews, may request supplemental information on an applicant's proposal.

These interviews and any supplemental information would clarify a laboratory's capacity or experience in one or more of the evaluation criteria or to explain other information contained in an applicant's proposal.

There will be no formal evaluation performed by a member of APHL staff. In cases where all other evaluation criteria are substantially similar, APHL will have the ability to advise the evaluation team on selections that would provide geographical spread or otherwise diversify APHL's funding allocations. In addition, the evaluation team may receive documentation from APHL staff on an applicant's past performance in other capacities noted in this RFP as part of the evaluation criteria.

Post-Evaluation Procedures

The selected laboratories will be notified by APHL staff within ten business days of the completion of the evaluation and the names of the three recipients will be posted to APHL's procurement website, www.aphl.org/rfp on the same day. Unsuccessful applicants will receive notification of these results by e-mail or by U.S. mail within 30 days of the date the name of the winning vendor is posted.

All applicant laboratories will be entitled to utilize APHL's RFP Appeals Process to formulate a protest regarding alleged irregularities or improprieties during the procurement process. Specific details of this policy are located on the procurement website.

Conditions of Award Acceptance

The eligible laboratories must be able to contract directly with APHL or have an existing relationship with a third-party organization that can contract directly with APHL on behalf of the laboratory. Laboratories must agree to comply with expectations outlined in [Appendix A](#).

Prior to making the official award, a group of individuals from CDC and APHL will be entitled to elect to tour the facilities to assess compliance with requirements for testing and/or have a teleconference with applicant laboratories. Post award, monitoring site visits may be conducted to include an assessment of continued compliance.

Proposal – Required Submissions

To submit a proposal for consideration please respond to the following questions. Responses should be limited to no more than four double-spaced pages (font size \geq 11pt and page margins of \geq 1 inch) and must comply with submission requirements set out in Additional Information and Deadlines for Application Submission Below.

1. **Please describe the laboratory's current HCV testing practices including methodologies and algorithms.**
 - a. Describe how long the methods have been in use, how often testing is performed, populations tested, and amount of experience laboratory staff have in using the methodology.
2. **Please describe the laboratory's experience for extracting high quality RNA including methodologies.**
 - a. Describe how long the methods have been in use, how often testing is performed, pathogens tested, and amount of experience laboratory staff have in using the methodology.
3. **Please describe the current methodologies for sequencing (using MiSeq) that are used in your laboratory.**
 - a. Describe which pathogens are being sequenced, any pertinent assays that are being used, how long the methodology has been performed, how often it is performed, your annual maximum volume, the amount of experience your laboratory staff has in using that methodology and any training your staff has received.
 - b. Please describe a description of the library and sequencing kits that will be used for this project.
4. **Please describe any existing infrastructure that could be utilized for this project including equipment.**
5. **Please describe the number of HCV positive cases in the jurisdiction affiliated with the laboratory and a specimen selection plan or criteria to select 24-96 HCV positive specimens.**
 - a. If specimens will be obtained from other laboratories, please describe this relationship and attach a letter of support.
 - b. If you do not have at least 24 HCV positive specimens please describe efforts to obtain HCV positive samples and clearly state that you will need specimens from CDC.
6. **Include a completed and signed copy of [Appendix B](#) as an attachment.**

Additional Information and Deadlines for Application Submission

All questions should be directed to Anne Gaynor at anne.gaynor@aphl.org. Questions received from interested PHLs, together with the answers provided by APHL or CDC staff will be posted to APHL's procurement website (www.aphl.org/rfp).

Applications should be submitted to Anne Gaynor at APHL (Anne.Gaynor@aphl.org; 8515 Georgia Ave Suite 700, Silver Spring, MD, 20910; telephone: 240-485-2739; fax: 240-485-2700).

Applications must be received at APHL, attention Anne Gaynor by close of business (5:00pm ET) September 9, 2016. Either electronic or physical submission is acceptable. APHL will send an email acknowledging the receipt of your application; if you do not receive an acknowledgement within 48 hours, call 240-485-2739 to confirm receipt.

Appendix A: Expectations for HCV Sequencing and GHOST Utilization Pilot Sites

Methods

- 1) Each site will receive a panel of up to 24 specimens for training and proficiency testing at the beginning of the project period. A total of 4 runs with different primer and sample combinations will be conducted on this panel at each site.
- 2) Labs are expected to isolate viral RNA and prepare samples for sequencing following the protocol provided in Appendix D.
- 3) The Illumina MiSeq is the selected sequencing platform for HCV sequencing. Paired end sequencing with read lengths of at least 200 base pairs is required using V2 (2x250) or V3(2x300) kits.
- 4) Each site is expected to perform an additional 3-4 sequence runs with at least 8-24 samples per run in the approximately 9 month time frame (24-96 additional specimens during the project period).
 - a) HCV Positive cases collected within the public health laboratories affiliated jurisdiction. Specimens should contain a minimum of 500ul and an aliquot of each of these specimens will also be sequenced by CDC.
 - b) If a site is unable to collect sufficient specimens, CDC will be able to provide specimens for the additional 3-4 sequence runs.
- 5) Sites will upload sequences to GHOST via an online portal within 1 week after completion of the sequencing run.

Procurement

Supplies, Reagents and Equipment can be procured using the funding for this project. Each site will only be funded up to \$25,000 and allocation of those funds is at the discretion of the awarded sites.

Data Management

FASTQC or a similar program should be used to calculate and report cluster density, Q30, total sequences per run and theoretical coverage. Minimum acceptable quality scores are those defined in the specifications for the sequencing kit to yield approx. 1000-1200 clusters/mm² and phasing/prephasing values < 0.25. Data and fastq files will be uploaded onto the GHOST portal through an online portal login. CDC will also analyze the data using our laboratory pipeline of additional bioinformatics tools to evaluate the data and identify any potential problems with the GHOST website-based analyses.

Performance Management and Evaluation

Performance will be monitored by timeliness of responses to CDC and APHL requests and successful completion of a proficiency panel. The HCV/GHOST pilot site must submit electronic notices of data transfer to APHL and CDC.

Reports

The laboratory will submit to APHL and CDC a monthly line listing of the samples tested and the following variables: sample name or species name, date of infection, date sample received and tested in the laboratory, date of RNA extraction and PCR processing, date sequencing complete, total sequences, theoretical coverage, percent coverage (calculation method available upon request if awarded) and date of fastq transfer to CDC.

The laboratory will submit to APHL and CDC a Sequence run report to include the following information: sequence run identifier, cluster density, clusters passing filter (%), bases >Q30 (%), estimated sequence yield.

Site visits and teleconferences

APHL, CDC and the HCV/GHOST Pilot Sites will participate in monthly teleconferences to review monthly reports, assess successes and challenges and discuss potential resolutions.

Appendix B: HCV/GHOST Pilot Site Minimum Requirements

| YES | NO | MINIMUM REQUIREMENT |
|-----|----|---|
| | | Does your laboratory have the infrastructure in place or the ability to adapt it to extract high quality HCV viral RNA? |
| | | Does your laboratory have the infrastructure currently in place or the ability to adapt it to perform next generation sequencing (NGS) of viral pathogens on a MiSeq? |
| | | Does your laboratory have sufficient workforce capacity for the testing volume proposed? |

Signature: _____

Date: _____

Printed Name:

Appendix C: HCV/GHOST Pilot Site RFP Score Card

The following table is a copy of the score card that will be used to evaluate RFP responses.

| Category | Maximum Value | Score | Comments (REQUIRED) |
|---|---------------|-------|--------------------------------|
| <p>Does the applicant have sufficient capacity and experience to perform RNA extraction and PCR testing of HCV? <i>Evaluate proposed methodology for preparing HCV amplicons. Consider the training and experience of existing staff.</i> Yes (20 points), No (0)</p> | 20 | | Type comments here. (REQUIRED) |
| <p>Does the applicant have sufficient capacity and experience performing post-PCR processing to comply with the requirements described in Appendix A of the RFP? <i>Evaluate proposed methodology for RNA extraction. Consider if the methodology provides RNA of sufficient quantity and quality for PCR processing, consider training and experience of existing staff.</i> High: routinely (>10 extractions/month) isolates HCV RNA, and has demonstrated (or others have demonstrated) that the RNA is of sufficient quality and quantity for the proposed sequencing method (21-30 points) Moderate: does not routinely isolate HCV RNA but has a validated protocol in place and existing staff with experience and training (11-20 points), Limited: does not have a validated protocol for isolating HCV RNA, but demonstrates potential capacity by modifying existing protocols (1-10 points), No experience = 0</p> | 30 | | Type comments here. (REQUIRED) |
| <p>Does the applicant have sufficient capacity and experience performing NGS of HCV or other RNA viruses to comply with the requirements described in Appendix A of the RFP? <i>Evaluate their experience with sequencing HCV or other RNA viruses. Consider number of HCV samples sequenced each month, experience and training of existing staff.</i> High: routinely sequences > 50 HCV samples/month (21 -30 points), Moderate: routinely sequences 10-50 HCV samples/month (11 – 20 points) Limited: does not routinely sequence HCV samples, but has some experience sequencing , and existing staff do have some training and experience (1-10 points), No experience (0 points).</p> | 30 | | Type comments here. (REQUIRED) |

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| | | |
|--|------------|--------------------------------|
| <p>Does the applicant have a clear plan for meeting the requirements? Has the applicant defined the number of HCV positive cases in the jurisdiction and described a specimen selection plan or criteria for selecting 24-96 HCV positive cases for sequencing? Will the proposal meet the expectations outlined in Appendix A? High likelihood of success (16-20points), Medium likelihood of success (8 – 15 points), Low likelihood of success (1-7 points), No (0 points)</p> | 20 | Type comments here. (REQUIRED) |
| TOTAL SCORE | 100 | |

Appendix D: HCV Sequencing Protocol

HCV_HVR1 PCR for NGS v1.2 (optimized for MiSEQ)

1. Extraction of NA

*Standard protocol on MagnaPure LC**

100-200µl serum (200 preferable)

Total NA _variable elution volume protocol – elute in 50ul;

Use Roche Total NA MagNAPure kit

*Alternative NA extraction equipment/procedure can be applied

Note: Include a blank as RNA Negative control (N1) for every column of 7 positive samples (a total of 8 samples).

2. cDNA Synthesis

SuperScript® VILO™ cDNA Synthesis Kit (cat# 11754-050- Invitrogen)

Random primer contained in kit

5X VILO™ Reaction Mix 6 µl

10X SuperScript® Enzyme Mix 3 µl

RNA (up to 2.5 µg) up to 21 µl

DEPC-treated water up to 30 µl

Incubation parameters (ABI7500 PCR machine)

25oC 10 min

42oC 90 min

85oC 5 min

4oC hold

Note: Include a cDNA reagent blank as negative control (N2)

3. 1st round PCR

Primers to use

1295F, TGGCTTGGGATATGATGATGAACT

1619R, GCAGTCCTGTTGATGTGCCA

Quanta PCR reagent - cat # 95072-012 - Quanta BioSciences

Quanta reagent 10 ul

Forward primer 1uM 1 ul

Reverse primer 1uM 1 ul

cDNA 3 µl (up to 5 ul)

DEPC-treated water up to 20 µl

Incubation parameters: (Roche LC480)

95°C 10 mins

(95°C 10 secs, 45°C 10 secs, 72°C 32 secs) x (40 cycles)

Note: Include a 1strd PCR reagent blank as negative control(N3)

4. Barcode PCR

Primers to Use

HVR1F1, GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACGAGTGCGT GGATATGATGATGAACTGGT
HVR1F2, GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACGCTCGACA GGATATGATGATGAACTGGT
HVR1F3, GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGACGCACTC GGATATGATGATGAACTGGT
HVR1F4, GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGCACTGTAG GGATATGATGATGAACTGGT
HVR1F5, GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATCAGACACG GGATATGATGATGAACTGGT
HVR1F6, GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATATCGCGAG GGATATGATGATGAACTGGT
HVR1F7, GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGTGTCTCTA GGATATGATGATGAACTGGT
HVR1F8, GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTCGCGTGTG GGATATGATGATGAACTGGT
HVR1R1, ACACTCTTCCCTACACGACGCTCTTCCGATCTACGAGTGCGT ATGTGCCAGCTGCCGTTGGTGT
HVR1R2, ACACTCTTCCCTACACGACGCTCTTCCGATCTACGCTCGACA ATGTGCCAGCTGCCGTTGGTGT
HVR1R3, ACACTCTTCCCTACACGACGCTCTTCCGATCTAGACGCACTC ATGTGCCAGCTGCCGTTGGTGT
HVR1R4, ACACTCTTCCCTACACGACGCTCTTCCGATCTAGCACTGTAG ATGTGCCAGCTGCCGTTGGTGT
HVR1R5, ACACTCTTCCCTACACGACGCTCTTCCGATCTATCAGACACG ATGTGCCAGCTGCCGTTGGTGT
HVR1R6, ACACTCTTCCCTACACGACGCTCTTCCGATCTATATCGCGAG ATGTGCCAGCTGCCGTTGGTGT
HVR1R7, ACACTCTTCCCTACACGACGCTCTTCCGATCTCGTGTCTCTA ATGTGCCAGCTGCCGTTGGTGT
HVR1R8, ACACTCTTCCCTACACGACGCTCTTCCGATCTCTCGCGTGTG ATGTGCCAGCTGCCGTTGGTGT

Upon synthesis the primers need to be diluted separately to 100 uM stocks.

Prepare 0.35uM dilution from each stock and dispense the primer pairs in strips of 8 in 200µl PCR tubes as follows:

F1 3.5µl + R1 3.5µl = final 7µl /tube

F2 3.5µl + R2 3.5µl

F3 3.5µl + R3 3.5µl

...

F8 3.5µl + R8 3.5µl

Use robotic dispensing if available.



F1/R1 F2/R2 F3/R3 F4/R4 F5/R5 F6/R6 F7/R7 F8/R8

Each “primer strip” will contain unique barcode combination in each well and is ready for PCR. Each strip is intended for single use. Store primer strips at -30 °C /-80°C

Quanta PCR reagent - cat # 95072-012 - Quanta BioSciences

To the strip with Forward/Reverse primer (7 µl)
add

| | |
|-------------------------------|-------|
| Quanta reagent | 10 µl |
| 1 st round product | 3 µl |
| <hr/> | |
| | 20 µl |

Incubation parameters: (Roche LC480)

95°C 10 mins,
(95°C 10 secs, 45°C 10 secs, 72°C 32 secs) x (30 cycles)

Note: Include a Barcode PCR reagent blank as negative control (N4)

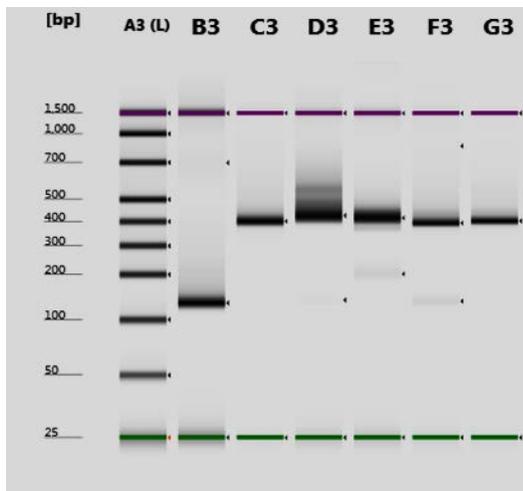
5. Verify fragment size and yield

(Agilent Technologies 2200 tape station) * D 1000 screen Tape cat# 5067-5582, D 1000 Reagents, cat # 5067-5583*

Using Agilent Tape station, verify that the resulting PCR product does not contain secondary smaller band (residual primers or primer-dimers) and if it does, purify with AmpureXP.

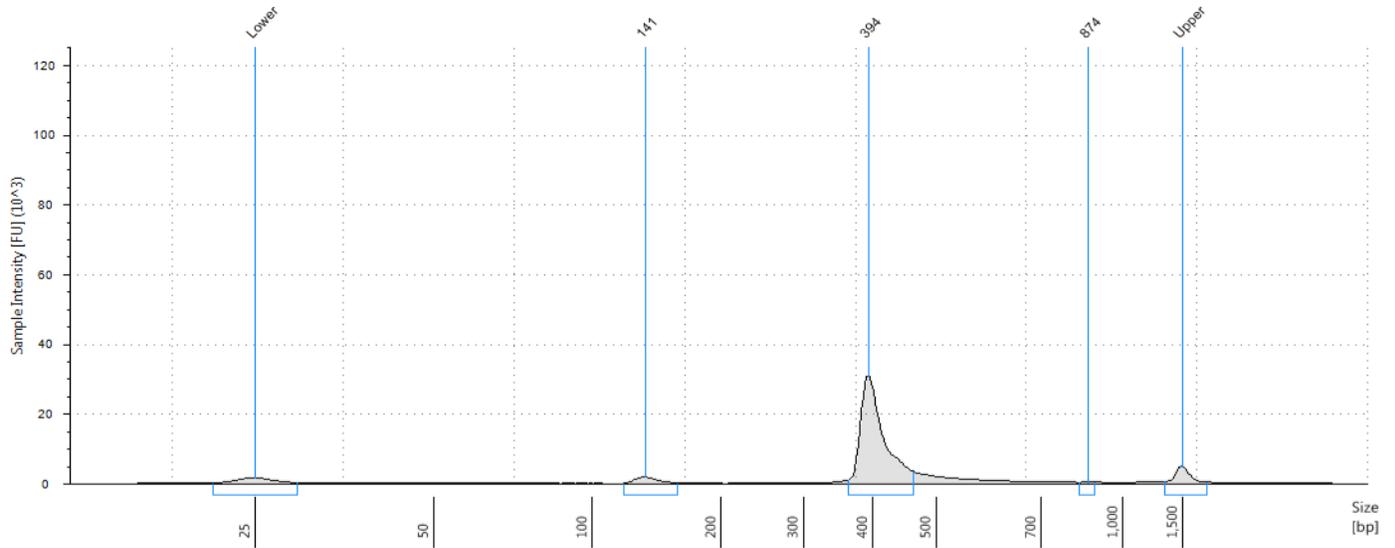
Images:

Product that needs purification (F3); does not require purification (G3)



F3 _ requires purification

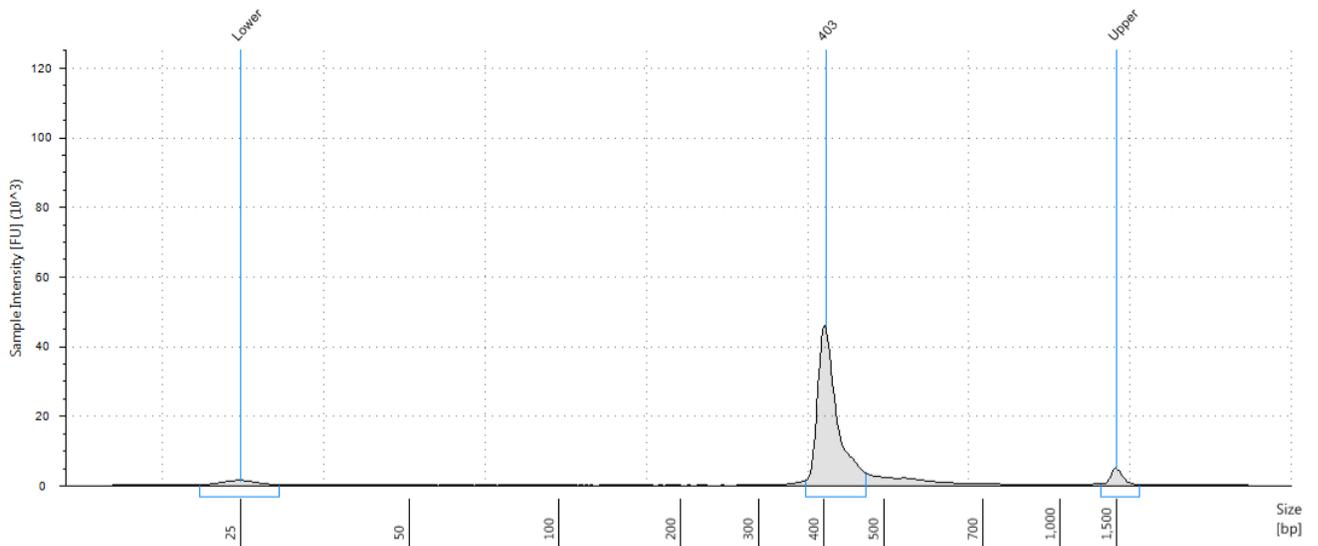
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F3 Peak Table

| Size [bp] | Calibrated Conc. [ng/μl] | Assigned Conc. [ng/μl] | Peak Molarity [nmol/l] | % Integrated Area | Peak Comment | Observations |
|-----------|--------------------------|------------------------|------------------------|-------------------|--------------|--------------|
| 25 | 5.25 | - | 323 | - | | Lower Marker |
| 141 | 3.58 | - | 39.2 | 5.61 | | |
| 394 | 59.8 | - | 233 | 93.72 | | |
| 874 | 0.427 | - | 0.751 | 0.67 | | |
| 1,500 | 6.50 | 6.50 | 6.67 | - | | Upper Marker |

G3_ does not require purification



G3 Peak Table

| Size [bp] | Calibrated Conc. [ng/μl] | Assigned Conc. [ng/μl] | Peak Molarity [nmol/l] | % Integrated Area | Peak Comment | Observations |
|-----------|--------------------------|------------------------|------------------------|-------------------|--------------|--------------|
| 25 | 5.45 | - | 335 | - | | Lower Marker |
| 403 | 92.8 | - | 354 | 100.00 | | |
| 1,500 | 6.50 | 6.50 | 6.67 | - | | Upper Marker |

5. Ampure XP clean up

Agencourt AMPure XP # A63881

Each fragment that require clean up should be purified separately prior to the Index PCR. Use 16 μl beads (x0.8) and fresh 80% ETOH to clean each fragment according to the manufacturer’s protocol. Use 5 minutes binding time, 5 minutes magnetic separation time, 2x200 μl ETOH/ sample, 10 minutes drying time. Add 30μl Elution Buffer and allow 5 minutes elution time before separation. Remove and store 25 μl of the eluate. Store short term (up to 2 weeks at -20oC) or indefinitely at -80oC.

6. Index PCR

Primers to use

UNIVERSAL,AATGATACGGCGACCACCGAGATCT ACACTCTTCCCTACACGACGCTCTTCCGATC
D501,CAAGCAGAAGACGGCATAACGAGATTATAGCCT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
D502,CAAGCAGAAGACGGCATAACGAGATATAGAGGC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
D503,CAAGCAGAAGACGGCATAACGAGATCCTATCCT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
D504,CAAGCAGAAGACGGCATAACGAGATGGCTCTGA GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
D505,CAAGCAGAAGACGGCATAACGAGATAGGCGAAG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
D506,CAAGCAGAAGACGGCATAACGAGATTAATCTTA GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
D507,CAAGCAGAAGACGGCATAACGAGATCAGGACGT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
D508,CAAGCAGAAGACGGCATAACGAGATGTACTGAC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC

Upon synthesis the primers need to be diluted separately to 100 uM stocks
Prepare 0.35uM dilution from each stock and dispense the primer pairs in strips of 8x 200μl PCR tubes as follows:

Universal 3.5μl + D501 3.5ul = final 7μl /tube
Universal 3.5μl + D502 3.5μl ...
Universal 3.5μl + D503 3.5μl ...
...
Universal 3.5μl + D508 3.5μl

Use robotic dispensing if available.



U/D501 U/D502 ...

U/D508

Each “primer strip” is intended for single use. Store primer strips at -30/—80°C

| | | |
|---|---------|------|
| To the strip with Forward/Reverse primer | (7 µl) | |
| add | | |
| Quanta reagent | 10 µl | |
| barcoded PCR product | 3 µl | |
| total | | 20µl |
| PCR conditions for the index PCR:(ABI7500 thermal cycler) | | |
| 95 °C for 3minutes (1 cycle) | | |
| (95°C for 30 “/ 55°C for 30 “/ 72°C for 30 “) (8 cycles) | | |
| 72°C for 5 minutes (1 cycle final extension) | | |
| 4°C hold | | |

Note: Include an Index PCR reagent blank as negative control (N5)

At this point, all negative controls (N1-N5) should not amplify any amplicon based on PCR results. Negative control N5, amplified with an index primer tag, should be submitted for sequencing as part of the library serving as the Library negative control along with the positive indexed amplicons.

6. Quantification and normalization

Use Agilent Tape station to measure the size and concentration of each indexed product. Ensure there are less than 5% of secondary smaller fragments (measured by molarity). Refer to the example described in item 4 above. The expected desired fragment size is approx. 450 nt and 96-100% of the signal. **IMPORTANT!** The molarity of any secondary smaller band should not exceed 5% of the molarity of the band of interest! At the same time verify the starting concentration of the PhiX control. It is expected to be approx. 10nM.

7. Normalize and create library pool

To normalize take appropriate volume (typically)1-5µl of the of each fragments that will go in one sequencing run and mix together to create a pooled library where every fragment has approximately the same molarity e.g.:

Fragment 1 - concentration of 12nM

Fragment 2 - concentration of 8nM

Fragment 3 - concentration of 20nM

Fragment 4 - concentration of 12nM

From:

| | | |
|---------------------|------|-------|
| Fragment 1(12nM) | take | 3.3ul |
| Fragment 2 (8nM) | | 5.0ul |
| Fragment 3(20nM) | | 2.0ul |
| Fragment 4(12nM)... | | 3.3ul |

8. Ampure XP clean up

Purify the resulting library with Ampure XP beads volume (x0.8) and fresh 80% ETOH. Follow the manufacturer's protocol. Use 5 minutes binding time, 5 minutes magnetic separation time, 2x200 µl ETOH/sample, 10 minutes drying time. Add 40µl Elution Buffer and allow 5 minutes elution time before separation. Remove and store 35 µl of the eluate. Store short term (up to 2 weeks at -20oC) or indefinitely at -80oC.

9. Illumina Amplicon Library for V2 2X250 (500) and v3 2x300 (600) kits

1. Take the Sequencing cartridge out to thaw in Room^TC water bath

2. Prepare Fresh 0.2N NaOH,

Use 2N NaOH sold by Illumina or other small aliquot source

*3. Prepare PhiX control and denature ***

3.1 Ensure PhiX initial concentration is 10nM

Use Agilent Tape Station

3.2 Prepare 4nM dilution of PhiX (5µl minimum)

3.3 Mix 5µl 4nMPhiX + 5 µl 0.2N NAOH µl

3.4 Incubate at Room^TC for 5 min

*3.5 Add 990 µlchilled HT1 buffer – results in 20pM denatured PhiX**

*The denatured 20pM PhiX could be stored at -20oC and used for up to 3 weeks

** Steps 3 and 4 could be done simultaneously

*4. Prepare Library and denature****

4.1 Accurately measure the library initial concentration

Use Agilent Tape Station

If there are more than 5% fragments (by molarity) of lower molecular weight than the desired fragment, repeat the library cleaning step (Ampure XP or size select protocol)

Minimum required concentration is 2nM

4.2 Prepare 2nM dilution of library (5µlminimum)*

*if using v3 kit, could start with 4nM dilution

- 4.3 Mix 5µl 2nM*library + 5 µl 0.2N NaOH
- 4.4 Incubate at RoomT°C for 5 min
- 4.5 Add 990 µl chilled HT1 buffer – results in 10pM denatured library.
*if using v3 kit, dilute to 12.5pM by adding 1590 µl HT1
- 4.6 Before loading heat at 95oC for 2min and chill on ice.

5. Prepare Illumina Instrument (MiSeq)

- 5.1 Ensure that all needed washes have been completed.
- 5.2 Prepare Sample Sheet and save in the appropriate Sample Sheet folder on the instrument desktop.
- 5.3 Wash the flow cell with copious amounts of mΩWater.
- 5.4 Wipe the flow cell glass surface with 70% ethanol and dry well.

6. Prepare Library + PhiX mix and run

- 6.1 Mix 500 µl of the denatured 10pM* library to 125 µl of the 20 pM denatured PhiX to achieve approx. 33% PhiX.
- 6.2 Load 600 µl on the cartridge.
- 6.3 Start sequence run, follow the prompts to load the needed components on the instrument.
- 6.4 Wait for instrument to check all parameters and start the run.

10-12pMol final library, 20-35% PhiX to yield approx. 1000-1200 clusters/mm2 and phasing/prephasing values < 0.25

V2 kit (500) -10pM

V3 kit (600) -12pM

The current barcode and index primers should be used only in the following configuration:

- F1-R1-D501
- F2-R2-D502
- F3-R3-D503
- F4-R4-D504
- F5-R5-D505
- F6-R6-D506
- F7-R7-D507
- F8-R8-D508

Allowing 8 specimens/run (7 serum specimens and one negative control)

The Illumina instrument will provide the index level demultiplexing.