Chapter Diagnostics Sexually Transmitted Infections (STI) NGS Kit Manual

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Research Use Only

Table of Contents

1. Definition of Symbols

Batch Code		Contains		Consult instructions for use	
Catalog Number		Manufacturer		Temperature limitation	
Use by		Sufficient for <n> tests		Caution	

2. Reagents by Catalog Number

Chapter Diagnostics STI kit is 4 X 96-well plates and can be used for up to 384 samples for each round of PCR amplification. Each tube numbered 1 to 20 for each plate contains polymerase master mix, target-specific primers and sequencing indexed primers.

Reagents for Plate 1 (Wells 1-96)

Tube Number	Reagents	Product Number	Fill Volume	Storage	Sufficient for
Tubes 1 to 12	HPV PHC1-01 Mix - HPV PHC1-12 Mix	P00101 - P00112	1325 µl	-20 °C	30 x 96 sample
Tubes 13 to 20	HPV PHC1-A Mix - HPV PHC1-H Mix	P0010A - P0010H	1914 µl		
Tubes 21 to 25	Buffer Q	P0001Q	1800 µl	-20 °C	30 x 96 sample

Reagents for Plate 2 (Wells 1-96)

Tube Number	Reagents	Product Number	Fill Volume	Storage	Sufficient for
Tubes 1 to 12	HPV PHC2-01 Mix - HPV PHC2-12 Mix	P00201 - P00212	1325 µl	-20 °C	30 x 96 sample
Tubes 13 to 20	HPV PHC2-A Mix - HPV PHC2-H Mix	P0020A - P0020H	1914 µl		
Tubes 21 to 25	Buffer Q	P0001Q	1800 µl	-20 °C	30 x 96 sample

Reagents for Plate 3 (Wells 1-96)

Tube Number	Reagents	Product Number	Fill Volume	Storage	Sufficient for
Tubes 1 to 12	HPV PHC3-01 Mix - HPV PHC3-12 Mix	P00301 - P00312	1325 µl	-20 °C	30 x 96 sample
Tubes 13 to 20	HPV PHC3-A Mix - HPV PHC3-H Mix	P0030A - P0310H	1914 µl		
Tubes 21 to 25	Buffer Q	P0001Q	1800 µl	-20 °C	30 x 96 sample

Reagents for Plate 4 (Wells 1-96)

Tube Number	Reagents	Product Number	Fill Volume	Storage	Sufficient for
Tubes 1 to 12	HPV PHC4-01 Mix - HPV PHC4-12 Mix	P00401 - P00412	1325 µl	-20 °C	30 x 96 sample
Tubes 13 to 20	HPV PHC4-A Mix - HPV PHC4-H Mix	P0040A - P0040H	1914 µl		
Tubes 21 to 25	Buffer Q	P0001Q	1800 µl	-20 °C	30 x 96 sample

qPCR Primer Mix 10x

Tube Number	Reagents	Product Number	Fill Volume	Storage	Sufficient for
Tube 26	HPV PHC Primer 57 (10x)	P0001P	1600 µl	-20 °C	-

3. Intended Use

Chapter Diagnostics STI NGS kit is intended for target-specific amplification and sequencing of 41 sexually transmitted infections (STI), including 28 human papillomaviruses (HPV) and 13 other sexually transmitted infections on Illumina NGS sequencing platforms. Chapter Diagnostics STI analysis software is intended for analyzing sequencing data for sequence results generated by Chapter Diagnostics STI NGS kit. The test is intended for utilization in molecular biology laboratories skilled in handling sexually transmitted infections specimens, DNA amplification and next-generation sequencing.

4. Summary and Explanation

Chapter Diagnostics STI NGS kit is a multiplex amplification and NGS-based method that detects 41 types and species in one single well and one-step PCR reaction. The assay uses Illumina sequencing platform and comes with customized software for data analysis.

5. List of HPVs and other STIs targeted by this kit

a. High-risk HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73, 26, 53, 82

b. Low-risk HPV: 6, 11, 40, 42, 43, 44, 55, 61, 81, 83

c. Other STIs

Chlamydia trachomatis (CT)	*Ureaplasma urealyticum* (UU)
Neisseria gonorrhoeae (NG)	*Ureaplasma parvum* (UP)
Mycoplasma genitalium (MG)	*Lymphogranuloma venereum* (LGV1 & 2)
Trichomonas vaginalis (TV)	Herpes simplex virus type 1 (HSV1)
Treponema pallidum (TP)	Herpes simplex virus type 2 (HSV2)
Haemophilus ducreyi (HD)	Varicella zoster virus (VZV)
Mycoplasma hominis (MH)	

6. Principles of this procedure

Chapter Diagnostics STI NGS kit is a highly multiplex single-well and single-step amplification and sequencing assay, wherein 41 HPVs and STIs are detected simultaneously. The assay consists of an internal control for each specimen tested and samples are simultaneously barcoded in the amplification step. Moreover, Chapter Diagnostics STI NGS kit is quantitative with target-specific primers for uniform amplification. It requires low amounts of input DNA and is suitable for medium- and high-throughput sample scales. Chapter Diagnostics STI NGS kit consists of the following steps; DNA extraction (not provided in this kit), amplification, pooling, cleanup and sequencing (not provided in this kit). The turnaround time for the assay is 24 hours (6 hours laboratory procedures, 17 hours of DNA sequencing and 1 hour of data analysis).

7. Reagents

a. Identification

See tables in Reagents by Catalog Number section for complete listing of products and catalog numbers.

b. Warnings and Cautions

1) For research use only (RUO)
2) For performing Chapter Diagnostics NGS STI assay, several separate enclosed lab spaces should be designated for pre-PCR, PCR, and post-PCR
3) Separate pipettes should be designated for Pre-PCR and Post-PCR settings
4) Biohazard: All biological samples should be treated as potentially infectious. Use universal precautions when handling biological samples
5) Never pipette by mouth. Avoid contact of reagents and specimens with skin and mucous membranes
6) Dispose of used materials in accordance with the Institution's and/or local regulations for disposal of potential bio-hazardous materials
7) PCR technology is susceptible to contamination, especially from its own product. Aerosols of amplicons that are generated during the post-PCR steps are a frequent source of contamination. Thus, care should be taken to prevent excessive splashing and

generation of aerosols. Standard PCR laboratory measures that include wiping of work surfaces with freshly prepared 10% bleach before processing or preparing PCR samples, use of ultraviolet (UV) light in hoods or biosafety cabinets in between use, space and time separation of pre- and post-PCR activities, use of aliquoted PCR reagents, use of positive and negative controls, etc. should also be followed during the use of the kit. Use of consistent, careful technique coupled with liberal incorporation and monitoring of controls will ensure a vigilant, proactive approach to control and monitoring of PCR contamination

8) Laboratories should validate their own cleaning procedures
9) Contamination of reagents or specimens may cause erroneous results. Therefore, care should be taken to avoid contaminating this product during use. Do not use contaminated reagents
10) Use the kit liquids as supplied. Dilution or alteration may generate erroneous results. Do not mix reagents between different lots.
11) Do not use leaking or unlabeled vials.
12) Previously frozen samples or reagents should be thoroughly mixed and then centrifuged after thawing prior to testing. Avoid generating foam and bubbles in the samples.
13) Keep all enzymes and master mixes on ice or cryo block (2 - 8°C) when thawing.
14) Ensure proper PCR plate or tube sealing prior to amplification to prevent evaporation.
15) Due to inherent differences in the mechanisms of thermal cycler performance, variation in results can occur when set thermal profiles are transferred between different makers and models of thermal cycler instruments. In some cases, reaction specificity and sensitivity can be compromised, leading to the false interpretation and reporting of data. Alternate thermal cyclers and profiles must be validated by the user
16) Incubation times or temperatures other than those specified may give erroneous results
17) Deviation from the recommended directions for use may result in less than optimal product performance. Depending upon the nature and severity of the deviation assay failure (individual sample as well as run failures) and/or erroneous results may occur
18) It is recommended to perform gel electrophoresis for the analysis of amplification products by size. PCR products should be visualized with ethidium bromide or Gel Red; other dyes may show weak bands
19) Use freshly diluted 80% ethanol for each bead clean
20) After each bead clean there is a safe stopping point during which the samples or library can be stored at -20°C for up to 4 days.
21) Amplified library should be purified within 1 hour of amplification.
22) Set-up for library quantification by qPCR or Qubit must be done in a PCR hood and with fresh dilution buffer for qPCR to avoid contamination.
23) Perform sample denaturation with fresh 0.2N sodium hydroxide.
24) Perform all sequencer post-run and maintenance washes and regular cleaning

c. Storage Instructions

1) Store Chapter Diagnostics STI NGS kit below -20ºC
2) Do not use components past their expiration date

d. Purification or Treatment Required for Use

1) See "**Specimen Collection and Preparation**"

8. Instrument requirements

a. Thermal Cycler equipped with a heated lid, adjustable ramp times, and a minimal thermal range of 2°C to 100°C and accuracy of at least +/- 0.5°C. Conditions for thermal cyclers may need to be altered in order to optimize the profiles. The Veriti thermal cycler from Applied Biosystems, when run in the default mode with the ramp rate of (3.9ºC/sec), has been validated
b. Appropriate method/apparatus for library quantification such as qPCR or Qubit
c. Illumina sequencing platform has been validated
d. Server and Software: Chapter Diagnostics STI Analysis Tool
e. The table below lists all the major instruments required:

Instruments	Manufacturer	Catalog Nr	Comments
Thermal Cycler Veriti	ThermoFisher	43-757-86	
Invitrogen™ Qubit™ Fluorometer	ThermoFisher	Q33226	
NanoDrop	ThermoFisher	13-400-519	Or other models
Pipette set 0.2 µl to 1 ml			
Multichannel pipette 12-Channel			
Real-Time PCR System			For library quantification
Fisherbrand™ Microplate Centrifuge	ThermoFisher	14-955-300	Or other makes
Illumina Sequencers (other models work as well)	Illumina	iSeq100	
	Illumina	MiniSeq	
	Illumina	MiSeq	

9. Specimen Collections and Preparation

a. Human genomic DNA can be extracted from samples from anogenital area such as endocervix with validated DNA extraction method
b. Absorbance measurements of the DNA sample at 260 and 280 nm should give a ratio of 1.65 to 2.0
c. DNA can be used immediately after isolation or stored at –20ºC for up to 1 year. Repeated freeze/thawing should be avoided since this can result in DNA degradation.

10. Procedure

a. **Materials Provided** (See table in Reagents by Catalog Number section for specific information)
   1) PCR Reagents
   2) Type/species specific primers
   3) Barcoding/indexing primers

b. Materials and Reagents *Required,* but Not Provided

1) The materials and reagents required but not provided are listed in the table below. Note that the Illumina sequencing kits listed in the table should be selected based on the instrument and/or throughput required. Illumina sequencing kits with the read length 2 X 150 from all Illumina platforms are suitable for the assay.

Reagents and Consumables	Manufacturer	Catalogue Nr
Qiagen MinElute PCR Purification Kit (50)	Qiagen	28004
Qubit 1x dsDNA HS Assay Kit (100 assays)	Thermo Fisher	Q33230
PowerUp™ SYBR™ Green Master Mix	Thermo fisher	A25742
PhiX Control v3	Illumina	FC-110-3001
TE buffer		
Tris-HCL		
Sodium Hydroxide		
Tween 20		
MiniSeq Mid Output Kit (300-cycles)	Illumina	FC-420-1004
TG MiSeq Reagent Micro Kit v2 (300 cycles)	Illumina	TG-142-1002
TG MiSeq Reagent Nano Kit, v2 (300 cycles)	Illumina	TG-142-1001
iSeq 100 iSeq 2 x 150 kit	Illumina	20021533
Molecular Grade Water		
Absolute Ethanol		
Applied Biosystems™ MicroAmp™ EnduraPlate™ Optical 96-Well	Thermo fisher	4483354
Applied Biosystems™ MicroAmp™ Clear Adhesive Film	Thermo fisher	4306311
PCR tube 0.2 ml		
Eppendorf tube 1.5 ml or 2 ml		
Fisherbrand™ 96-Well DeepWell™ Polypropylene Microplates	Fisherbrand	12-566-120
Pippin Prep and 1.5% Pre-Cast Agarose Gel Cassettes for DNA Size Selection	Sage Science, Inc	

11. Directions for use

The assay is intended for up to 384-samples and can be performed using the manual protocol described below.

NOTES:

1) Take extreme caution in the aliquot process. Use calibrated pipettes. Failure to do so may result in reagent loss and assay failure.
2) All temperatures must be precisely maintained.
3) The amplified product can be stored up to 4 days at -20ºC prior to use. The amplified product can only be frozen and thawed once. Repeated freezing and thawing may result in degradation of amplified product and may yield poor results if used to generate library.

a. Prepare plates

1) From box 1, aliquot 59.8 µl of HPV PHC1-**A** Mix to the **first** tube of an 8-strip 200 µl PCR tubes, aliquot 59.8 µl of HPV PHC1-**B** Mix to the **second** tube of the 8-strip PCR tubes, and so on, and finally aliquot 59.8 µl of HPV PHC1-**H** Mix to the **last** tube of the 8-strip PCR tubes (Figure 11.1)

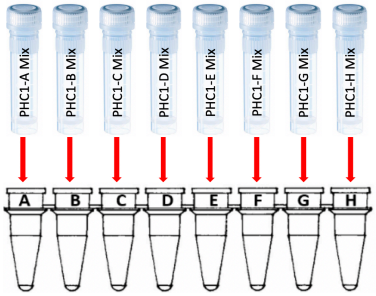


Figure 11.1. Aliquot HPV PHC1-A Mix – HPV PHC1-H Mix to an 8-strip PCR tubes.

2) From box 1, aliquot 41.4 µl of HPV PHC1-**01** Mix to the **first** tube of a 12-strip 200 µl PCR tubes, aliquot 41.4 µl of HPV PHC1-**02** Mix to the **second** tube of the 12-strip PCR tubes, and so on, and finally aliquot 41.4 µl of HPV PHC1-**12** Mix to the **last** tube of the 12-strip PCR tubes (Figure 11.2)

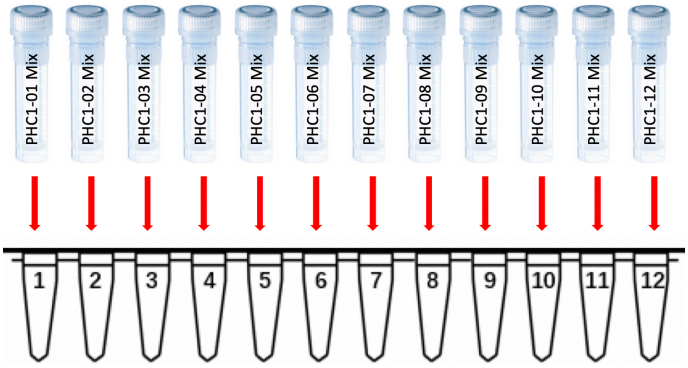


Figure 11.2. Aliquot HPV PHC1-01 Mix – HPV PHC1-12 Mix to a 12-strip PCR tubes.

3) Aliquot to 30 sets of 8-strip tubes and 30 sets of 12-strip tubes and freeze at -20ºC prior to first time use are recommended to avoid multiple times of freeze and thaw.

4) AS step 1-3, aliquot 30 sets of 8-strip tubes PHC2 A-H and 30 sets of 12-strip tubes PHC2 1-12 from box 2

5) AS step 1-3, aliquot 30 sets of 8-strip tubes PHC3 A-H and 30 sets of 12-strip tubes PHC3 1-12 from box 3

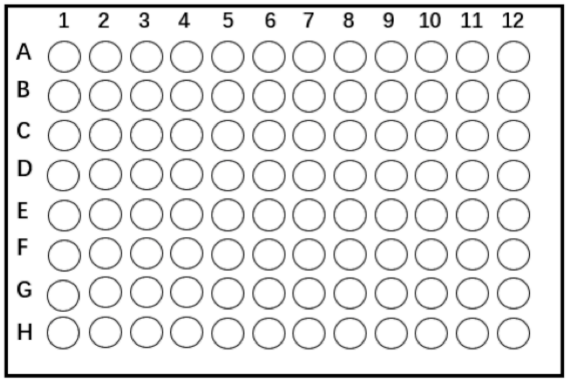
6) AS step 1-3, aliquot 30 sets of 8-strip tubes PHC4 A-H and 30 sets of 12-strip tubes PHC4 1-12 from box 4

b. DNA Extraction

Purify genomic DNA using method of choice. The DNA sample requirements are as following:

1) Genomic DNA samples must be extracted from samples that may carry STIs such as anogenital area, cervical scrapings, urethral, nasopharyngeal aspiration, vaginal, semen, inguinal lymphadenopathy, genital ulcer, first void urine, oral ulcer, condyloma and oropharynx.
2) The DNA must be diluted in TE buffer and stored at -20°C or below.

c. PCR Set-up and Amplification



	A	B
1	plate & barcode ID	sample ID
2	P1-A1	hpv01
3	P1-A2	hpv02
4	P1-A3	hpv03
5	P1-A4	hpv04
6	P1-A5	hpv05
7	P1-A6	hpv06
8	...	...
9	P1-H7	hpv91
10	P1-H8	hpv92
11	P1-H9	hpv93
12	P1-H10	hpv94
13	P1-H11	hpv95
14	P1-H12	hpv96

Figure 1. Sample Barcode File Example and sample plate preparation

1) Fill out Sample Barcode Excel File with sample names and barcode assignments as shown in Figure 1. Multiple plates can be used if more samples need to be sequenced in the same run.
2) Generate a project in the Chapter Diagnostics analysis software after creating a sample barcode file with the sample IDs and barcodes. The test/experiment name is needed for the sequencer run name.
3) Prepare a 96-well sample plate. Add at least 10µl of extracted DNA per well to a 96-well sample plate.
4) Thaw one aliquoted PHC1-A-H 8-strip tubes, mix well, spin down. Thaw one aliquoted PHC1-1-12 12-strip tubes, mix well, spin down.
5) Use multichannel Pipette to aliquot 4.6 µl PHC1-A-H to a 96 well PCR plate (Figure 2)

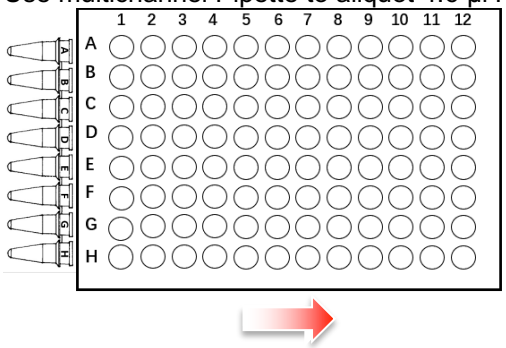


Figure 2. Dispense PHC1 A-H Mix to 96-well plate

6) Use multichannel Pipette to aliquot 4.6 µl PHC1-1-12 to the same 96 well plate from step 4 (Figure 3), and change tips in between for each row dispensation. After the two dispensations, each well contains 9.2 µl in total. Figure 3 shows the arrangement for PCR plate and PCR tubes mixes.

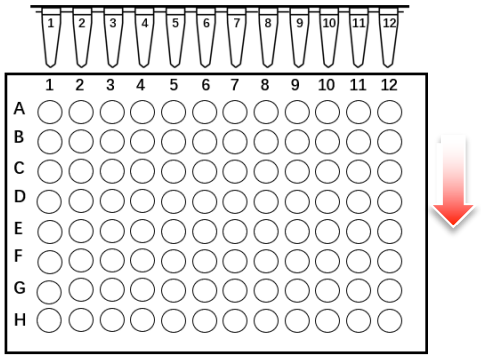


Figure 3. Dispense PHC1 01-12 Mix to 96-well plate

7) Transfer 5.8 µl extracted DNA from sample plate to PCR plate according to the well position. Seal the plate, agitate the plate on a vortex for 15 seconds, spin the plate in a plate centrifuge, and then start the PCR.

Stage 1	95 °C	2 min	
Stage 2	95 °C	30 s	10 cycles
	59 °C	2 min	
	72 °C	1 min	
Stage 3	95 °C	30 s	5 cycles
	68 °C	30 s	
	72 °C	1 min	
Stage 4	95 °C	30 s	15 cycles
	72 °C	90 s	
Stage 5	72 °C	10 min	
Stage 6	4 °C	∞ hold	

8) If two plates are going to be used for one sequencing run, take one set of box2 aliquots PHC2 A-H 8-strip tubes and PHC2 01-12 12-strip tubes and repeat step 1-7; another option is use one set form box3 and box4 instead.
9) If four plates are going to be used for one sequencing run, use one set from box 1-4 which will be 384 samples.

d. PCR product pooling and purification

1) Prepare a 12-strip PCR tubes for each plate of PCR reaction, add 15 µl buffer Q to each tube. Transfer all PCR product to the 12-strip tubes with buffer Q (provided by Chapter Diagnostics) with a multichannel pipette, mix well each time by pipetting. Then, finally pool the PCR products from the 12-strip tubes into a 1.5 ml (or into a bigger tube for multiple PCR plates) and mix well
2) SAFE STOPPING POINT. PCR products may be stored at -20ºC
3) Take 300 µl of all pooled PCR (keep the rest of the pooled PCR products at -20°C as backup). Perform purification with Qiagen MinElute PCR purification kit according to manufacturer's instructions and at final step, elute with 31 µl Elute Buffer. Proceed to cleanup step. If not proceeding to the beads cleanup step immediately, store at -20°C

e. Library purification with Pippin Prep

If Pippin is used for library purification, load 1ug of purified PCR product from previous step to Pippin Prep with 1.5% gel cassette and collect 350-500 bp product. Pippin elutes DNA sample with TAE buffer. Use Qiagen MinElute PCR purification kit to change TAE buffer to TE buffer. Purify according to manufacturer's instruction, and elute with 15 µL TE buffer.

f. (Optional) Library purification with beads

1) Prepare fresh 80% ethanol
2) Transfer 30 µl purified PCR product to a 0.2 µl PCR tube
3) Add 22.5 µl beads, mix well by pipetting up and down at least 15 times. Incubate the DNA and bead mix at room temperature for 5 minutes. Place the tube in the magnet for 1 minute. Carefully remove the supernatant without disturbing the beads while the tube is in the magnet.
4) Leave the tube in the magnet for the ethanol wash steps. Add 150 µl of freshly prepared 80% ethanol to the beads, incubate for at least 30 seconds then carefully remove and discard ethanol. Perform the ethanol wash step one more time. Spin and remove the all traces of ethanol. Then, open the lid and allow the beads to air-dry, this usually takes 3-5 minutes. Re-suspend beads with 20 µl TE buffer.
5) Add 15 µl beads, mix well by pipetting up and down at least 15 times. Incubate the DNA and bead mix at room temperature for 5 minutes. Place the tube in the magnet for 1 minute. Carefully remove the supernatant without disturbing the beads while the tube is in the magnet. Wash with 150 µl 80% ethanol, air dry and re-suspend with 20 µl TE buffer as described in step 4.
6) Repeat step 5) 3 more times (total 5 times beads purification). Elute with 15 µl TE buffer.
7) Use Qubit to measure the concentration, check library DNA size distribution on Bioanalyzer or by gel imaging

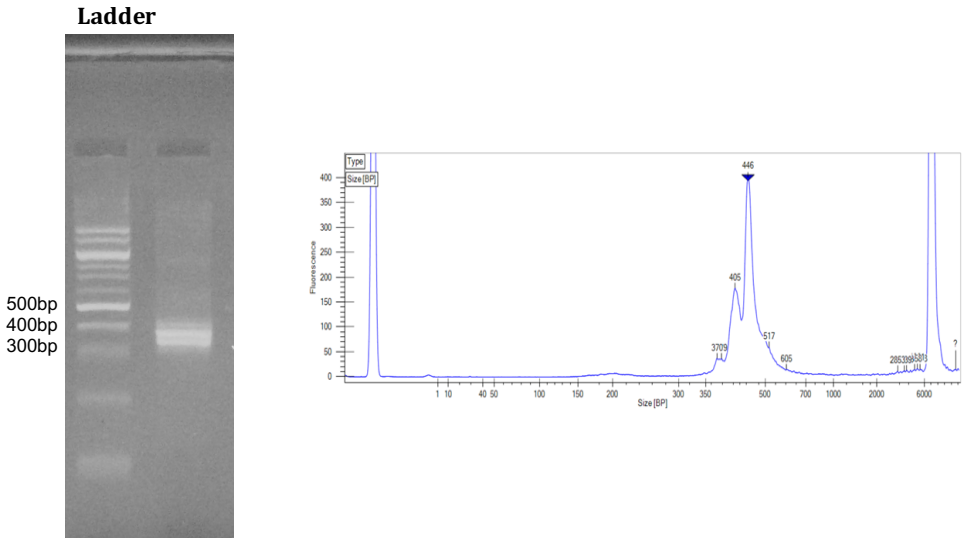


Figure 4. Examples of library gel picture and Bioanalyzer

g. Library quantification

1) Determine the concentration of Library using methods that accurately measure DNA concentration. We recommend first use Qubit, and then use qPCR to accurately determine effective library concentration
2) First dilute library to 1nM based on Qubit defined library concentration and average library size of 380 bp

$$\frac{(\text{concentration in ng}/\mu\text{l})}{(660\ \text{g/mol} \times \text{average library size in bp})} \times 10^6 = \text{concentration in nM}$$

3) qPCR to further determine the effective library concentration comparing with Phix standard library from Illumina. qPCR primers are provided.
4) Real-time PCR conditions for library quantification; dilute the library and Illumina Phix Sequencing Control to 0.02 nM. Prepare the reaction volume for 2-3 replicates for both the library and Phix control. The real-time PCR components, volumes and conditions are listed in the tables below:

Component	Volume
qPCR master mix	10 µl
Primer mix 10X	2 µl
Library/Phix control	2 µl
RNAse free water	6 µl
Total	20 µl

Stage	Temperature	Time	Cycles
Stage 1	50°C	2 min	1
	95°C	10 min	
Stage 2	95°C	15 s	25
	60°C	1 min	

5) Calculate the library/Phix ratio (qPCR results) to determine the amount of library for sequencing (for example, illumine recommend to load 1.8pM phix. If library/ Phix=2, load 0.9pM library)
6) Follow Illumina instructions for sequencing the library