Pathogen- and Matrix-independent Metagenomics Workflow

Harmonized Step-by-step Protocols

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This document contains the following SOPs:

Standard Operating Procedure

Disintegration of liquid and solid samples using the Covaris cryoPREP CP02 Impactor

29th Aug 2018

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SOP-Version: 5

Attachments: none

Introduction

The cryoPREP CP02 impactor (Covaris) allows a rapid and efficient sample disintegration of samples deeply frozen in liquid nitrogen in order to avoid RNA degradation during the disintegration process. The system delivers repeatable high impact mechanical force in order to cryofracture sample material for improved lysis with extraction buffers. This protocol describes the disruption using the automated system combined with Covaris Tissue Tubes. The pulverized sample is resuspended in lysis buffer AL (Qiagen) and should either be used directly in downstream applications e.g. nucleic acid extraction (SOP_020104_NaE_RNA_Trizol_FLI, SOP_020203_NaE_RNA_ViralRNAKit_FLI, SOP_020304_NaE_DNA_DNAMini Kit_FLI) or can be stored at 4°C over night.

Sample Material

Solid and liquid sample material.

Equipment and Reagents

Successful RNA extraction demands an RNase-free environment, therefore only RNase-free tubes, aerosol-free pipette tips, and only DEPC-treated (Nuclease-free) water must be used. Gloves have to be worn throughout the procedure and changed regularly in order to avoid RNase contamination.
In general only use reagents that do not exceed the expiration date. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the manufacturer.

Equipment

- Covaris cryoPREP CP02 Impactor
- Tissue Tubes TT1 extra thick (520007), plug (520006) and special handle (all Covaris)
- Liquid nitrogen
- Container suitable for safe transport of liquid nitrogen (e.g. Plastic Dewar vessel, neoLab; cat. no. 1-0042 [4 l])
- Ice pans suitable for liquid nitrogen (e.g. Magic Touch Ice pans; Thomas Scientific, cat no. 1224J40)
- Laminar flow hood
- Chemical fume hood
- Thermal block for 1.5 ml reaction tubes
- Dissection instruments (e.g. forceps, scissors, petri dishes)
- Plastic lab ware (2 ml tubes, pipettes and pipette filter tips)
- Disposable gloves
- Cotton gloves
- Personal protective clothing (protective goggles)

Reagents

- Lysis buffer AL (Qiagen; cat. no. 19075)
- Disinfectant for dissection instruments (e.g. gigasept FF, Schülke, cat. no. 125502)

General remarks

ONLY use the Covaris cryoPREP-System after having been instructed by trained personnel
Before handling liquid nitrogen, read the appropriate material safety data sheet and operating instructions!

Literature

- Covaris Protocol Tissue dry Pulverization using the cryoPREP™ CP02 (Part number: 010303, Revision: A, 22 June 2015)
- Wylezich C, Papa A, Beer M, Höper D (2018) A versatile sample processing workflow for metagenomic pathogen detection. Scientific Reports DOI:10.1038/s41598-018-31496-1

Before getting started

- Preheat 2 – 3 ml buffer AL for each sample in a thermal block at 56°C.
- Pull on cotton gloves (to protect your hands from freezing) and two pairs of disposable gloves
- Fill liquid nitrogen in the ice pan and put the pan in a laminar flow for the subsequent sample processing

Procedure

1. - Prepare approx. 20 mg solid or 200 µl liquid sample

2. - Place the sample to the centre of the Tissue Tube through the top opening using forceps or tweezers

3. - After the sample is loaded, seal the Tissue Tube using a plug but **do not screw tightly**

4. - While holding the tube using the special handle, freeze the sample by immersing the flexible pouch into liquid nitrogen; avoid dipping the plug or transfer tube

5. - Verify that the pouch is not swelled (a sign of trapped air) and that the sample remains centred in the pouch

6. - Open the cryoPREP lid and insert the frozen Tissue Tube using the handle

7. - Close the cover, select impact level 6, and press green “ACTIVATE” button

8. - Remove Tissue Tube from the cryoPREP instrument using the l handle

9. - Repeat steps 4 – 8 once

10. - Unscrew the plug and resuspend the sample in 1 - 2 ml preheated lysis buffer AL

11. - Transfer the sample solution into two fresh 2 ml reaction tubes

For subsequent nucleic acid isolation, continue with SOP_020104_NaE_RNA_Trizol_FLI, SOP_020203_NaE_RNA_ViralRNAKit_FLI, or SOP_020304_NaE_DNA_DNAMiniKit_FLI

Standard Operating Procedure

Disintegration of solid or liquid samples in liquid nitrogen using the Mikro-Dismembrator S

12th Sept 2017

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SOP-Version: 3

Attachments: none

Introduction

The procedure described here is used for the disintegration of sample material. For the disintegration the “Mikro-Dismembrator S” (Sartorius) is used. Mikro-Dismembrator is a laboratory grinding mill which can be used for efficient and reproducible fine grinding and disintegration of samples for the isolation of DNA and RNA. The disintegration is performed in liquid nitrogen within PTFE shaking flasks and chrome steel or tungsten carbide grinding balls enabling a continuously cooling chain of the samples and thus prevents degradation of the RNA. The Mikro-Dismembrator is operated with a selected shaking frequency for a defined time. The pulverized sample is resuspended in lysis buffer AL (Qiagen) and should be used directly in downstream applications e.g. nucleic acid isolation (e.g. SOP_020104_NaE_RNA_Trizol_FLI, SOP_020203_NaE_RNA_ViralRNAKit _FLI, SOP_020304_NaE_DNA_DNAMiniKit_FLI) or can be stored at 4°C over night.

Sample Material

Solid sample material (tissue) or liquid samples.

Equipment and Reagents

Successful RNA extraction demands an RNase-free environment, therefore only RNase-free tubes, aerosol-free pipette tips, and only DEPC-treated (Nuclease-free) water must be used. Gloves have to be worn throughout the procedure and changed regularly in order to avoid RNase contamination.
In general only use reagents that do not exceed the expiration date. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the manufacturer.

Equipment

- Mikro-Dismembrator S (Sartorius, cat. no. 8531609)
- Holder for shaking flasks (Sartorius, cat. no 38240343)
- Grinding balls
- Tungsten carbide (Sartorius, cat. no 8547203 [5 mm], cat. no. 8547408 [7 mm], or cat. no. 8547300 [10 mm])
- Chrome steel (Sartorius, cat. no. 8546703 [5 mm], cat. no. 8546916 [9 mm], or cat. no. 8546800 [10 mm])
- PTFE Shaking flasks (Sartorius, cat. no. 8531838 [3 ml], cat. no. 8531846 [5 ml], or cat. no. 8531854 [7 ml])
- Ice pans suitable for liquid nitrogen (e.g. Magic Touch Ice pans; Thomas Scientific, cat. no. 1224J40)
- Laminar flow hood
- Chemical fume hood
- Liquid nitrogen
- Container suitable for safe transport of liquid nitrogen (e.g. Plastic Dewar vessel, neoLab cat. no. 1-0042)
- Thermal block
- Sterilised dissection instruments (e.g. forceps, scissors, petri dishes)
- RNase-free plastic lab ware (2 ml tubes, pipettes and pipette filter tips)

- Disposable gloves
- Cotton gloves
- Personal protective clothing (protective goggles)

Reagents

- Lysis buffer AL (Qiagen; cat. no. 19075)
- Disinfectant (e.g. gigasept FF, Schülke, cat. no. 125502)
- Ethanol (denatured)

General remarks

ONLY use the Mikro-Dismembrator S after having been instructed by trained personnel!
Before handling liquid nitrogen, read the appropriate material safety data sheet and operating instructions!

Literature

- Operating Manual Mikro-Dismembrator S (Satorius, publication no.: SB-1001-e05021)

Before getting started

- Sort shaking flasks (wear disposable gloves!) and grinding balls according to their size.
- Grinding ball:
    - 5 ml shaking flask: one 5 mm grinding ball
    - 7 ml shaking flask: one 7 mm or 10 mm grinding ball
- Instrument settings: frequency 2000 rpm, duration 2 min
- Preheat 2 – 3 ml buffer AL for each sample in a thermal block at 56°C
- Pull on cotton gloves (to protect your hands from freezing) and two pairs of disposable gloves
- Fill liquid nitrogen in the ice pan and put the pan in a laminar flow for the subsequent sample processing

Procedure

1. - Take the flask cap, the beaker and the grinding ball with a forceps, put them into the liquid nitrogen (opening upwards) and let them cool down until no bubbles rise up anymore

2. - Prepare approx. 20 mg tissue or 200 µl liquid sample

3. - Carefully remove the beaker of the liquid nitrogen (make sure that the beaker is filled with liquid nitrogen)

4. - Place the tissue sample or pipette the liquid sample into the beaker

5. - Add the grinding ball and add further liquid nitrogen in case the beaker is not completely filled anymore

6. - Quickly close the beaker with the flask cap

7. - Place the closed shaking flask into the holder and disintegrate at 2000 rpm for 2 min

8. - Take the flask out of the holder and open it in a laminar flow

9. - Add 1 ml lysis buffer AL pre-heated to 56 °C, mix thoroughly by pipetting

10. - Pipette the pulverized sample into a 2-ml tube and repeat the previous step until the disintegrated sample is completely removed from the beaker

11. - Cleaning
    - Clean and disinfect the shaking flasks and grinding balls in gigasept FF (diluted)
    - Subsequently clean it with sterile water
    - Rinse with 99.9% Ethanol (denatured) and let dry under UV light

For subsequent nucleic acid isolation, continue with SOP_020104_NaE_RNA_Trizol_FLI, SOP_020203_NaE_RNA_ViralRNAKit_FLI, or SOP_020304_NaE_DNA_DNAMiniKit_FLI

Standard Operating Procedure

Extraction of total RNA from liquid or disintegrated solid samples using TRIZOL and RNeasy Mini Kit spin columns with on-column DNase digestion

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SOP-Version: 5

Attachments: none

Introduction

The here described method allows extraction of total RNA from liquid or disintegrated samples. This protocol combines a phenol/guanidine-isothiocyanate-based lysis with silica-membrane-based spin column RNA purification. RNA can be purified from either lysed liquid or disintegrated and lysed solid samples; in both cases, Trizol LS Reagent is used. Also for liquid samples, a disintegration is recommended if they are to be used for pathogen detection. For on-column isolation of the RNA, the QIAamp RNeasy Mini Kit columns are used. Additionally, to avoid co-purification of DNA, DNA is digested on-column with the RNase-Free DNase Set. The RNA is eluted in RNase-free buffer or water.

Sample Material

Liquid samples (e.g., serum/plasma, cell culture supernatant, EDTA-blood diluted 1:2 in PBS, supernatant from swabs, liquor) or samples disintegrated and lysed (tissue and stool homogenates) according to SOP_010104_Disintegration_Cryoprep_FLI or SOP_010203_Disintegration_Mikrodismembrator_FLI.

Equipment and Reagents

Successful RNA extraction demands an RNase-free environment, therefore only RNase-free tubes, aerosol-free pipette tips, and only DEPC-treated (nuclease-free) water must be used. Gloves have to be worn throughout the procedure and changed regularly in order to avoid RNase contamination.
In general only use reagents that do not exceed the expiration date. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the manufacturer.

Equipment

- Refrigerated centrifuge (for 1.5 ml and 2 ml tubes)
- Vortexer
- Chemical fume hood
- Pipettes
- Disposable gloves
- 1.5 ml and/or 2 ml tubes
- Sterile, RNase-free pipet tips (with aerosol barriers to prevent cross contamination)

Reagents

- Trizol LS Reagent (Life Technologies; cat. no. 10296-028)
- RNeasy Mini Kit (Qiagen, cat. no. 74106)
- RNase-Free DNase Set 50 (Qiagen cat. no. 79254)
- Ethanol (96 – 100%, undenatured)
- Ethanol (75%, undenatured, diluted in DEPC-treated water)
- Chloroform
- RNase-free water (DEPC-treated)

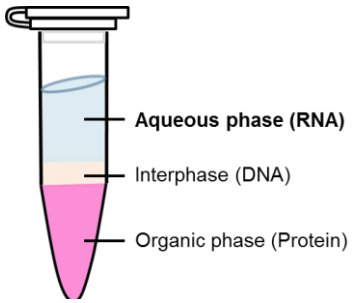
General remarks

Trizol contains high amounts of chaotropic salts and phenol!

WARNING Toxic in contact with skin and if swallowed. Causes burns. After contact with skin, wash immediately with plenty of detergent and water. If you feel unwell, seek medical advice (show label where possible). Phenol (108-95-2) and other components (NJTSRN 80100437-5000p).

CAUTION When working with Trizol use gloves and eye protection (shield, safety goggles). Avoid contact with skin or clothing. Use in a chemical fume hood. Avoid breathing vapor. Preparation of reagents supplied with the kits.

After phase separation of solutions containing Trizol by centrifugation, the RNA remains exclusively in the aqueous (upper) phase:



Literature

- Trizol LS Reagent Manual (Invitrogen, Life Technologies, Part no. 10296010.PPS, MAN0000806, 15 Nov 2010)
- RNeasy Mini Kit Handbook (Qiagen, cat. no. 1064941, 09/2010)
- Wylezich C, Papa A, Beer M, Höper D (2018) A versatile sample processing workflow for metagenomic pathogen detection. Scientific Reports DOI:10.1038/s41598-018-31496-1

Before getting started

- Pre-cool the refrigerated centrifuge at 4°C
- Equilibrate samples to room temperature (RT)
- If intending to perform an on-column DNase digest, prepare 80 µl DNaseI working solution per sample (on ice!):
    - Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD
    - Mix by gently inverting the tube and centrifuge briefly
    - Do not vortex as DNase I is sensitive to physical denaturation
- Prepare buffer RPE from the QIAamp RNeasy Mini Kit:
    - Before first use, add the appropriate amount of ethanol (96 – 100%) to the buffer concentrate as indicated on the bottle
    - Stable for 1 year when stored closed at RT
    - Shake before use

Procedure

This protocol is suitable for the purification of RNA from 250 µl sample volume. Larger starting volumes can be processed by increasing the initial sample volume proportionally to the volumes of Trizol and chloroform and loading the complete volume successively onto the column (step 5).

1. - Pipet 750 µl Trizol LS Reagent into a 2 ml tube

2. - Add 250 µl sample
- Thoroughly shake manually for 15 s
- Briefly centrifuge for 5 s at 8,000 rpm
- Incubate 5 min at RT

3. - Add 200 µl chloroform
- Thoroughly shake manually for 15 s
- Incubate 10 min at RT
- Centrifuge 10 min at 13,000 rpm at 4°C

4. - Transfer the upper aqueous phase into a new sterile 2 ml tube
- Add 600 µl ethanol (75%)
- Mix by pulse-vortexing
- Briefly centrifuge for 5 s at 8,000 rpm

5. - Apply 600 µl of the sample from step 4 to the column
- Centrifuge at 10,000 rpm for 20 s
- Discard the collection tube and place the column into a clean 2 ml collection tube

6. - Apply residual sample from step 4 to the column
- Centrifuge at 10,000 rpm for 20 s
- Discard the collection tube and place the column into a clean 2 ml collection tube

7. - Add an equal volume (600 µl) buffer RW1 to the column
- Centrifuge at 10,000 rpm for 20 s
- Discard the collection tube and place the column into a clean 2 ml collection tube

8. - Apply 80 µl DNase working solution to the centre of the membrane
- Incubate for 15 min at RT
- Add 500 µl buffer RW1 to the column
- Centrifuge at 10,000 rpm for 20 s
- Discard the collection tube and place the column into a clean 2 ml collection tube

9.
- Add 500 µl buffer RPE
- Centrifuge at 10,000 rpm for 20 s
- Discard the collection tube and place the column into a clean 2 ml collection tube

10.
- Add 500 µl buffer RPE
- Centrifuge 1 min at 13,000 rpm
- Discard the collection tube and place the column into a clean 2 ml collection tube
- Centrifuge 2 min at 13,000 rpm (to let the column dry)
- Discard the collection tube and place the column into a clean 1.5 ml tube

11.
- Add 50 µl RNase-free water to the centre of the membrane
- Incubate 1 min at RT
- Centrifuge 1 min at 10,000 rpm

12.
- Repeat step 11 twice, elute into the same tube

RNA is stable for up to one year when stored at -70°C.
When the RNA will be used for NGS it is recommended to subsequently continue with quantification of RNA (SOP_030102_NAQuant_ND1000_FLI and SOP_030202_RNA Quant_RiGreen_FLI) and ds cDNA synthesis (SOP_060104_cDNA-synthesis_FLI).

Standard Operating Procedure

Isolation of viral RNA for whole genome sequencing from liquid or disintegrated samples using the QIAamp Viral RNA Mini Kit

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SOP-Version: 3

Attachments: none

Introduction

QIAamp Viral RNA Mini Kits enable the purification of viral RNA from liquid cell-poor samples. To obtain high-quality RNA it is recommended to use fresh samples. In case of frozen samples they should not be thawed more than once. Repeated freeze–thawing of samples will lead to reduced viral titres and degradation of the RNA. In principle, the sample is first lysed under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA. Buffering conditions are then adjusted to provide optimum binding of the RNA to a silicagel-based membrane, and the sample is loaded onto the QIAamp Mini spin column. The RNA binds to the membrane, and contaminants are washed away. Viral RNA is not separated from DNA so that both will be purified in parallel. To avoid co-purification of DNA, DNA can be digested on-column with the RNase-Free DNase Set. The RNA is eluted in RNase-free buffer or water and can be stored for up to for up to one year at –70°C.

Sample Material

Liquid samples (serum/plasma, cell culture supernatant, EDTA-blood diluted 1:2 in PBS, supernatant from swabs, liquor) or samples disintegrated and lysed (tissue and stool homogenates) according to SOP_010104_Disintegration_Cryoprep_FLI or SOP_010203_ Disintegration_Mikrodismembrator_FLI.

Equipment and Reagents

Successful RNA extraction demands an RNase-free environment, therefore only RNase-free tubes, aerosol-free pipette tips, and only DEPC-treated water must be used. Gloves have to be worn throughout the procedure and changed regularly in order to avoid RNase contamination.
In general only use reagents that do not exceed the expiration date. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the manufacturer.

Equipment

- Microcentrifuge (for 1.5 ml and 2 ml tubes)
- For use of the vacuum protocol vacuum manifold (e.g. EveryPrep Universal Vacuum Manifold, ThermoFisher Scientific cat. no. K2111-01)
- Vortexer
- Laminar flow hood
- Pipettes
- Disposable gloves
- 1.5 ml and/or 2 ml tubes
- 2 ml collection tubes (Qiagen, cat. no. 19201)
- Sterile, RNase-free pipet tips (with aerosol barriers to prevent cross contamination)

Reagents

- QIAamp Viral RNA Mini Kit (Qiagen, cat. no. 52904 or 52906)
- RNase-Free DNase Set (Qiagen, cat. no. 79254)
- Ethanol (96 – 100%, undenatured)
- RNase-free water (DEPC-treated)

General remarks

Carrier-RNA included in the Viral RNA Mini Kit consists of poly-A sequences (100 bp – 10 kbp in length). Therefore, for whole genome shotgun sequencing or metagenomics no carrier-RNA is used to avoid compromising the DNA library for sequencing!

Literature

- QIAamp Viral RNA Mini Kit Handbook (1090245, 12/2014)
- RNeasy Mini Kit Handbook (on-column DNase Digestion)

Preparation of reagents supplied with the QIAamp Viral RNA Mini kit

Buffer AW1

- Before first use, add the appropriate amount of ethanol (96 – 100%) as indicated on the bottle to the buffer concentrate
- Stable for 1 year when stored closed at room temperature (RT)

Buffer AW2

- Before first use, add the appropriate amount of ethanol (96 – 100%) as indicated on the bottle to the buffer concentrate
- Stable for 1 year when stored closed at RT

Before getting started

- Equilibrate samples to room temperature (RT)
- Check that buffers AW1 and AW2 are prepared as described above
- Shake all buffers for mixing
- If intending to perform an on-column DNase digest, prepare DNaseI working solution (on ice!):
    - Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD
    - Mix by gently inverting the tube and centrifuge briefly
    - Do not vortex as DNase I is sensitive to physical denaturation
- Prepare buffer RPE from the QIAamp RNeasy Mini Kit:
    - Before first use, add the appropriate amount of ethanol (96 – 100%) to the buffer concentrate as indicated on the bottle
    - Stable for 1 year when stored closed at RT

Procedure

This protocol is for the purification of viral RNA from 140 µl liquid sample. Larger starting volumes, up to 560 µl (in multiples of 140 µl) can be processed by increasing the initial volumes proportionally and loading onto the QIAamp Mini spin column.
The spin protocol is suitable for all situations. When handling several samples in parallel or for larger sample volumes, the vacuum protocol is recommended (below).
All centrifugation steps are carried out at RT

A. Spin Protocol

1.
- Pipet 560 µl of Buffer AVL per 140 µl sample into a suitable tube (note the final volume is 9-fold the sample volume)

2.
- Add sample
- Mix by pulse-vortexing for 15 s
- Incubate at room temperature for 10 min and briefly centrifuge

3.
- Add 560 µl ethanol (96 – 100%) per 140 µl sample
- Mix by pulse-vortexing for 15 s and briefly centrifuge

4.
- Apply 630 µl of the sample solution to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim
- Centrifuge at 8000 rpm for 1 min
- Discard the collection tube and place the column into a clean 2 ml collection tube

5.
- Repeat step 4 until the complete sample has been applied to a single column

6.
- Add 650 µl Buffer AW1 to the column
- Centrifuge at 8000 rpm for 1 min

- Discard the collection tube and place the column into a clean 2 ml collection tube

7.
- Add 80 µl DNase working solution to the column
- Incubate for 15 min at RT
- Add 650 µl Buffer AW1
- Centrifuge at 8000 rpm for 1 min
- Discard the collection tube and place the column into a clean 2 ml collection tube

8.
- Add 650 µl Buffer AW2 to the column
- Centrifuge at full speed 14,000 rpm for 3 min
- Discard the collection tube and place the column into a clean 2 ml collection tube
- Centrifuge at full speed 14,000 rpm for 1 min

9.
- Place the column in a clean 1.5 ml tube
- Add 50 µl of Buffer AVE
- Incubate at room temperature for 1 min
- Centrifuge at 8000 rpm for 1 min

10.
- For higher RNA recovery and recovery of long RNAs, repeat step 11 twice and elute into the same tube

B. Vacuum protocol

1.
- Pipet 560 µl of Buffer AVL per 140 µl sample into a suitable tube (note the final volume is 9-fold the sample volume)

2.
- Add sample
- Mix by pulse-vortexing for 15 s
- Incubate at room temperature for 10 min and briefly centrifuge

3.
- Add 560 µl ethanol (96 – 100%) per 140 µl sample
- Mix by pulse-vortexing for 15 s and briefly centrifuge

4.
- Mount one spin column per sample onto the vacuum manifold, attach the manifold to the vacuum pump

5.
- Apply 630 µl of the sample solution to the QIAamp Mini column without wetting the rim.
- Apply vacuum to the manifold until all samples have completely been drawn through the column. After all lysates have been drawn through the QIAamp Mini column, close the main vacuum valve and open the screw cap valve to vent the manifold. Close the screw cap valve after the vacuum is released from the manifold.

6.
- Repeat step 5 until the complete sample has been applied to a single column

7.
- Add 750 µl Buffer AW1 to the column
- By applying vacuum as described in step 5, draw the complete buffer AW1 through the columns

8.
- Add 80 µl DNase working solution to the column
- Incubate for 15 min at RT

9.
- Add 750 µl Buffer AW1 to the column
- By applying vacuum as described in step 5, draw the complete buffer AW1 through the columns

10.
- Add 750 µl Buffer AW2 to the column

- By applying vacuum as described in step 5, draw the complete buffer AW2 through the columns

11.
- Place each column in a clean 2 ml collection tube
- Centrifuge at full speed 14,000 rpm for 1 min to dry the membrane
- Discard the collection tube

12.
- Place each column in a clean 1.5 ml tube
- Add 50 µl of Buffer AVE
- Incubate at room temperature for 1 min
- Centrifuge at 8000 rpm for 1 min

13.
- For higher RNA recovery and recovery of long RNAs, repeat step 12 twice and elute into the same tube

RNA is stable for up to one year when stored at -70°C.
When the RNA will be used for NGS it is recommended to subsequently continue with quantification of RNA (SOP_030102_NAQuant_ND1000_FLI and SOP_030202_RNA Quant_RiGreen_FLI) and ds cDNA synthesis (SOP_060104_cDNA-synthesis_FLI).

Standard Operating Procedure

Isolation of DNA from liquid or disintegrated samples using the QIAamp DNA Mini Kit

14th Jun 2018

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SOP-Version: 5

Attachments: none

Introduction

The QIAamp DNA Mini Kit enables the purification of total DNA using proteinase K. In principle the purification procedure comprises four steps and is carried out using QIAamp Mini spin columns in a standard microcentrifuge. In a first step, the sample is lysed. Afterwards, the sample is loaded to the spin column on which the DNA is adsorbed onto the silica membrane. The bound DNA is washed in two centrifugation steps to remove any residual contaminants. Finally, the DNA is eluted in Buffer AE or Nuclease-free water ready for direct downstream applications. Alternatively, it can be stored at -20°C for later use. The purified DNA is up to 50 kb in size, with predominating fragments of approx. 20-30 kb. When the DNA will be used for NGS, it is recommended to directly proceed to the next step.

Sample Material

Liquid samples (e. g., whole blood, plasma, serum, buffy coat, other body fluids, bone marrow, lymphocytes, cultured cells) or samples disintegrated and lysed (tissue and stool homogenates) according to SOP_010104_Disintegration_Cryoprep_FLI or SOP_010203_ Disintegration_Mikrodismembrator_FLI.

Equipment and Reagents

Successful DNA extraction demands a nuclease-free environment, therefore only Nuclease-free tubes, aerosol-free pipette tips, and only nuclease-free water must be used. Gloves have to be worn throughout the procedure and changed regularly in order to avoid nuclease contamination.
In general only use reagents that do not exceed the expiration date. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the manufacturer.

Equipment

- Microcentrifuge (for 1.5 ml and 2 ml tubes)
- Vortexer
- Laminar flow hood
- Pipettes
- Disposable gloves
- 1.5 ml and/or 2 ml tubes
- 2 ml collection tubes (Qiagen, cat. no. 19201)
- Sterile, DNase-free pipet tips (with aerosol barriers to prevent cross contamination)
- Thermal mixer, heating block or water bath

Reagents

- QIAamp DNA Mini Kit (Qiagen cat. no. 51304 or 51306)
- Ethanol (96 – 100%, undenatured)
- RNase-free water (DEPC-treated)

Literature

- QIAamp DNA Mini Kit Handbook (1090246, 02/2015)

Preparation of reagents supplied with the QIAamp DNA Mini kit

Buffer AW1 and AW2

Before first use, add the appropriate amount of ethanol (96 – 100%) as indicated on the bottle to the buffer concentrate

Buffer AL

Do not add proteinase K directly to buffer AL

All Buffers are stable for 1 year when stored closed at room temperature (RT).

Before getting started

- Equilibrate samples to RT
- Check that buffers AW1 and AW2 are prepared as described above
- Heat thermal mixer to 56°C

Procedure

This protocol is for the purification of DNA from 200 µl liquid sample or tissue homogenates. All centrifugation steps are carried out at RT

1. - Pipet 20 µl Proteinase K in a 1.5 ml tube

2. - Add 200 µl of the sample and 200 µl buffer AL
- Mix by pulse-vortexing for 15 s
- Incubate at 56°C for 10 min and briefly centrifuge

3. - Add 200 µl ethanol (96 – 100%)
- Mix by pulse-vortexing for 15 s and briefly centrifuge

4. - Apply 700 µl of the sample solution to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim
- Centrifuge at 8,000 rpm for 1 min
- Discard the collection tube and place the column into a clean 2 ml collection tube

5. - Add 700 µl Buffer AW1 to the column
- Centrifuge at 8,000 rpm for 1 min
- Discard the collection tube and place the column into a clean 2 ml collection tube

6. - Add 700 µl Buffer AW2 to the column
- Centrifuge at 14,000 rpm for 1 min
- Discard the collection tube and place the column into a clean 2 ml collection tube

7. - Repeat step 6 once

8. - Centrifuge at full speed 14,000 rpm for 3 min
- Place the column in a clean 1.5 ml reaction tube

9. - Add 50 µl of Nuclease-free water directly on the membrane
- Incubate at RT for 2 min
- Centrifuge at 8,000 rpm for 1 min

10. - For higher DNA recovery, repeat step 8 and elute into the same tube

Double-stranded DNA is stable for up to one year when stored at -20°C. When the DNA will be used for NGS, it is recommended to subsequently continue with quantification of DNA (SOP_030102_NAQuant_ND1000_FLI).

Standard Operating Procedure

Nucleic acid quantification using NanoDrop ND1000 Spectrophotometer

8th Sept 2017

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SOP-Version: 2

Attachments: none

Introduction

The NanoDrop 1000 Spectrophotometer (Thermo Scientific) measures concentration and purity of nucleic acids in minute volumes (1-2 µl) with high accuracy and reproducibility. Due to the wide concentration range that can precisely be measured (lower limit 2 ng/µl, upper limits dsDNA 3700 ng/µl, RNA 3000 ng/µl, ssDNA 2400 ng/µl) prior dilution of the samples is not necessary. The spectrophotometer utilizes a patented sample retention technology that employs surface tension alone to hold the sample in place without the need for cumbersome cuvettes allowing for clean-up in seconds.
The sample is pipetted onto the end of a fiber optic cable (the receiving fiber). A second fiber optic cable (the source fiber) is then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. The gap is controlled to both 1 mm and 0.2 mm paths. A pulsed xenon flash lamp provides the light source and a spectrometer utilizing a linear CCD array is used to analyze the light after passing through the sample. The instrument is controlled by PC based software, and the data is logged in an archive file on the PC.

Sample Material

RNA extracted according to protocols SOP_020104_NaE_RNA_Trizol_FLI, or SOP_020203_NaE_RNA_ViralRNAKit_FLI, DNA extracted according to protocol (SOP_020304_NaE_DNA_DNAMiniKit_FLI).

Equipment and Reagents

Successful RNA extraction demands an RNase-free environment, therefore only RNase-free tubes, aerosol-free pipette tips, and only DEPC-treated water must be used. Gloves have to be worn throughout the procedure and changed regularly in order to avoid RNase contamination.
In general only use reagents that do not exceed the expiration date. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the manufacturer.

Equipment

- NanoDrop 1000 Spectrophotometer connected with a computer running the NanoDrop control software
- 2.5 µl or 10 µl Pipette
- Sterile, RNase-free pipet tips (with aerosol barriers for preventing cross-contamination)
- Soft laboratory wipes for cleaning
- Disposable gloves

Reagents

- Sample solvent
- Nuclease-free water (DEPC-treated)

General remarks

ONLY use the NanoDrop ND1000 after having been instructed by trained personnel!

Literature

- NanoDrop 1000 Spectrophotometer V3.7 User's Manual (Thermo Scientific)

Before getting started

No remarks

Procedure

1. Switch on the computer and start software ‘ND’

2. While the computer and software are starting up, clean the sample pedestals of the ND1000 using nuclease-free water and laboratory wipes

3. After software start-up, click the button “Nucleic Acid”, apply 2 µl of nuclease free water to the lower sample pedestal when requested, close the upper pedestal, and initialise the instrument

4. After initialisation, choose the sample type right-hand pull-down menu

5. Open the sampling arm and pipette 2 µl of a blank sample (the buffer, solvent, or carrier liquid used with your samples) direct onto the lower measurement pedestal

6. Close the sampling arm and initiate a measurement using the **Blank** button

7. Open the sampling arm, wipe the lower and upper pedestals using a soft laboratory wipe

8. Enter the sample name into the sample ID field (make sure not to press <ENTER> in this field)

9. Pipette 2 µl of the sample directly onto the lower pedestal, close the sampling arm, and initiate a measurement using the **Measure** button

10. Immediately after the measurement is complete, open the sampling arm and wipe the sample from both the upper and lower pedestals to prevent sample carryover

11. Repeat steps 8 – 10 for all samples

12. After the last sample measurement, perform a measurement of your solvent according to steps 7 – 9 to make sure the instrument is clean; enter “blank” or “solvent” as sample ID

Standard Operating Procedure

RNA Quantification using Quant-iT RiboGreen RNA Assay Kit

8th Sept 2017

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SOP-Version: 2

Attachments: none

Introduction

Quant-iT RiboGreen RNA reagent is an ultrasensitive fluorescent nucleic acid stain for quantifying RNA in solution and enables quantification of as little as 1 ng/ml RNA using a fluorescence microplate reader. The RiboGreen reagent is non-fluorescent when free in solution; upon binding to nucleic acids (single- or double stranded), the fluorescence of the RiboGreen reagent increases more than 1000-fold. The nucleic acid-bound RiboGreen reagent has an excitation maximum of approximately 500 nm and an emission maximum of approximately 525 nm. The fluorescence is directly proportional to the amount of nucleic acids over a range of approx. 10 pg/µl – 400 pg/µl. This linearity is maintained in the presence of several compounds commonly found to contaminate nucleic acid preparations, including nucleotides, salts, urea, ethanol, chloroform, detergents, proteins, and agarose. Since RiboGreen reagent also binds to DNA, pre-treatment of mixed RNA/DNA samples with DNase has to be used to generate an RNA-selective assay.

Sample Material

RNA extracted according to protocols SOP_020104_NaE_RNA_Trizol_FLI, or SOP_020203_NaE_RNA_ViralRNAKit_FLI.

Equipment and Reagents

Successful RNA extraction demands an RNase-free environment, therefore only RNase-free tubes, aerosol-free pipette tips, and only DEPC-treated (nuclease-free) water must be used. Gloves have to be worn throughout the procedure and changed regularly in order to avoid RNase contamination.
In general only use reagents that do not exceed the expiration date. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the manufacturer.

Equipment

- Multimode Microplate Reader (e.g., Tecan Infinite F200 incl. fluorescence optical kit - Blue, 490/515-580 nm)
- 2 PCR workstations (one master mix box, one template box)
- Mini centrifuge
- Black 96-well microplates (e.g. Greiner, cat. no. 655900)
- Pipettes
- Sterile, RNase-free pipet tips (with aerosol barriers to prevent cross contamination)
- Sterile, disposable polypropylene plasticware for reagent preparation
- RNase-free water (DEPC-treated)
- Disposable gloves

Reagents and consumables

- Quant-iT RiboGreen RNA Reagent (*Component A*) (Invitrogen; cat. no. R11491, R11490)
- 20X TE Buffer, RNase-free (*Component B*) (Invitrogen; cat. no. R11490)
- Ribosomal RNA standard, 16S and 23S rRNA from E. coli (*Component C*) (Invitrogen; cat. no. R11490)

General remarks

Caution: No data are available addressing the mutagenicity or toxicity of Quant-iT RiboGreen RNA reagent. Because this reagent binds to nucleic acids, **treat the reagent as a potential mutagen and handle with appropriate care**.
Handle the DMSO stock solution with particular caution (double gloves) as DMSO is known to facilitate the entry of organic molecules into tissues.

Literature

- Quant-iT™ RiboGreen® RNA Reagent and Kit User Manual (no. MP 11490, 10 June 2008) (https://tools.thermofisher.com/content/sfs/manuals/mp11490.pdf)

Before getting started

- Allow the Quant-iT RiboGreen RNA Reagent (*Component A*) to warm to room temperature before opening the vial (keep dark).

Procedure

Mastermix box

1. Prepare the 1X TE working solution
  - Calculate 1800 µl 1X TE for the standards
  - Calculate 200 µl 1X TE per sample
  - Dilute 100 µl of 20X TE (*Component B*) in 1900 µl nuclease-free water. To reduce the pipetting error, use the same pipette for all steps, if possible

2. Prepare RiboGreen working solution
  - Dilute RiboGreen reagent (*Component A*) in 1X TE; add 1µl RiboGreen to 999 µl 1X TE

Template box

3. Prepare the quantification standard
  - Prepare 260 µl RNA standard working solution 0,5 ng/µl (dilute *Component C* 1:200 in 1X TE)
  - Serially dilute RNA standard 1:2 in 1X TE (e.g. add 105 µl RNA standard to 105 µl 1X TE) up to a final dilution of 1:64

4. Dilute samples with RNA concentrations >20 ng/µl (NanoDrop measurement) in 1X TE to fit in the linear measurement range of the assay (approx. 10 pg/µl – 400 pg/µl)

5. Pipet each standard concentration in duplicates of 50 µl into the microtiter plate according to the scheme below (table 1); pipet 50 µl of the undiluted standard into an additional well

6. Pipet duplicates of each sample into the microtiter plate and add 1X TE to a final volume of 50 µl

7. Add 50 µl RiboGreen working solution to every standard and sample, mix and incubate for 3 min at RT, protected from light

Measurement

8. Measure the fluorescence with the microplate reader; for the Tecan Infinite F200 use the parameters specified in table 2, below. For other microplate readers use equivalent parameters

9. Save the results to an Excel-File

Calculation

10. Check the raw fluorescence data for plausibility

11. Calculate mean values per well from the multiple measurements

12. Check that the duplicate measurements do not deviate more than 5%

13. Calculate the linear regression from the standards, check that $R^2 \geq 0.985$

14. Using the linear regression formula, calculate the amount of RNA in the samples; mind the sample dilutions!

Table 1 Pipetting scheme

Standard 1:1 50 µl 25 ng	**Standard** 1:1 50 µl 25 ng	**RiboRef** Standard 1:1 50 µl 25 ng	A4	A5	A6	A7	A8	A9	A10	A11	A12
Standard 1:2 50 µl 12,5 ng	**Standard** 1:2 50 µl 12,5 ng	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
Standard 1:4 50 µl 6,25 ng	**Standard** 1:4 50 µl 6,25 ng	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
Standard 1:8 50 µl 3,125 ng	**Standard** 1:8 50 µl 3,125 ng	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
Standard 1:16 50 µl 1,5625 ng	**Standard** 1:16 50 µl 1,5625 ng	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
Standard 1:32 50 µl 0,78125 ng	**Standard** 1:32 50 µl 0,78125 ng	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
Standard 1:64 50 µl 0,390625 ng	**Standard** 1:64 50 µl 0,390625 ng	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
Standard 1X TE 50 µl 0 ng	**Standard** 1X TE 50 µl 0 ng	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

Table 2 Measurement parameters for Tecan Infinite 200

Parameter	**Value**
Plate	Greiner 96 Flat Bottom Black Polystyrol [GRE96fb_chimney.pdfx]
Shake	Orbital, 3 mm Amplitude, 20 s
Temperature	25 °C ± 0,5 °C
Mode	Fluorescence, top measurement
Measurements per well	Circle (filled), 5 x 5, frame 350 µm
Excitation wavelength	485 nm
Emissions wavelength	535 nm
Excitation bandwidth	20 nm
Emission bandwidth	25 nm
Amplification	automatic determination from well A3 (RiboRef)
Number flashes	25
Integration time	20 µs
Delay	0 µs
Idle time	0 ms

FLI *SOP_040102_RNAQual-check_RNAPico-chip_FLI*	**Quality check of RNA** **(RNA Pico Chip)**	

Standard Operating Procedure

Quality check of extracted RNA using Agilent RNA 6000 Pico Kit

8th Sept 2017

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SOP-Version: 2

Attachments: none

Introduction

High quality input RNA is essential for successful preparation of cDNA shotgun sequencing libraries. The Agilent RNA 6000 Pico assay is suitable to analyze total RNA or mRNA using only 1 µl of sample containing 200 – 5000 pg/µl RNA with a size between 25 and 6000 bases. For samples with a higher concentration of RNA, the samples may be diluted or the Agilent RNA 6000 Nano Kit for samples with RNA concentrations ranging from 25 ng/µl to 500 ng/µl may be used. The dye labelled nucleic acids are separated electrophoretically based on their size in a microfluidic system. Internal standards ensure assay accuracy and reproducibility. From the generated electropherogram, the software calculates an RNA Integrity Number (RIN) that can help in assessing RNA quality.
Quality checked RNA can be accurately quantified according to SOP_030202_RNAQuant_RiGreen_FLI or be directly used for cDNA synthesis (SOP_060104_cDNA-synthesis_FLI).

Sample Material

RNA extracted according to protocols SOP_020104_NaE_RNA_Trizol_FLI, or SOP_020203_NaE_RNA_ViralRNAKit_FLI.

Equipment and Reagents

Successful RNA extraction demands an RNase-free environment, therefore only RNase-free tubes, aerosol-free pipette tips, and only DEPC-treated (nuclease-free) water must be used. Gloves have to be worn throughout the procedure and changed regularly in order to avoid RNase contamination.
In general, only use reagents that do not exceed the expiration date. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the manufacturer.

Equipment

- Agilent 2100 Bioanalyzer
- Chip Priming Station (Agilent, cat. no. 5065-4401, supplied with Bioanalyzer)
- IKA Vortexer Model MS3 with chip adapter (supplied with Bioanalyzer)
- Vortexer, Microcentrifuge
- Pipettes

Reagents and consumables

- Agilent RNA 6000 Pico Kit (Agilent, cat. no. 5067-1513)
- Nuclease-free water (DEPC-treated)
- 0.5, 1.5 ml tubes (sterile, Nuclease-free, low bind)
- Disposable gloves
- Sterile, Nuclease-free pipet tips (with aerosol barriers to prevent cross contamination)

General remarks

- The RNA concentration should be between 200-5000 pg/µl for total RNA; if necessary, dilute samples with Nuclease-free water

Literature

- Agilent RNA 6000 Pico Kit Quick Start Guide (Part no. G2938-90049, 08/2013)

Before getting started

- Prepare the RNA ladder:
  - Briefly centrifuge the ladder and pipette tube content in an RNase-free tube
  - Incubate for 2 min at 70°C for heat denaturing; immediately cool on ice
  - Add 90 µl RNase-free water, mix well and spin down
  - Prepare 2 µl-aliquots in 0.5 µl RNase-free tubes
  - Store aliquots at -70°C and thaw on ice if needed
- Prepare the RNA gel matrix:
  - Pipette 550 µl of RNA gel matrix into a spin filter
  - Centrifuge at 1500 × *g* for 10 min
  - Prepare aliquots á 65 µl into 0.5 µl RNase- free tubes
  - Store at 4°C and use the filtered gel within 4 weeks
- Equilibrate the RNA dye concentrate to room temperature 30 min before use (protect from light)
- Dilute RNA samples to ≤ 5 ng/µl if concentration is higher and denature the samples 2 min at 70°C, store on ice until use
- Set up the chip priming station:
  - Screw on the syringe to the lid of the chip priming station
  - Adjust the syringe clip to the top position
  - Ensure that the base plate is inserted in position C

Procedure

1. Vortex the RNA dye concentrate for 10 sec, spin down, add 1 µl of dye into an aliquot of filtered gel

2. Vortex well and centrifuge at 13000 × *g* for 10 min; use this mix within one day

3. Put a new RNA chip on the chip priming station

4. Pipette 9 µl of gel-dye mix in the well marked G

5. Ensure that the plunger is positioned at 1 ml, close the chip priming station and press the plunger until it is held by the clip

6. After exactly 30 sec release the clip

7. After further 5 sec slowly pull back the plunger to the 1 ml position

8. Open the chip priming station; pipette 9 µl of gel-dye mix in wells marked **G**

9. Pipette 9 µl of RNA conditioning solution into the well marked **CS**

10. Pipette 5 µl of RNA marker in all sample wells (1-11) and the well marked "**ladder**"

11. Pipette 1 µl of heat denatured ladder aliquot in the well marked "**ladder**"

12. Load all sample wells: pipette 1 µl of sample in each used sample well (1-11); pipette 1 µl of RNA marker in each sample well not used for a sample

13. Vortex the chip with the IKA Vortexer MS3 for 1 min at 2400 rpm (as indicated)

14. Within 5 min, run the chip in the Agilent 2100 Bioanalyzer with the assay "Eukaryote Total RNA Series II.xsy" for RNA

15. After running the chip, apply a cleaning chip filled with 350 µl of fresh Nuclease-free water for about 30 sec to clean electrodes; let the electrodes air dry with the lid open

Standard Operating Procedure

Concentrating RNA for cDNA synthesis using Agencourt RNAClean XP Beads

29th Aug 2018

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SOP-Version: 4

Attachments: none

Introduction

For cDNA synthesis, up to 500 ng high quality RNA in a maximum volume of 17 µl is needed. Low concentrated RNA solutions need to be concentrated with the Agencourt RNAClean XP Beads. This system utilizes solid-phase paramagnetic bead technology for purification and concentration of RNA. Excess oligonucleotides, nucleotides, salts, and enzymes are removed using a simple washing procedure. Total RNA bound to magnetic beads will be separated from contaminants and washed with ethanol. Afterwards, the bound RNA will be eluted in an appropriate amount of RNase-free water. Concentrated RNA can directly be used for cDNA synthesis (SOP_060104_cDNA-synthesis_FLI).

Sample Material

RNA extracted according to protocols SOP_020104_NaE_RNA_Trizol_FLI, or SOP_020203_NaE_RNA_ViralRNAKit_FLI.

Equipment and Reagents

Successful working with RNA demands an RNase-free environment, therefore only RNase-free tubes, aerosol-free pipette tips, and only DEPC-treated water must be used. Gloves have to be worn throughout the procedure and changed regularly in order to avoid RNase contamination.
In general, only use reagents that do not exceed the expiration date. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the manufacturer.

Equipment

- PCR workstations (template box)
- Magnetic particle concentrator (MPC; e.g. Life Technologies cat. no. 12321D)
- Shaker for 1.5 ml tubes
- Vortexer
- Pipettes

Reagents and consumables

- Agencourt RNAClean XP beads (Beckman Coulter, cat. no. A63987)
- Un-denatured ethanol (80% (v/v), RNase-free
- RNase-free water (DEPC-treated)
- 1.5 ml tubes (sterile, RNase-free, low-bind)
- Disposable gloves
- Sterile, RNase-free pipet tips (with aerosol barriers to prevent cross contamination)

General remarks

- Make sure the Agencourt RNAClean XP beads are at room temperature and are thoroughly mixed. The reagent (magnetic particle solution) should appear homogenous and consistent in colour.
- Do not freeze.

Literature

- Agencourt RNAClean XP manual (Beckman Coulter, Protocol 001298v001)
- Wylezich C, Papa A, Beer M, Höper D (2018) A versatile sample processing workflow for metagenomic pathogen detection. Scientific Report DOI:10.1038/s41598-018-31496-1

Before getting started

Resuspend an aliquot of the magnetic particle solution via shaking or vortexing.

Procedure

Template box

1. Add 1.8 volumes of Agencourt RNAClean XP Beads to an RNA sample in a low-bind tube and mix the RNA-bead-solution thoroughly by pipette mixing (15x)

2. Incubate 7 min at room temperature (RT) on a shaker (550 rpm)

3. Place the reaction tube onto the MPC for 5 min to separate the beads from supernatant (until the beads have concentrated on the tube wall)

4. Slowly aspirate the cleared solution from the reaction tube without disturbing the beads and discard the solution; keep the reaction tube in the MPC while aspirating the solution

5. Depending on pellet size, dispense 500 – 1000 µl 80% ethanol to reaction tube and incubate 30 s at RT

6. Slowly aspirate the ethanol from the reaction tube and discard while keeping the tube on the MPC

7. Repeat step 5-6 once while the reaction tube keeps in the MPC

8. Air-dry the pellet for 10 min with the cap open (keeping on MPC); do not over-dry!

9. Take reaction tube from MPC, elute RNA with 20 µl RNase-free water by careful pipette mixing and incubate for 2 min

10. Place the reaction tube onto the MPC, incubate for 2 min and pipet 17 µl of the concentrated RNA solution into a clean reaction tube

Continue directly with the cDNA synthesis (SOP_060104_cDNA-synthesis_FLI).

Standard Operating Procedure

cDNA synthesis
for the generation of double stranded cDNA from purified RNA

29th Aug 2018

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SOP-Version: 5

Attachments: none

Introduction

The here described one-tube procedure is dealing with the synthesis of double stranded (ds) cDNA from purified total RNA using the cDNA Synthesis System from Roche. The ds cDNA is required for the subsequent construction of non-directional cDNA libraries for next-generation sequencing. If necessary, prior to cDNA synthesis the purified RNA has to be concentrated with Agencourt RNAClean XP Beads. This purified and eventually concentrated RNA is then used in the ds cDNA synthesis. Firstly, a first-strand synthesis reaction has to be performed and in order to generate ds cDNA, a subsequent second strand synthesis is done. The first and second strand syntheses are performed in the same tube, which speeds up the synthesis procedure and maximizes recovery of cDNA. The last step in the cDNA synthesis is to ensure that the termini of the cDNA are blunt. cDNA can either be used for a subsequent DNA fragmentation step (SOP_070104_DNA_Frag_Covaris_FLI) or stored at -20°C.

Sample Material

Five-hundred ng high quality RNA in a maximum volume of 17 µl. In case the concentration is less, RNA needs to be concentrated with the Agencourt RNAClean XP Beads (SOP_050103_Concentrating-RNA_FLI).

Equipment and Reagents

Successful cDNA synthesis demands an RNase-free environment, therefore only RNase-free tubes, aerosol-free pipette tips, and only DEPC-treated water must be used. Gloves have to be worn throughout the procedure and changed regularly in order to avoid RNase contamination.
In general only use reagents that do not exceed the expiration date. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the manufacturer.

Equipment

- 2 PCR workstations (one master mix box, one template box)
- Microcentrifuge
- Thermal Cycler
- NanoDrop Spectrophotometer for nucleic acid quantification
- Magnetic particle concentrator (MPC; e.g. DynaMag™-2, Life Technologies)
- Vortexer
- Pipettes

Reagents and consumables

- cDNA-Synthesis System (Roche, cat. no. 11 117 831 001)
- Hexanucleotide Mix (Roche, cat. no. 11277081001)
- RNase-free water (DEPC-treated)
- 0.2 M EDTA pH 8.0
- 0.2 ml, 1.5 ml and 2.0 ml tubes (sterile, RNase-free)
- Disposable gloves
- Sterile, RNase-free pipet tips (with aerosol barriers to prevent cross contamination)

General remarks

To avoid contaminations:

- Tubes containing purified template RNA/DNA must exclusively be opened and handled in a **template PCR workstation**.
- Buffers and reagents for preparing master mixes must exclusively be opened and handled in a clean **master mix box**.
- **Change gloves after handling template RNA/DNA** before working in the master mix box.
- Buffers and reagents once opened in the template box must never be used and opened in the master mix box.

Literature

- Roche cDNA-Synthesis System (cat. no. 11 117 831 001) Manual
- Wylezich C, Papa A, Beer M, Höper D (2018) A versatile sample processing workflow for metagenomic pathogen detection. Scientific Reports DOI:10.1038/s41598-018-31496-1

Before getting started

- Preheat thermal cycler to 95°C.

Procedure

A. First strand synthesis

Master mix box

1. Prepare master mix for the first strand synthesis:

Components	1 x		x	
Vial 1 (5× RT-buffer AMV)	8.0	µl		µl
Vial 3 (DTT, 0.1 M)	4.0	µl		µl
Vial 7 (dNTPs, 10 mM)	4.0	µl		µl
Vial 4 (Protector RNase Inhibitor, 25 U/µl)	1.0	µl		µl
Vial 2 (AMV RT, 25 U/µl)	2.0	µl		µl
Total volume	**19.0**	**µl**		**µl**

2. Pipette 4 µl of the Hexanucleotide Mix in a 0.2 ml PCR-Tube.

Template box

3. Add a maximum of 500 ng template RNA in a total volume of 17 µl to the Hexanucleotide Mix

4. Briefly vortex and centrifuge

5. Incubate 2 min at 95°C and subsequently cool down the reaction for 2 min on ice

6. Add 19 µl of the first-strand master mix

7. Incubate 10 min at 25°C, 60 min at 42°C with hot-lid on (110°C)

8. Briefly before the incubation is finished start preparing the master mix for the second-strand synthesis (see below)

9. After the incubation is finished, keep on ice

10. Before proceeding to the master mix box, change gloves

B. Second strand synthesis

Master mix box

11. Prepare master mix for the second strand synthesis:

Components	1 x		x	
Vial 9 (5× 2nd strand synthesis buffer)	30.0	µl		µl
Vial 12 (Water, PCR Grade)	72.0	µl		µl
Vial 7 (dNTPs, 10 mM)	1.5	µl		µl
Vial 10 (2nd strand enzyme)	6.5	µl		µl
Total volume	**110.0**	**µl**		**µl**

Template box

12. Add 110 µl second strand synthesis master mix to the first-strand reaction, briefly mix and centrifuge

13. Incubate 2 h at 16°C (hot-lid off)

14. Add 20 µl of **vial 11** (T4 DNA Polymerase), carefully mix and centrifuge briefly

15. Incubate 5 min at 16°C

16. Stop reaction by adding 17 µl 0.2 M EDTA, pH 8.0

Store cDNA at -20°C or continue directly with the DNA fragmentation (SOP_070104_DNA_Frag_Covaris_FLI).

Standard Operating Procedure

Fragmentation of dsDNA by hydrodynamic shearing using the Covaris M220 Focused-ultrasonicator

28th Aug 2017

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SOP-Version: 4

Attachments: none

Introduction

The fragmentation of DNA is a critical sample preparation step required by all second-generation sequencing platforms. The quality and diversity of the final sample library depends on this fragmentation step. A random process to generate unbiased libraries is required that must also be robust to ensure consistent and reproducible performance without operator induced variation. The M220 Focused-ultrasonicator uses the Adaptive Focused Acoustic (AFA) process to apply hydrodynamic shearing forces to the DNA to randomly fragment it. It is conducted under isothermal conditions ensuring both unbiased fragmentation and high recovery of double-stranded DNA. The M220 generates DNA fragments adjustable from 150 to 5000 bp. Depending on the sequencing platform, different DNA shearing protocols are used to obtain the optimal fragment length.

Sample Material

The complete volume of the cDNA synthesised according to *SOP_060104_cDNA-synthesis_FLI* or 500 ng DNA isolated according to *SOP_020304_NaE_DNA_DNAMini Kit_FLI* and quantified following the procedure of *SOP_030102_NAQuant_ND1000_FLI*.

Equipment and Reagents

In general only use reagents that do not exceed the expiration date. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the manufacturer.

Equipment

- Covaris M220 Focused-ultrasonicator with M220 microTUBE holder (cat. no. 500301)
- Fragmentation tubes (microTUBE AFA Fiber Screw Cap 6 x 16 mm, 130 µl; Covaris, cat. no. 520096)
- Wash bottle (supplied with starter Kit)

Reagents

- AFA-grade water (Covaris, cat. no. 520101) or highly purified water, at least ASTM Type III or ISO grade 3
- Nuclease-free water (DEPC-treated)

General remarks

- **ONLY** use the M220 after having been instructed by trained personnel
- **NEVER** run a method without AFA-grade water in the acoustic assembly; this could damage the transducer
- **DO NOT** leave water in the water bath for an extended time. Empty the water bath and wipe it dry after use with a lint-free cloth
- Do not employ isopropyl alcohol, ammonia-based or abrasive cleaners on the acoustic assembly
- Store the tube holder in a dry place

DNA shearing protocols: Use the following pre-installed DNA shearing protocol:

DNA_0500_bp_130_ul_Snap_Cap_microTUBE

Literature

- User manual M220 (Covaris, publication no. P/N 010157, 10/2014)
- Wylezich C, Papa A, Beer M, Höper D (2018) A versatile sample processing workflow for metagenomic pathogen detection. Scientific Reports DOI:10.1038/s41598-018-31496-1

Before getting started

- Switch on the M220 Focused-ultrasonicator and the computer
- Start software ‘SonoLab’
- Carefully place the microTube holder in the Acoustic Assembly
- Fill the Acoustic Assembly with approx. 15 ml AFA-grade water

Procedure

1. Pipette 130 µl sample in the microTUBE (if the sample volume is less than 130 µl fill up to 130 µl with water)

2. Place the microTUBE in the microTube holder and fix it with the sample weight

3. Start protocol via the start button, repeat fragmentation once by pressing the repeat button

4. Pipette sample into a clean 1.5 ml tube

5. If the sample volume is greater than 130 µl, repeat steps 1-4 using the same microTube

Use the fragmented DNA for library preparation (*SOP_090102_Library-prep_automated_IlluminaMiSeq_FLI* or *SOP_080102_Purification_fragmented-cDNA_FLI* followed by *SOP_090202_Library-prep_IonTorrent_FLI*).

Standard Operating Procedure

Purification and concentration of fragmented DNA using Agencourt AMPure XP beads

29th Aug 2018

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SOP-Version: 3

Attachments: none

Introduction

For manual library preparation for the Ion Torrent PGM according to LOP_Library-prep_IonTorrent_FLI_v1, a maximum volume of 20.5 µl DNA sample is needed. The cDNA fragmented according to LOP_DNA_Frag_Covaris_FLI_v3 usually has a volume of 260 µl and therefore needs to be concentrated with the Agencourt AMPure XP beads. This system utilizes solid-phase paramagnetic bead technology for purification of DNA. Excess short nucleotides, salts, and enzymes are removed using a simple washing procedure. DNA bound to magnetic beads will be separated from contaminants and washed with ethanol. Afterwards, the bound DNA will be eluted in an appropriate amount of Nuclease-free water. Concentrated and purified cDNA can directly be used for library preparation (*SOP_090202_Library-prep_IonTorrent_FLI*).

Sample Material

DNA fragmented according to *SOP_070104_DNA_Frag_Covaris_FLI*.

Equipment and Reagents

Successful working with DNA demands a Nuclease-free environment, therefore only Nuclease-free tubes, aerosol-free pipette tips, and only DEPC-treated water must be used. Gloves have to be worn throughout the procedure and changed regularly in order to avoid Nuclease contamination.
In general only use reagents that do not exceed the expiration date. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the manufacturer.

Equipment

- PCR workstation (template box)
- Magnetic particle concentrator (MPC; e.g. Life Technologies cat. no. 12321D)
- Shaker for 1.5 ml tubes
- Vortexer
- Pipettes

Reagents and consumables

- Agencourt AMPure XP beads (Beckman Coulter, cat. no. A63881)
- Un-denatured ethanol (80% (v/v)), Nuclease-free
- Nuclease-free water (DEPC-treated)
- 1.5 ml tubes (sterile, Nuclease-free, low-bind)
- Disposable gloves
- Sterile, Nuclease-free pipet tips (with aerosol barriers to prevent cross contamination)

General remarks

- Aliquot the AMPure XP beads in order to prevent repeated warming and cooling as this will degrade the buffer and render the beads non-functional.
- When opening a new lot of AMPure XP beads, perform a bead calibration before first use (*SOP_000102_AMPureBead-calibration_FLI*).

- Make sure the Agencourt AMPure XP beads are at room temperature and are thoroughly mixed. The reagent (magnetic particle solution) should appear homogenous and consistent in colour.
- Do not freeze.

Literature

- DeAngelis MM, Wang DG, Hawkins TL. 1995. Solid-phase reversible immobilization for the isolation of PCR products. Nucleic Acids Res 23:4742-4743.
- AGENCOURT AMPURE XP PCR PURIFICATION (Beckman Coulter, protocol no. 000387v001)
- Wylezich C, Papa A, Beer M, Höper D (2018) A versatile sample processing workflow for metagenomic pathogen detection. Scientific Reports DOI:10.1038/s41598-018-31496-1

Before getting started

Allow an aliquot of the magnetic particle solution to come to room temperature before use. Resuspend via vortexing until it appears homogenous and consistent in colour.

Procedure

Template box

1. Add 1.8 volumes of Agencourt AMPure XP beads to each cDNA sample in a low-bind tube and mix the DNA-bead-solution thoroughly by pipette mixing (15x)

2. Incubate 7 min at room temperature (RT) on a shaker (550 rpm)

3. Place the reaction tube onto the MPC for 5 min to separate the beads from supernatant (until the beads have concentrated on the tube wall)

4. Slowly aspirate the cleared solution from the reaction tube without disturbing the beads and discard the solution; keep the reaction tube in the MPC while aspirating the solution

5. Dispense 1 ml 80% ethanol to reaction tube and incubate 30 s at RT

6. Slowly aspirate the ethanol from the reaction tube and discard

7. Repeat step 5-6 while the reaction tube keeps on MPC

8. Air-dry the pellet for 10 min with the cap open (keeping on MPC); do not over-dry!

9. Take reaction tube from MPC, elute DNA with 25 µl Nuclease-free water by careful pipette mixing and incubate for 2 min

10. Place the reaction tube onto the MPC, incubate for 2 min, and pipet 22 µl of the concentrated DNA solution into a clean reaction tube

Continue directly with the library preparation (*SOP_090202_Library-prep_IonTorrent_FLI*).

Standard Operating Procedure

Automated library preparation with the SPRIworks Fragment Library System II for sequencing with Illumina MiSeq

29th Aug 2018

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SOP-Version: 2

Attachments: none

Introduction

The here described procedure is dealing with the automatic preparation of libraries that are for use on Illumina MiSeq instruments. The SPRIworks Fragment Library System II is an automated system which can process up to 10 samples in parallel in less than 4 hours and is working absolutely contamination-free. Reagents required to prepare a single library are provided pre-dispensed in disposable cartridges. As input, fragmented DNA is loaded onto the SPRI-TE instrument which performs the subsequent steps in the library construction process. The SPRIworks Fragment Library System II employs enzymatic reactions (end-repair and ligation of barcode adapters containing a unique identifying sequence), SPRI-based reaction purifications to automatically generate fragment libraries. Except the adapters, all reagents required to construct a single library are contained within the reagent cartridge. End-repaired and adapter-ligated DNA fragments can directly be used for size selection (*SOP_100102_SizeSelection_Library_FLI*) or stored at -20°C.

Sample Material

DNA fragmented according to *SOP_070104_DNA_Frag_Covaris_FLI*.

Equipment and Reagents

Only Nuclease-free tubes, aerosol-free pipette tips, and only DEPC-treated water must be used. Gloves have to be worn throughout the procedure and changed regularly in order to avoid Nuclease contamination.
In general, only use reagents that do not exceed the expiration date. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the manufacturer.

Equipment

- SPRI-TE Nucleic Acid Extractor (Beckman Coulter)
- SPRIworks Method Card for Instrument Library II (REF A85410)
- 2 workstations (one master mix box, one template box)
- Rotator
- Microcentrifuge
- Vortexer
- Pipettes

Reagents

- Cartridges, tubes and tips: provided with SPRIworks System II for Roche GS FLX DNA Sequencer (Beckman Coulter, cat. no A84806)
- Barcode Adapters (25 µM, e.g. Bioo Scientific NEXTFlex 96 DNA Adapters, cat. no. 514106)
- Nuclease-free water (DEPC-treated)
- Disposable gloves
- Sterile, Nuclease-free pipet tips (with aerosol barriers to prevent cross contamination)

General remarks

- Blunt end adapters are not compatible with SPRIworks Fragment Library System II
- Do not change the method card while instrument is powered on

To avoid contaminations:

- Tubes containing fragmented and concentrated DNA or cDNA synthesis products must exclusively be opened and handled in a **template PCR workstation**.
- Buffers and reagents for preparing master mixes must exclusively be opened and handled in a clean **master mix box**.
- **Change gloves after handling template cDNA** before working in the master mix box.
- Buffers and reagents once opened in the template box must never be used and opened in the master mix box.
- Do not use the same barcode as in the sequencing run before.

Literature

- SPRIworks Fragment Library System II (Beckman Coulter, Part Number A87326B, 2015)
- Wylezich C, Papa A, Beer M, Höper D (2018) A versatile sample processing workflow for metagenomic pathogen detection. Scientific Reports DOI:10.1038/s41598-018-31496-1

Before getting started

- Thaw one cartridge per library at room temperature on a rotator until all contents are completely thawed (30 – 60 min)
- Shake the cartridges thoroughly until all beads are dissolved
- Make sure the liquid drop is at the bottom of the tube/cartridge without air bubbles

Procedure

Master mix box

1. Prepare 3 labelled screw cap tubes provided with the kit for each library: **sample** tube, **adapter** tube, **library** tube

2. Take off all caps of the tubes; keep the caps of the library tubes

3. Pipet 2,5 µl of one of the different barcode adapters to the **adapter** tube and dilute with 2,5 µl Nuclease-free water; *open only one barcode adapter tube at a time and change gloves between pipetting the different adapters to avoid cross-contamination*

Template box

4. Transfer 500 ng fragmented DNA in a maximum of 400 µl to the sample tube, fill up to 400 µl with Nuclease-free water

SPRI-TE Nucleic Acid Extractor

5. Set up the instrument
   - Turn off the instrument
   - Press the EJECT button, remove any method card if necessary and carefully insert the SPRIworks method card for Fragment Library System II (REF A85410)
   - Close the instrument door
   - Turn the power on

6. Open the door only when the screen displays the TOP MENU

7. When cartridges have completely thawed, ensure that all solutions including magnetic beads are at the bottom of the wells by gently tapping on the bench top

8. Remove tip/tube rack and reagent rack (in this order) from the instrument

9. Insert one cartridge per library into the grooved channel of the reagent rack by grasping the labelled lip and sliding it to the back of the channel

10. Arrange the tubes and tips provided with the kit as displayed in the Table below

Position in the tip tube rack	Consumables
1	Sheath with **Piercing Tip**
2	Sheath with **1 ml Tip**
3	Sheath with **200 µl Tip**
4	Uncapped 2 ml **Adapter Tube** with Adapters
5	Uncapped 2 ml **Library Tube**

11. Load reagent rack and tip/tube rack (in this order) into the instrument; the back edge of the tip/tube rack overlaps the reagent rack when loaded correctly

12. Load the uncapped **sample** tube containing the fragmented DNA into the circular hole in position 13 of the reagent rack

13. Start the run
- Close the instrument door
- Select the green START button
- Specify 1 = no size selection and select ENTER to confirm
- After a heat block test, the run begins automatically

14. The run is finished when the instrument displays “Run Complete”
- Open the door
- Retrieve and cap the **library** tubes
- Discard used sheaths, tips and cartridges

15. To clean the instrument, select UV-Clean from the TOP MENU

Store the libraries at 4°C for up to 6 hours or -20°C for long time storage or continue directly with size selection (*SOP_100102_SizeSelection_Library_FLI*).

Standard Operating Procedure

Library preparation including end-repair and adapter ligation for sequencing with Ion Torrent PGM

29th Aug 2018

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SOP-Version: 2

Attachments: none

Introduction

The here described one-tube procedure is dealing with the preparation of shotgun sequencing libraries for use on Life Technologies instruments (e.g. Ion Torrent PGM). This procedure uses fragmented DNA as input. After fragmentation, the ends of the DNA fragments have to be repaired. Then, platform specific adaptors, either barcoded or non-barcoded, have to be ligated to both ends of the DNA fragments. The resulting libraries can directly be used for purification and subsequent size selection (*SOP_090302_ Purification_IonTorrent-Library_FLI*, *SOP_100102_SizeSelection_Library_FLI*) or stored at -20°C. Libraries prepared according to this SOP are also suitable for sequencing using the Ion Torrent S5/S5XL instruments using Ion 520 or Ion 530 chips but not with Ion 540 chips.

Sample Material

Double-stranded DNA fragmented according to *SOP_070104_DNA_Frag_Covaris_FLI* and concentrated and purified according to *SOP_080102_Purification_fragmented-DNA_FLI*.

Equipment and Reagents

Only Nuclease-free tubes, aerosol-free pipette tips, and only DEPC-treated water must be used. Gloves have to be worn throughout the procedure and changed regularly in order to avoid Nuclease contamination.
In general only use reagents that do not exceed the expiration date. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the manufacturer.

Equipment

- 2 PCR workstations (one master mix box, one template box)
- Microcentrifuge
- Thermal Cycler
- Magnetic particle concentrator (MPC; e.g. DynaMag™-2, Life Technologies)
- Vortexer
- Pipettes

Reagents and Consumables

- GeneRead DNA Library L Core Kit (Qiagen, cat. no. 180432)
- Barcode Adapters (e.g. Life Technologies, Ion Xpress Barcode Adapters 1-16, cat. no. 4471250)
- Nuclease-free water (DEPC-treated)
- 0.2 ml tubes (sterile, Nuclease-free)
- Disposable gloves
- Sterile, Nuclease-free pipet tips (with aerosol barriers to prevent cross contamination)

General remarks

To avoid contaminations:

- Tubes containing fragmented and concentrated DNA must exclusively be opened

and handled in a **template PCR workstation**.

- Buffers and reagents for preparing master mixes must exclusively be opened and handled in a clean **master mix box**.
- **Change gloves after handling template DNA** before working in the master mix box.
- Buffers and reagents once opened in the template box must never be used and opened in the master mix box.
- Do not use the same barcode as in the sequencing run before.

Literature

- QIAGEN GeneRead™ Library Prep (L) Handbook (cat. no. 1090330, 12/2014)
- Wylezich C, Papa A, Beer M, Höper D (2018) A versatile sample processing workflow for metagenomic pathogen detection. Scientific Reports DOI:10.1038/s41598-018-31496-1

Before getting started

- Enter and save the following temperature profile for **end-repair** in the thermocycler

Temperature	Time
25°C	20 min
70°C	10 min
8°C	∞

- Enter and save the following temperature profile for **adapter ligation** in the thermocycler

Temperature	Time
25°C	10 min
72°C	5 min
8°C	∞

- Thaw, mix, and shortly centrifuge both end repair and ligation buffer, adapters and dNTPs and keep them on ice.
- Keep DNA samples on MPC some minutes before pipetting to avoid transfer of residual AMPure beads.

Procedure

A. End-repair

Master mix box

1. Pipette 2.5 µl of End-Repair Buffer in one 0.2 ml PCR tube per DNA sample

2. Add 2 µl End-Repair Enzyme Mix and keep the PCR tube on ice

Template box

3. Add 20.5 µl DNA sample to buffer and enzyme mix for a final total volume of 25 µl

4. Briefly vortex and centrifuge

5. Incubate in the thermocycler with pre-set temperature profile **end-repair**

6. Briefly before the incubation is finished, start preparing the master mix for the adapter ligation (see step 8, below)

7. Before proceeding to the master mix box, change gloves

B. Apapter ligation

Master mix box

8. Prepare master mix for the adapter ligation:

Components	1 x		x	
RNase-free water	8.0	µl		µl
Ligation Buffer (2x)	40.0	µl		µl
dNTP Mix (10 mM)	1.0	µl		µl
Universal adapter P1 (to 0.5 µM final conc.)	1.0	µl		µl
Ligation and Nick Repair Mix	4.0	µl		µl
Total volume	**54.0**	**µl**		**µl**

9. Briefly vortex and centrifuge

10. Pipette 54 µl of master mix in one 0.2 ml PCR tube per DNA sample

11. Add 1 µl of the chosen barcode adapter to each master mix aliquot; *open only one barcode adapter tube at a time and change gloves between pipetting the different adapters to avoid cross-contamination*

Template box

12. Add 25 µl end-repaired DNA (from part A End-repair, above), briefly mix and centrifuge

13. Incubate in the thermocycler with pre-set temperature profile **adapter ligation**

Continue directly with purification of the library (*SOP_090302_Purification_IonTorrent-Library_FLI*).

Standard Operating Procedure

Purification and concentration of the end-repaired and adapter-ligated Ion Torrent Library using Agencourt AMPure XP beads

29th Aug 2018

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SOP-Version: 3

Attachments: none

Introduction

The end-repaired and adapter-ligated library has to be purified to remove excess of polyethylene glycol (PEG), salts, and enzymes. Otherwise, the high concentration of PEG in the ligation mix will interfere with proper size selection (see *SOP_100102_ SizeSelection_Library_FLI*). To this end, Agencourt AMPure XP beads are used. This system utilizes solid-phase paramagnetic bead technology for purification and concentration of DNA. DNA bound to magnetic beads will be separated from contaminants and washed with ethanol. Afterwards, the bound DNA will be eluted in an appropriate amount of Nuclease-free water. Concentrated and purified cDNA can directly be used for size selection (*SOP_100102_SizeSelection_Library_FLI*).

Sample Material

End-repaired and adapter-ligated libraries prepared according to *SOP_090202_Library-prep_IonTorrent_FLI*.

Equipment and Reagents

Successful working with DNA demands a Nuclease-free environment, therefore only Nuclease-free tubes, aerosol-free pipette tips, and only DEPC-treated water must be used. Gloves have to be worn throughout the procedure and changed regularly in order to avoid Nuclease contamination.
In general, only use reagents that do not exceed the expiration date. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the manufacturer.

Equipment

- PCR workstations (template box)
- Magnetic particle concentrator (MPC; e.g. Life Technologies cat. no. 12321D)
- Shaker for 1.5 ml tubes
- Vortexer
- Pipettes

Reagents and consumables

- Agencourt AMPure XP beads (Beckman Coulter, cat. no. A63881)
- Un-denatured ethanol (80% (v/v), RNase-free
- Nuclease-free water (DEPC-treated)
- 1.5 ml tubes (sterile, Nuclease-free, low-bind)
- Disposable gloves
- Sterile, Nuclease-free pipet tips (with aerosol barriers to prevent cross contamination)

General remarks

- Aliquot the AMPure XP beads in order to prevent repeated warming and cooling as this will degrade the buffer and render the beads non-functional.
- When opening a new lot of AMPure XP beads, perform a bead calibration before first use (LOP_AMPureBead-calibration_FLI_v1).

- Make sure the Agencourt AMPure XP beads are at room temperature and are thoroughly mixed. The reagent (magnetic particle solution) should appear homogenous and consistent in colour.
- Do not freeze.

Literature

- DeAngelis MM, Wang DG, Hawkins TL. 1995. Solid-phase reversible immobilization for the isolation of PCR products. Nucleic Acids Res 23:4742-4743.
- AGENCOURT® AMPURE® XP PCR PURIFICATION (Beckman Coulter, protocol no. 000387v001)
- Wylezich C, Papa A, Beer M, Höper D (2018) A versatile sample processing workflow for metagenomic pathogen detection. Scientific Reports DOI:10.1038/s41598-018-31496-1

Before getting started

Allow an aliquot of the magnetic particle solution to come to room temperature before use. Resuspend via vortexing until it appears homogenous and consistent in colour.

Procedure

Template box

1. Pipette 144 µl (1.8 volumes) of Agencourt AMPure XP beads to 80 µl of end-repaired and adapter-ligated DNA library in a low-bind tube and mix the DNA-bead-solution thoroughly by pipette mixing (15x)

2. Incubate 7 min at room temperature (RT) on a shaker (550 rpm)

3. Place the reaction tube onto the MPC for 5 min to separate the beads from supernatant (until the beads have concentrated on the tube wall)

4. Leaving the tube in the MPC, slowly aspirate the supernatant from the tube without disturbing the beads and discard the solution

5. Dispense 500 µl 80% ethanol to reaction tube and incubate 30 s at RT

6. Leaving the tube in the MPC, slowly aspirate the ethanol from the tube without disturbing the beads and discard the solution

7. Repeat step 5-6 twice while the reaction tube keeps in the MPC

8. Air-dry the pellet for 10 min with the cap open (keeping on MPC); do not over-dry!

9. Take reaction tube from MPC, elute DNA with 27 µl Nuclease-free water by careful pipette mixing and incubate for 2 min

10. Place the reaction tube onto the MPC, incubate for 2 min and pipet about 25 µl of the concentrated DNA solution into a clean low-bind tube

11. Repeat step 9-10 to increase the yield of library (final volume 50 µl)

Continue directly with size selection (*SOP_100102_SizeSelection_Library_FLI*).

FLI *SOP_100103_SizeSelection_Library_FLI*	**Size selection of Library (magnetic beads)**	

Standard Operating Procedure

Size selection of end-repaired and adapter-ligated library using Agencourt AMPure XP beads

29th Aug 2018

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SOP-Version: 3

Attachments: none

Introduction

After DNA fragmentation, the obtained fragments are of different sizes. For optimal sequencing results, the fragment size should be within the specified range of the used sequencing platform and sequencing protocols. For current PGM or MiSeq sequencing protocols, a peak size of 550 bp with a size range of 300 – 1000 bp is recommended. Fragments out of this range can be removed using Agencourt AMPure XP beads applying solid-phase paramagnetic bead technology. Due to the high concentration of polyethylene glycol (PEG) and NaCl, DNA binds reversibly to the bead surface. By adjusting the PEG concentration, the size of fragments binding to the beads can be controlled. Unbound DNA will be separated from DNA fragments of the desired size and be discarded by washing with ethanol. After the washing steps, the bound DNA is eluted in an appropriate amount of Nuclease-free water. Size-selected libraries have to be quality checked and quantified (*SOP_110102_LibQual-check_HS-chip_FLI*, *SOP_120102_LibQuant_Kapa_FLI*).

Sample Material

End-repaired and adapter-ligated libraries prepared according to *SOP_090202_Library-prep_IonTorrent_FLI* and purified according to *SOP_090302_Purification_IonTorrent-Library_FLI* or prepared according to *SOP_090102_Library-prep_automated_Illumina MiSeq_FLI*.

Equipment and Reagents

Successful working with DNA demands a Nuclease-free environment, therefore only Nuclease-free tubes, aerosol-free pipette tips, and only DEPC-treated water must be used. Gloves have to be worn throughout the procedure and changed regularly in order to avoid Nuclease contamination.
In general, only use reagents that do not exceed the expiration date. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the manufacturer.

Equipment

- PCR workstations (template box)
- Magnetic particle concentrator (MPC; e.g. Life Technologies cat. no. 12321D)
- Shaker for 1.5 ml tubes
- Vortexer
- Pipettes

Reagents and Consumables

- Agencourt AMPure XP beads (Beckman Coulter, cat. no. A63881)
- Un-denatured ethanol 80% (v/v), RNase-free
- Nuclease-free water (DEPC-treated)
- Buffer EB (QIAGEN, cat. no. 19086)
- 1.5 ml tubes (sterile, Nuclease-free, low-bind)
- Disposable gloves
- Sterile, Nuclease-free pipet tips (with aerosol barriers to prevent cross contamination)

General remarks

- Aliquot the AMPure XP beads in order to prevent repeated warming and cooling as this will degrade the buffer and render the beads non-functional.
- When opening a new lot of AMPure XP beads, perform a bead calibration before first use (*SOP_000102_AMPureBead-calibration_FLI*).
- Make sure the Agencourt AMPure XP beads are at room temperature and are thoroughly mixed. The reagent (magnetic particle solution) should appear homogenous and consistent in colour.
- Do not freeze.

Literature

- DeAngelis MM, Wang DG, Hawkins TL. 1995. Solid-phase reversible immobilization for the isolation of PCR products. Nucleic Acids Res 23:4742-4743.
- AGENCOURT® AMPURE® XP PCR PURIFICATION (Beckman Coulter, protocol no. 000387v001)
- GS FLX Titanium General Library Preparation Method Manual (USM-00048.B, April 2009)
- GS FLX System Short Fragment Removal Procedure (Technical Bulletin TCB No. 2011-002, February 2011)
- TruSeq® DNA PCR-Free Library Prep - Reference Guide (Part # 15036187 Rev. D, June 2015)
- Wylezich C, Papa A, Beer M, Höper D (2018) A versatile sample processing workflow for metagenomic pathogen detection. Scientific Reports DOI:10.1038/s41598-018-31496-1

Before getting started

- Allow an aliquot of the magnetic particle solution to come to room temperature before use. Resuspend via vortexing until it appears homogenous and consistent in colour.
- For the upper size selection, dilute Agencourt AMPure XP beads per the table:

	1 x		x
AMPure XP beads	104.0	µl	µl
Water (PCR grade)	80.0	µl	µl
Sum	**184.0**	**µl**	**µl**

Procedure

Template box

1. Fill the generated library up to 100 µl with Nuclease-free water (low-bind tube)

2. Add 160 µl **diluted** Agencourt AMPure XP beads (see Table above) per DNA library to remove large fragments and mix the DNA-bead-solution thoroughly by pipette mixing (15x)

3. Incubate 7 min at room temperature (RT) on a shaker (550 rpm)

4. Place the reaction tube onto the MPC for 5 min to separate the beads from supernatant (until the beads have concentrated on the tube wall)

5. Slowly aspirate the cleared solution from the reaction tube without disturbing the beads; pipette into a clean 1.5 ml low-bind tube in two steps á 125 µl

6. Add 30 µl **undiluted** Agencourt AMPure XP beads per 250 µl supernatant from step 5 to remove small fragments and mix the DNA-bead-solution thoroughly by pipette mixing (15x)

7. Incubate 7 min at room temperature (RT) on a shaker (550 rpm)

8. Place the reaction tube onto the MPC for 5 min to separate the beads from supernatant (until the beads have concentrated on the tube wall)

9. Leaving the tube in the MPC, slowly aspirate the supernatant in two steps á 138 µl from the tube without disturbing the beads and discard the solution

10. Leaving the tube in the MPC, dispense 200 µl 80% ethanol to reaction tube and incubate 30 s at RT

11. Slowly aspirate the ethanol from the reaction tube and discard

12. Repeat step 10-11 while the reaction tube keeps on MPC

13. Air-dry the pellet for 3-5 min with the cap open (keeping on MPC); do not over-dry!

14. Take reaction tube from MPC, elute DNA with 17.5 µl Buffer EB by careful pipette mixing and incubate for 2 min

15. Place the reaction tube onto the MPC, incubate for 2 min and pipet 15 µl of the DNA solution into a clean safe-lock tube

16. Repeat step 14-15 to increase the yield of library

Store the completed library at 4°C or continue directly with quality check (*SOP_110102_ LibQual-check_HS-chip_FLI*)

Standard Operating Procedure

Quality check of Library using Agilent High Sensitivity DNA Kit

11th Sept 2017

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SOP-Version: 2

Attachments: none

Introduction

High quality sequencing libraries are essential for successful sequencing. Therefore, the size distribution, especially the absence of too short or too long fragments and of primer dimers, must be ensured. The High Sensitivity DNA assay is suitable for this analysis. The dye labelled nucleic acids are separated electrophoretically based on their size in a microfluidic system. Internal standards ensure assay accuracy and reproducibility. Quality checked libraries can be accurately quantified according to *SOP_120102_LibQuant_Kapa_FLI*.

Sample Material

Sequencing library generated and size selected according to *SOP_100102_SizeSelection_Library_FLI*.

Equipment and Reagents

Successful working with DNA demands a Nuclease-free environment, therefore only Nuclease-free tubes, aerosol-free pipette tips, and only DEPC-treated water must be used. Gloves have to be worn throughout the procedure and changed regularly in order to avoid Nuclease contamination.
In general, only use reagents that do not exceed the expiration date. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs) for DMSO, available from the manufacturer.

Equipment

- Agilent 2100 Bioanalyzer
- Chip Priming Station (Agilent, cat. no. 5065-4401, supplied with Bioanalyzer)
- IKA Vortexer Model MS3 with chip adapter (supplied with Bioanalyzer)
- Vortexer, Microcentrifuge
- Pipettes

Reagents and consumables

- High Sensitivity DNA Kit (Agilent, cat. no. 5067-4626)
- Nuclease-free water (DEPC-treated)
- 1.5 ml tubes (sterile, Nuclease-free, low bind)
- Disposable gloves
- Sterile, Nuclease-free pipet tips (with aerosol barriers to prevent cross contamination)

General remarks

- While not in use, keep all reagents at 4°C and protect from light
- The DNA sample should be between 5 – 500 pg/µl for accurate determination

Literature

- Agilent High Sensitivity DNA Kit Quick Start Guide (Part no. G2938-90320, 05/2009)

Before getting started

- Prepare the gel-dye mix:
  - Equilibrate the HS DNA dye concentrate and one vial of the HS DNA gel matrix to room temperature (at least 30 min; protect from light!)
  - Add 15 µl HS DNA dye concentrate to the HS DNA gel matrix vial
  - Vortex, briefly centrifuge, and transfer solution to a spin filter
  - Centrifuge at 2240 × *g* for 10 min
- Equilibrate reagents and gel-dye mixes to room temperature 30 min before use; protect from light!
- Dilute DNA samples to 5 – 500 pg/µl if necessary
- Set up the chip priming station:
  - Screw on the syringe to the lid of the chip priming station
  - Adjust the syringe clip to the lowest position
  - Ensure that the base plate is inserted in position C

Procedure

1. Put a new HS chip on the chip priming station

2. Pipette 9 µl of gel-dye mix in the well marked **G**

3. Ensure that the plunger is positioned at 1 ml, close the chip priming station and press the plunger until it is held by the clip

4. After exactly 60 sec release the clip

5. After further 5 sec slowly pull back the plunger to the 1 ml position

6. Open the chip priming station; pipette 9 µl of gel-dye mix in wells marked **G**

7. Pipette 5 µl of marker in all sample wells (1-11) and the well marked "**ladder**"

8. Pipette 1 µl of HS DNA ladder in the well marked "**ladder**"

9. Load all sample wells: pipette 1 µl of sample in each used sample well (1-11); pipette 1 µl of marker in each sample well not used for a sample

10. Vortex the chip with the IKA Vortexer MS3 for 1 min at 2400 rpm (as indicated)

11. Within 5 min, run the chip in the Agilent 2100 Bioanalyzer using the assay "High Sensitivity DNA.xsy" for dsDNA

12. After running the chip, apply a cleaning chip filled with 350 µl of fresh Nuclease-free water for about 30 sec to clean electrodes; let the electrodes air dry with the lid open

In case the size distribution is out of the suitable range, repeat the size selection procedure according to *SOP_100102_SizeSelection_Library_FLI*. Otherwise, continue directly with quantification of the library (*SOP_120102_LibQuant_Kapa_FLI*).