# RABIES SEQUENCING PROTOCOL: SAMPLE TO SEQUENCE

Version 1.0

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# SAMPLE PREPARATION

*Reference: Zymo kit protocol*

Homogenised samples stored in DNA/RNA shield

- Bring samples to room temperature
- Transfer 350ul of homogenised sample to a new 2ml screw cap tube
- Add 350ul of RNA Lysis Buffer (1:1) and mix well.

Samples stored in RNA later/glycerol-saline

- Remove the tissue* from RNA later/glycerol, dab excess on filter paper
- Add tissue to 1ml DNA/RNA shield and a measure of 1.5mm ceramic beads in a 2ml reinforced tube
- Use Terralyzer to homogenise sample
- Transfer 350ul of homogenised sample to a new 2ml screw cap tube
- Add 350ul of RNA Lysis Buffer (1:1) and mix well.

*If the sample has liquefied:

- Transfer 200ul of liquid to a new 2ml screw cap tube
- Add 200ul of RNase-free water or PBS to the sample (1:1). Then add 4 volumes RNA Lysis Buffer (4:1) and mix. Proceed to step 5

# RNA EXTRACTION AND PURIFICATION

*Reference: Zymo kit protocol*

All centrifugation steps should be performed at 10,000 – 16,000 x g for 30 seconds unless specified.

1. Transfer the sample lysed in RNA Lysis Buffer into a Spin-Away™ Filter[1] (yellow) in a Collection Tube and centrifuge to remove the majority of gDNA. Save the flow-through.

   [1] To process samples >700 µl, Zymo-Spin™ columns may be reloaded

2. Add 1/2 volume ethanol (95-100%) to the sample flow-through and mix well.

3. Transfer the mixture to a Zymo-Spin™ IIICG Column[1] (green) in a Collection Tube and centrifuge. Discard the flow-through.

4. DNase I treatment (in-column)[2]

   [2] Prior to use, reconstitute the lyophilized DNase I as indicated on the vial. Store frozen aliquots.

   - (D1) Wash the column with 400 µl RNA Wash Buffer and centrifuge. Discard the flow-through.
   - (D2) In an RNase-free tube, add 5 µl DNase I to 75 µl DNA Digestion Buffer and mix. Add the mix directly to the column matrix (take care not to pierce filter with tip).
   - (D3) Incubate the column at room temperature (20-30oC) for 15 minutes. Proceed to step 5.

   [2] Prior to use, reconstitute the lyophilized DNase I as indicated on the vial. Store frozen aliquots.

5. Add 400 µl RNA Prep Buffer to the column and centrifuge. Discard the flow- through.

6. Add 700 µl RNA Wash Buffer to the column and centrifuge. Discard the flow- through.

7. Add 400 µl RNA Wash Buffer and centrifuge the column for 2 minutes to ensure complete removal of the wash buffer. Transfer the column carefully into an lobind 1.5ml tube.

8. Add 50 µl DNase/RNase-Free Water directly to the column matrix and centrifuge.

9. Quantify the RNA using Qubit RNA kit. The eluted RNA can be used immediately or stored at ≤-70oC.

# DNA PREPARATION

**Important**

You must have AT LEAST ONE negative control in EVERY library. Ideally this should be an extraction/cDNA/PCR control taken right through to sequencing.

## Step 1: cDNA synthesis

**NOTE ON HOOD PREPARATION:**

To prevent cross contamination of both the sample and other reagents, this should be carried out in the MASTERMIX HOOD, which is pre-sterilised with UV and treated with MediPal wipes, DNAway and RNAseZap reagent. Wipe down the hood with each sequentially, allowing 5 minutes for drying between each. Pipettes should also be treated in the same way, and UV treated for 30 mins between library preparations.

**General advice**

- Aliquot reagents to prevent cross-contamination and reduce potential freeze/thaw cycles
- Make mastermix solutions where possible to reduce variability between reactions.
- Always use filter tips!
- Always do a negative control (if possible a positive control too!)

**cDNA set-up**

1. In the MASTERMIX HOOD: prepare a mastermix of the reagents below according to the number of samples and controls to be prepared (plus 1 extra).

*Note*: The Lunascript reagent is quite viscous so take care pipetting, pipette slowly and pause before final purge to ensure reagent is in the tip before dispensing.

| Component | Volume (x1 reaction) |
|---|---|
| **5x LunaScript Master Mix** | 2µL |
| **Nuclease Free Water (NFW)** | 3µL |

2. Still in the MASTERMIX HOOD: aliqout 5µL of mastermix for each sample reaction into labelled 0.2ml PCR strip tubes.
3. Take the prepared tubes to the TEMPLATE HOOD. Add 5µL* of RNA to each tube. Mix gently by pipetting or gently flicking tube (do not vortex) and pulse centrifuge to collect liquid at the bottom of the tube. Run on a thermocycler with the following conditions:

| | Temperature | Time | Cycles |
|---|---|---|---|
| Primer annealing | 25°C | 2 mins | 1 |
| cDNA synthesis | 55°C | 10 mins | |
| Heat inactivation | 95°C | 1 min | |

**Can adjust volume of RNA and compensate with more water*

## Step 2: Multiplex PCR reaction

**NOTE:**
Primers need to be used at a final concentration of 0.015µM per primer in the PCR reaction. Use the table below to identify the correct volume for the primer scheme being used- for you simplicity can add the same volume of primer to each pool.

| Scheme | Geographic area | Pool 1 | Pool 2 |
|---|---|---|---|
| rabvTanzDg | East Africa | 1.8 | 1.72 *(can add 1.8)* |
| rabvSEasia | South East Asia | 1.6 | 1.5 *(can add 1.6)* |

### 2A. PCR set-up

1. Set up two PCR mastermixes in the MASTERMIX HOOD with the following components, with enough for the number of reactions + negative control + approx. 10%. In one tube, add primers for primer pool A, in the other tube, add primers for primer pool B. (cDNA should not be taken anywhere near the MASTERMIX HOOD at any stage.)

| Component | Amount (µL) |
|---|---|
| **NEB Q5® Polymerase 2X MasterMix** | 12.5 |
| **Primer Pool 1 or 2 (10µM)** | From table |
| **Water** | Up to *21* |
| ***TOTAL*** | *21* |

} x2

2. Label 0.2ml PCR strip tubes according to sample number and primer pool. It is best to use one strip for pool 1 and one strip for pool 2.
3. Add 21µL of the correct mastermix into the labelled PCR tubes in the MASTERMIX HOOD.
4. In the TEMPLATE AREA add 4µL of cDNA to each Pool1 and Pool2 reaction mix and mix well. Include the negative control from cDNA synthesis. Pulse centrifuge the tubes to remove any contents from the lid. Set up the cycling conditions as follows:

| | Temperature | Time | Cycles |
|---|---|---|---|
| Heat Activation | 98°C | 30 seconds | 1 |
| Denaturation | 98°C | 15 seconds | 35** |
| Annealing | 65°C | 300 seconds | |
| Hold | 4°C | Indefinite | 1 |

***Can be lowered if CT values are high*

**PAUSE POINT: DNA can be stored in the fridge for a few days or longer term at -20oC.**

### 2B. SPRI bead clean-up

**SPRI handling tips:**

- Vortex beads before use.
- Store beads at the correct temperature (4°C) but bring to room temperature before use

**PCR clean up method**

1. Clean-up the amplicon pools using the following protocol in the TEMPLATE AREA:
2. Prepare fresh 75% (70-80%) ethanol (bring to room temperature).
3. Ensure SPRI beads are well resuspended by thoroughly mixing prior to addition to the sample. Mixture should be a homogenous brown colour.

4. Add an equal volume of SPRI beads to the tube (1:1 ratio) and mix gently by either flicking or pipetting. There should be ~25µL of sample, so add 25µL of beads.
5. Incubate for 5 mins at room temperature (25°c), occasionally inverting or flicking tubes.
6. Gently pulse-centrifuge the tubes to make sure everything is at the bottom. Do not centrifuge too hard, or you will separate the beads to the bottom and have a clear liquid above. If this occurs, re-mix the tube, lower the centrifuge speed, and re-centrifuge at lower speed.
7. Place on magnetic rack and incubate for 2 mins or until the beads have pelleted against the magnet and the solution is completely clear. (Tip: sometimes if you are unsure if it is clear, putting a white piece of paper behind the tube, or behind a pipette tip with some solution in, helps you to see more easily).
8. Carefully remove and discard the solution, being careful not to displace the bead pellet.
9. Add 200µL of room-temperature 75% ethanol to the pellet.
10. Wait for 30secs to ensure the beads are washed properly. Optional: some people like to rotate the tube 180° whilst in the rack so beads go through the ethanol, then rotate back to starting position.
11. Carefully remove and discard ethanol, being careful not to displace the bead pellet.
12. Repeat steps 7 to 9 to wash the pellet again.
13. Carefully remove as much ethanol as possible using a 10µL tip.
14. Keep the lids open and allow the pellet to air dry, being careful not to overdry (if the pellet is cracking, then it is too dry).
15. Remove the tube from the magnet. Resuspend pellet in 30µL of nuclease-free water, and incubate at room temperature for 5 mins (this is to allow the DNA to unbind from the beads into the water).
16. Place on magnet. DO NOT DISCARD THE WATER- this has the DNA in it! Carefully transfer the water elution to a clean 1.5mL Eppendorf tube. MAKE SURE that no beads are transferred into this tube. In some cases a pulse centrifugation can be used to pellet residual beads.
17. Quantify the amplicon library using Qubit machine following the dsDNA protocol.

**PAUSE POINT: DNA can be stored in the fridge for a few days or longer term at -20oC.**

### 2C. DNA Quantification

**Qubit quantification: High sensitivity dsDNA assay**

Set up the required number of 0.5mL tubes for standards and samples. The Qubit™ 1X dsDNA HS Assay requires 2 standards.

**NOTE:** Use only thin-wall, clear, 0.5mL PCR tubes. Acceptable tubes include Qubit™ assay tubes (Cat. No. Q32856)

1. Label the tube lids. Do not label the side of the tube as this could interfere with the sample read. Label the lid of each standard tube correctly. Calibration of the Qubit™ Fluorometer requires the standards to be inserted into the instrument in the right order
2. Add 10µL of each Qubit™ standard to the appropriate tube.
3. Add 1–20µL of each user sample to the appropriate tube.
4. **NOTE:** If you are adding 1–2µL of sample, use a P-2 pipette for best results.
5. Add the Qubit™ 1X dsDNA 1X buffer to each tube such that the final volume is 200µL.
6. **NOTE:** The final volume in each tube must be 200µL. Each standard tube requires 190µL of Qubit™ working solution, and each sample tube requires anywhere from 180–199µL. Ensure that you have sufficient Qubit™ working solution to accommodate all standards and samples.

7. To avoid any cross-contamination, we recommend that you remove the total amount of working solution required for your samples and standards from the working solution bottle and then add the required volume to the appropriate tubes instead of pipetting directly from the bottle to each tube.
8. Mix each sample vigorously by vortexing for 3–5 seconds.
9. Allow all tubes to incubate at room temperature for 2 minutes, then proceed to "Read standards and samples".
10. On the Home screen of the Qubit™ 3 Fluorometer, press DNA, then select 1X dsDNA HS as the assay type. The Read standards screen is displayed. Press Read Standards to proceed.
11. **NOTE:** If you have already performed a calibration for the selected assay, the instrument prompts you to choose between reading new standards and running samples using the previous calibration. **If you want to use the previous calibration, skip to step 11**. Otherwise, continue with step 9.
12. Insert the tube containing Standard #1 into the sample chamber, close the lid, then press Read standard. When the reading is complete (~3 seconds), remove Standard #1.
13. Insert the tube containing Standard #2 into the sample chamber, close the lid, then press Read standard. When the reading is complete, remove Standard #2.
14. The instrument displays the results on the Read standard screen. For information on interpreting the calibration results, refer to the Qubit™ Fluorometer User Guide, available for download at thermofisher.com/qubit.
15. Press Run samples.
16. On the assay screen, select the sample volume and units:
17. Press the + or – buttons on the wheel, or anywhere on the wheel itself, to select the sample volume added to the assay tube (from 1–20µL).
18. From the unit dropdown menu, select the units for the output sample concentration (in this case choose ng/µL).
19. Insert a sample tube into the sample chamber, close the lid, then press Read tube. When the reading is complete (~3 seconds), remove the sample tube.
20. **The top value (in large font) is the concentration of the original sample and the bottom value is the dilution concentration**. For information on interpreting the sample results, refer to the Qubit™ Fluorometer User Guide.
21. Repeat step 14 until all samples have been read.
22. Carefully **record all results** and store run file from the Qubit on a memory stick.
23. If you're negative controls have any DNA in, some contamination has occurred and you must redo previous steps. All negative controls should ideally be 'too low' on Qubit machine, but MUST be < 1ng per ul (or less if positive samples have low DNA concentration).

### Step 3: Normalization.

The amplification of DNA products varies between pools and between samples. Therefore it is recommended that the measured concentration is used to normalise each pool of DNA before combining for each sample.
*Note*: read more about molarity and input mass in the Oxford Nanopore protocol "1d-gDNA-sqk-lsk109" protocol in ONT-protocols folder and "Normalisation-the details.doc".

We will start with 5ng per pool from each sample, so a total of 10ng per sample once pooled together.
Keep PCR products separate at this stage; add the appropriate volume of each pool from each sample from to individual 1.5-ml Eppendorf tubes and then adjust the volume in each Eppendorf to 20 µl with nuclease-free water.

# LIBRARY PREPARATION

**Before starting:**
- Label the 1.5mL LoBind tubes with the ID of the previously quantified pooled samples.
- Mix by inversion and spin down all reagent tubes before using.
- Prepare 70-80% EtOH in a falcon tube.
- Take AMPure beads out the fridge and mix by vortexing. Use at room temperature.
- Make 1ml stocks of AMPure beads and nuclease-free water to avoid contamination.

## Step 4: Barcoding and adaptor ligation: One-pot protocol.

**NOTE:**
The following steps assume that you are using the following kit versions:
- Native barcoding kit EXP-NBD104/EXP-NBD114
- Ligation sequencing kit SQK-LSK109

If older (or newer) versions of these kits are used there may be modifications to the protocol below, please refer to ONT protocols to identify differences.

**NOTE:**
This is a 'one-pot ligation' protocol for native barcoded ligation libraries, which combines end repair and barcode ligation steps, thus eliminating a clean-up step. We have seen no reduction in performance compared to standard libraries, and is made faster by using the Ultra II® ligation module which is compatible with the Ultra II® end repair/dA-tailing module removing a clean-up step.

**Important**
You must include a negative control. If this has not been prepared already as an extraction/cDNA/PCR control then you should include a water control at this stage.

### 4A. End repair and dA-tailing

1. Set up the following end-prep reaction for each biological sample (i.e. the normalised pooled sample from previous step):

*Note:* The reagents are viscous so take care when pipetting and prepare enough mastermix for the total no of samples +2.

| Component | Volume (1x) | Volume (mastermix) |
|---|---|---|
| **PCR product (10ng per sample)** | 20uL | |
| **UltraII End Prep Reaction Module** | 2.8uL | |
| **UltraII End Prep enzyme mix** | 1.2uL | |
| **TOTAL** | 24uL | |

2. Incubate at a) room temperature (~20°C) for 10 mins; and then b) incubate at 65°C for 5 mins
3. Place on ice for ~30 secs (this is just to make sure the reaction is not still at 65°C when you add the next enzyme, or you will inactivate the next enzyme immediately).

### 4B. Barcoding

4. Add the following directly to the previous reactions. Use a SINGLE barcode per biological sample and remember to write down which barcode is assigned to which sample!

| Component | Volume (1x) |
|---|---|
| **NBXX Barcode** | 2.5uL |
| **UltraII Ligation mastermix** | 27.5uL |
| **UltraII Ligation enhancer** | 1uL |
| **Sample** | 24uL |
| **TOTAL** | 55uL |

5. Incubate at a) room temperature for 20 mins; and then at b) 70°C for 5 mins to denature the ligase

### 4C. Pool barcoded samples and clean-up

6. If you have prepared 12 samples combine all the barcode ligation reactions (all the tubes) into a single 1.5ml Eppendorf tube (you can use the same tip). If you have prepared more than 12 put half in one tube and half in another.
7. For each barcode (including the negative control) in the pooled tube add 55µL SPRI beads (e.g., if you have 10 barcodes, this is 55x10= 550ul of beads). Mix gently by inversion and incubate for 10 mins.
8. Perform a SPRI clean-up:
9. Place on a magnet rack until clear.
10. Remove and dispose of supernatent.
11. Add 400ul of 80% ethanol to the tube still on the magnetic rack (or add enough to cover the beads- this will change according to how many samples you had but could be more e.g. ~1000ul),
12. Incubate 30 secs.
13. Remove solution.
14. Repeat last three steps (5-7).
15. Pulse spin, remove residual 80% ethanol and air dry for 1 min.
16. Resuspend in 45µL nuclease-free water.
17. Incubate off the magnetic rack at 37oC for 10 mins.
18. Replace on magnetic rack. Leave to settle for ~2 mins at room temperature and then carefully remove solution and transfer to a clean 1.5mL Eppendorf tube.
19. Remove 1µL and assess concentration by Qubit as described in previous section.

**PAUSE POINT:** As long as you have not yet ligated the sequencing adapter, the library can be stored at 4 °C and continue with the prep at a later point. It is better to store at 4°C, as freezing and thawing can introduce nicks or breaks in the DNA. Several days to weeks in the fridge are possible. For longer-term storage, the library can be placed at -20 °C, though unnecessary freeze-thaw cycles should be avoided for best results.

## Step 5: Adapter ligation

Note: Ethanol must not be used in the following steps so hide it away to prevent accidental use!

1. Set up the following adapter ligation reaction:

| Component | Volume uL (1x) |
|---|---|
| **Cleaned-up barcoded fragment (from previous step)** | 44 |
| **AMII Adaptor** | 5 |
| **UltraII Ligation mastermix** | 50 |
| **UltraII Ligation enhancer** | 1 |
| **TOTAL** | 100 |

2. Incubate at room temperature for 20mins.

**Note: The following clean-up steps are different from previous clean-ups. Do not use ethanol!**

3. Add 100µL SPRI beads (a 1:1 ratio)
4. Incubate for 10 mins, mix be inversion to promote binding of the library to the beads
5. Spin down and place on a magnetic rack until clear
6. Remove supernatant
7. Wash the beads by adding 250µL SFB and resuspend fully by flicking. (Optional:Incubate for 1min.)
8. Place on magnetic rack until clear
9. Remove supernatant
10. Repeat SFB wash (Optional: do a 3$^{rd}$ wash if you are concerned about unligated adapter in the library)
11. Spin down and remove residual SFB
12. Add 13µL EB and resuspend by flicking or pipetting, make sure all beads have been eluted from the wall tube (use a pipette to scrap down from the tube walls if necessary)
13. Incubate at 37°C for 10 mins
14. Spin down, then place on magnetic rack and let beads pellet.
15. Carefully transfer eluate to a clean 1.5mL Eppendorf tube.
16. Quantify 1 ul of your eluted library using the Qubit.

**Note:** With the new R9.4.1 you only need 10-20 ng to sequence. So after quantifying, dilute the library to 10-20 ng in 12 ul of EB.

**PAUSE POINT: For best results it is best to proceed immediately to sequencing but if necessary the library can be stored at -20°C.**

## Step 6: Priming and loading the SpotON flow cell

1. Thaw the following:
   For library:
   • Sequencing Buffer (**SQB**) at RT, then place on ice
   • Library loading beads (**LLB**) at RT, then place on ice
   For priming mix:
   • Flush Tether (**FLT**) at RT, then place on ice.
   • **FLB** (flush buffer) at RT, then place on ice. Thoroughly mix the contents of the **RBF** and **LLB** tubes by pipetting.
2. Mix the Sequencing Buffer (**SQB**) and Flush Buffer (**FLB**) tubes by vortexing, spin down and return to ice.
3. Spin down the Flush Tether (**FLT**) tube, mix by pipetting, and return to ice.
4. Priming mix: add 30 µl of thawed and mixed Flush Tether (**FLT**) directly to the tube of thawed and mixed Flush Buffer (**FLB**), and mix by pipetting up and down.
5. Flip back the MinION lid and slide the priming port cover clockwise so that the priming port is visible.

**IMPORTANT:** Care must be taken when drawing back buffer from the flow cell. The array of pores must be covered by buffer at all times. Removing more than 20-30µL risks damaging the pores in the array.

6. After opening the priming port, check for small bubble under the cover. Draw back a small volume to remove any bubble (a few µLs):
7. Set a P1000 pipette to 200µL
8. Insert the tip into the priming port
9. Turn the wheel until the dial shows 220-230µL, or until you can see a small volume of buffer entering the pipette tip.
10. Load 800µL of the priming mix into the flow cell via the priming port, using the dial-down method described in step 9, avoiding the introduction of air bubbles.
11. Wait for 5 minutes.
12. In a new tube prepare the library dilution for sequencing (vortex **SQB** before use):
13. Note: the Loading Beads (**LB**) tube contains a suspension of beads. These beads settle very quickly, so it is vital that they are mixed immediately before use.

| Component | Volume |
|---|---|
| **SQB (vortex first)** | 37.5µL |
| **LB (mix with pipette first)** | 25.5µL |
| **DNA Library (10-20ng)** | 12µL |
| **Total** | 75µL |

14. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
15. Load 200µL of the priming mix into the flow cell via the priming port (**NOT** the SpotON sample port), avoiding the introduction of air bubbles.
16. Mix the prepared library gently by pipetting up and down just prior to loading.
17. Add 75µL of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.
18. Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid.
19. Double–click the MinKNOW icon located on the desktop to open the MinKNOW GUI.
20. If your MinION was disconnected from the computer, plug it back in.
21. MinKNOW should automatically detect the flowcell, if not select the correct flowcell from the dropdown menu.
22. Check the flow cell as Selected.
23. Click the New Experiment button at the bottom left of the GUI.

24. On the New experiment popup screen, select the running parameters for your experiment from the individual tabs:
    - Name your sequencing run. It is useful to include the location of the sequencing run (e.g. UNITID) and flowcell type (e.g. R941) in the title. Do not include spaces in the name, use an underslash _ if you want to space words.
    - Turn off live basecalling
    - Output settings - FAST5 : The number of files that MinKNOW will write to a single folder. By default this is set to 4000
25. Click Begin Experiment.
26. The MinKNOW Experiment page will indicate the progression of the run; this can be accessed through the Experiment tab that will appear at the top right of the screen
27. Monitor messages in the Message panel in the MinKNOW GUI

Note: The starting voltage can vary depending on how long you have previously run that flow cell for. Approximate run voltages are: for the first 10 hours -180 v, after 10 hours -190 v, after 200v. An 'hours_voltage_1' file and 'hours_voltage_2' in the Dropbox folder will give you more precise guidance.

## Step 7: Washing the flowcell

### The normal way

1. After sequencing, set up the following digestion buffer, mixing between each addition.

| Component | Amount (µl) |
|---|---|
| **Running buffer (RBF)** | 100 |
| **Nuclease free water** | 100 |
| **CaCl2 (0.1M – dilute 1M stock 1/10)** | 4 |
| **DNase I** | 4 |
| **Total volume** | 208 |

2. Mix gently by pipetting and spin down.
3. Remove the waste from the flow cell (priming port and spoton port closed).
4. Open priming port and remove the air bubble. **Extremely slowly and carefully** (best to do this using the turn-dial on pipette) add 200ul of diluted RBF (ratio 576 **RBF** + 672 NFW) to the port, then 150 ul of the digestion buffer to the port as well. It is extremely important not to introduce or push any air bubbles into the flow cell.
5. Start a new sequencing run to check that it is digesting. Wait 30-60 min or until no more reads are being produced and the number of in strand pores is zero.

Note: you can keep the flow cell in the fridge or RT overnight

6. Before loading the new library, start a new experiment to make sure there is no remnant of the previously library. Check the reads and the active pores (usually you can see a high active pores number, but check if these are just the adapters).
7. Prime the flow cell and load the new library as normal.

### The "nuclease bomb"

This version of the nuclease flush mixture uses a high concentration DNase I (Invitrogen dnase 20,000 unit kit (260U/ul)). It will work pretty much immediately but you should wait ~10mins to ensure no new reads are produced.

| Component | Amount (µl) |
|---|---|
| **Running buffer (RBF)** | 100 |
| **Nuclease free water** | 106 |
| **CaCl2 (0.1M)** | 1 |
| **Invitrogen DNase I** | 1 |
| **Total volume** | 208 |