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# Protocol for micro-sheet pocket 1: Detailed operation manual of six requirements

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This protocol details micro-sheet pocket 1 and detailed operation manual of six requirements.

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protocol

Iwaya K, Arai H, Takatou N, Morita Y, Ozeki R, Nakaoka H, Sakamoto M, Kouno T, Soma M (2022) A sheet pocket to prevent cross-contamination of formalin-fixed paraffin-embedded block for application in next generation sequencing. PLoS ONE 17(5): e0266947. doi: [10.1371/journal.pone.0266947](https://doi.org/10.1371/journal.pone.0266947)

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- Test environment: A horizontal worktable in a laboratory in accordance with JIS 8703-1983, ISO 554-1976 (temperature 23°C, relative humidity 50%)

#### Water permeability test:

##### Equipment / Consumables

- 1 x  50 mL plastic measuring pipette
- P1000 Pipette Tips (without filter) (Gilson) 1 pc
- P200 Pipette Tips (without filter) (Gilson) 2 pcs
- Container 1 (at least 8-10 cm in diameter and 15 cm deep, made of a material that can be easily pierced) for example, 1 L-PET bottle.
- Container 2 (at least 15 cm in diameter and at least 5 cm in depth)
- Burette stand
- Parafilm
- stopwatch (measures up to 2 decimal places)
- funnel
-  500 mL beaker
- Scissors and/or cutter
- plastic cutter
- drill

##### Solution :





 500 mL distilled water

Test sheets (cut with scissors and/or cutter to 3cm x 3cm square).

#### Cell transit test:

##### Equipment / Consumables

- P1000 Pipette Tips (without filter) (Gilson) 1 pc
- P100 Pipette (Gilson) 2 pcs
- P100 Pipette

- P20 Pipette
- P20 Pipette Tips
-  **2 mL** micro tube
-  **1.5 mL** /  **2 mL** microtube rack
- microscope slide
- alcohol-resistant marking pen
- optical microscope (400x)
- aspirator
-  **50 mL** plastic centrifuge tube
- cell counterplate (Watson, Neubauer Improved)
- Parafilm
- Scissors


#### Solution/Reagent:

- 10% neutral buffered formalin solution
- Residual specimen (ascites or pleural effusion) or cultured cells ( $1 \times 10^6$ - $10^7$  cells)
- PBS 70mL
- 0.4% Trypan blue solution
- 95% Ethanol
- Papanicolaou staining solution

### Average diameter of filter opening

1 Examine the average diameter of filter opening by referring to the catalog of the sheet.






2 

Observe under a polarized light microscope for sheets with a diameter of filter opening of less than  **10  $\mu$ m** .




3 

Confirm opening size by using scanning electron microscopy (SEM), if possible.

## Thickness

- 4 Examine the thickness by referring to the catalog of the sheet.
- 5 Prepare 10 test sheets (cut with scissors to 20 cm x 20 cm square ).
- 6 Put 10 test sheets on a horizontal worktable.
- 7 Lay a glass plate (200 ± 2 mm square, a mass of 82 ± 2 g, a thickness of 0.7 mm ) on the test sheets.
- 8 After about 00:00:10 , measure the test pieces to 0.1 mm using a vertical ruler. 10s

## Chemical resistance

- 9 Cut in half the sheet in order to compare each other.
- 10 Immerse the whole part of one cut sheet in the test solution (xylene).
- 11 Cover the sheet with a lid and kept at  Room temperature for about 08:00:00 . 8h
- 12   
Wash with water, dried, and observe both samples.
- 13 If no obvious change is observed, repeat the procedure 9-12 by changing the test solution (formalin, methanol).

## Water permeability test

### 14 Make-up for water permeability test device

#### 14.1 Create a water storage tank

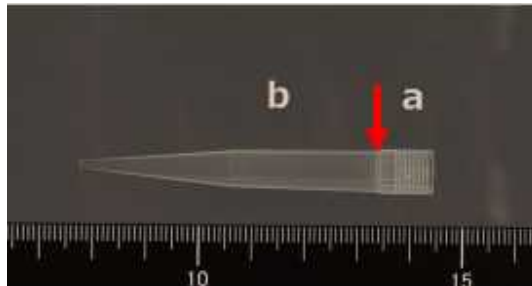
Cut off the suction opening of the **50 mL** disposable measuring pipette with a plastic cutter.

#### 14.2 Create drainage tank 1

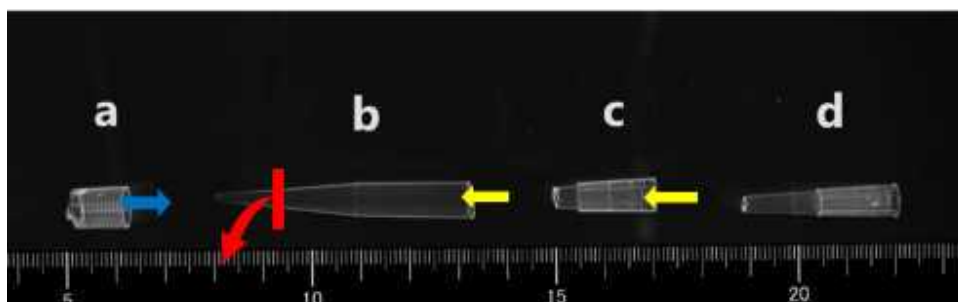
Drill a hole to make an overflow port at a height of about **12 cm** from the bottom of the container 1 (inner diameter of hole, about **15 mm**).

### 15 Create a sample reservoir

#### 15.1 Cut the attachment part (a) of the tip of the P1000 pipette with scissors from the tip body (b) indicated by red arrow.



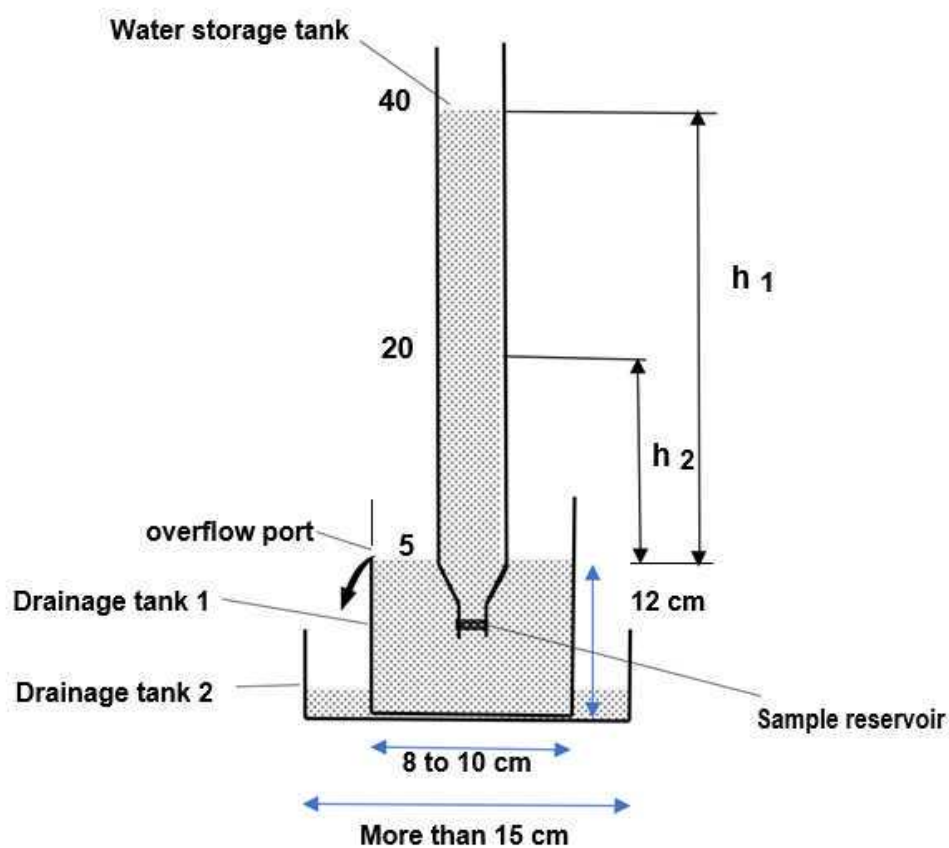
#### 15.2 For one sheet, cut off at **2 cm** from the tip of (b). In the case of two or more sheets, cut off **1.5 cm** from the tip. (Red line).



- 15.3 Measure the inner diameter (cm) of the cut side of (b) and calculate the cross-sectional area (inner diameter x inner diameter x 3.14) (Cross-sectional area of test sheet).
- 15.4 Cut a 2 cm area from the opening of the tip of the P200 pipette with scissors (c).
- 15.5 Cut a 3 cm area from the opening of the other P200 pipette tip with scissors (d).
- 15.6 Connect (b) Connect (c) and (d) (yellow arrow).
- 15.7 Cover (b) with the test sheet (3cm×3cm) on the red line.
- 15.8 Fix the sheet by fitting (a) (blue arrow).
- 15.9 Cut the sheet that protrudes from (a).
- 15.10 Seal and fix the connected parts with Parafilm.





Complete view of the sample reservoir





Assembly diagram of permeability test device

## 16 Vertical installation of a water storage tank on the workbench using a Burette stand.

- 17 Assemble water storage tank, drainage tank 1, drainage tank 2, according to the reference above diagram.
- 18 Adjust the height of the overflow opening to "  5 mL " on the scale of a  50 mL disposable measuring pipette.
- 19 Attach the sample reservoir to the end of the reservoir.
- 20 Wrap the parafilm to prevent liquid leakage.

## 21 Operation Procedure

- 21.1 Fill drainage tank 1 with water until it flows out of the overflow port.
- 21.2 Fill water storage tank with water by using a funnel.
- 21.3 Measure transit time ( $t_2-t_1$  value) it takes for the water in a water storage tank to pass from the "  40 mL " scale to the "  20 mL " scale using a stopwatch.
- 21.4 Repeat the operation in steps 21.1 and 21.2 and confirm that the  $t_2-t_1$  value was almost constant.
- 21.5 Measure  $t_2-t_1$  value 5 times.

## 22 Calculation




The permeability coefficient was calculated by the following formula, applying JIS A 1218:2020.


$$k = 2.303 \frac{aL}{A(t_2 - t_1)} \log_{10} \frac{h_1}{h_2}$$

$\kappa$ :	permeability coefficient	cm/s		
a:	cross-section area of storage tank	cm <sup>2</sup>		
L:	thickness of test sheet	cm		
A:	cross-section area of test sheet	cm <sup>2</sup>		
$t_2 - t_1$ :	measurement time	s		
$h_1$ :	water level difference at time t1	cm		
$h_2$ :	water level difference at time t2	cm		

### Water retention test

23 Prepare 3 sheets cut in 100 mm x 100 mm sized square.

24 Measure the mass of a cut sheet in the standard condition to  1 mg -level.

25 Immerse 3 sheets in the water for at least  00:15:00 .

15m

26 Remove 3 sheets from the water with tweezers.

27 Allow the water to drip off for at least  00:01:00

1m

28 Measure the mass of a cut sheet after wetting and dripping off the water (m2).

29 Calculate the water retention rate by the following formula

$$m=(m_2-m_1)/m_1 \times 100$$

m: water retention rate (%)

m<sub>1</sub>: mass of the specimen in the standard condition (mg)

m<sub>2</sub>: mass of the specimen after wetting and dripping off the water (mg)


- 30 Calculate the average value in accordance with JIS A 5209:2014, Rule B (rounding method).

#### Cell transit test

20m 5s

#### 31 Preparation of sample reservoir

Preparation of sample reservoir (consisting of part (a) and (b), we named “filter assembly”) is explained in step 15.1

- 32 Set sample reservoir onto  2 mL microtube.



### 33 Preparation of cell suspension (collection of cells from ascites and/or pleural fluid)

33.1



5m

Centrifugation residual specimens (ascites and/or pleural effusion) at **2000 rpm** for **00:05:00**.

33.2

Gently remove the supernatant with an aspirator.

33.3

Collect more than **1 mL** of cell pellet.

33.4



Add **30 mL** to **35 mL** of PBS and mix by inverting.

33.5

Stand still for **00:15:00**.

15m

33.6

Gently remove the supernatant with an aspirator.

33.7



Repeat PBS wash (step 32.4 - 32.6).

33.8



5s

Counts all cells in a cell suspension

1. Add **90 µL** of 0.4% trypan blue solution to **10 µL** of cell suspension (10-fold dilution).
2. Gentle vortex for **00:00:02** - **00:00:03**.
3. Inject **6 µL** samples with a P20 pipette through the sample inlet of the cell counter plate.

4. Count the number of cells in 4 compartments using the cell counter plate.
5. Calculate the number of cells per **1  $\mu\text{L}$**  of stock solution {(average number of cells of 4 compartments)  $\times 10 \times$  (dilution factor)}.

Note : The count of one plot should be about 100.

If the number of cells is large, dilute the cell suspension 10 to 100 times with PBS. If the number of cells is small, reduce the amount of 0.4% trypan blue solution added to the cell suspension **10  $\mu\text{L}$** .

34 Adjust to  $1 \times 10^6$ - $10^7$  cells/ml in 10% neutral buffered formalin solution.

## 35 Operation Procedure

### 35.1

Apply **600  $\mu\text{L}$**  of cell suspension ( $1 \times 10^6$ - $10^7$  cells/ml) to the sample reservoir (filter assembly) with a P1000 pipette.

### 35.2 Allow to natural filtration.

### 35.3

Measure the number of cells in the filtrate

1. Write an enclosure on a glass slide with an alcohol-resistant marking pen.
2. Add **15  $\mu\text{L}$**  drops of 95% ethanol in the enclosure, followed promptly by **15  $\mu\text{L}$**  drops of filtrate.
3. Allow to natural drying.
4. Perform Papanicolaou staining.
5. Count cells in 10 fields of view at 400x under the microscopy.