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〈For Research Use Only〉

Code No. 294-67001 (for 60 tests)

(For enzyme activity staining of tartrate-resistant acid phosphatase and alkaline phosphatase)

TRAP/ALP Stain Kit
for Pathology Research

【Introduction】

Normal bone metabolism is based on the balance between bone formation by osteoblasts and bone resorption by osteoclasts. When the balance is disturbed and bone resorption by osteoclasts is abnormally increased, bone mass is reduced leading to osteoporosis.
Therefore, various researches have been carried out to understand the mechanism of osteoclast and osteoblast metabolism, and to utilize this knowledge for the treatment of the diseases and for the development of effective drugs.
Today, alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) are known as marker enzymes for osteoblasts and osteoclasts, respectively, and these enzymes are used as one of the markers showing the presence of osteoblasts and osteoclasts in tissue sections or cultured cells.
This kit enables you to examine the state of differentiation of bone cells and the cell distribution in bone tissues by observation of the stained images of osteoblasts and osteoclasts in the tissues and cultured cells using ALP/TRAP enzyme activities in the tissue sections and cultured cells.

【Features】

(1) Just mix three solutions for the preparation of the colorimetric substrate solution required for staining of TRAP activity.
(In case of the staining of acid phosphatase including tartrate-sensitive acid phosphatase, mix two solutions except for Tartaric acid soln.)
(2) Simple steps for staining of ALP activity by using ALP substrate soln. (premixed).
(3) Double staining can be performed, reddish-purple for active region of acid phosphatase and bluish brown for alkaline phosphatase.
(4) Usable in cultured cells and bone tissue sections (GMA resin embedded section).

【Kit Contents】

Reagent *	Pkg. Size	Stain number	
		bone tissue sections	cell culture in 24- or 96-well multiplate
(1) Tartaric acid soln. (×10)	1×3 mL	60 pieces (0.5 mL/slide)	24-well : 5 plates (120 wells) 96-well : 6 plates (576 wells)
(2) TRAP substrate soln. A **	1×30 mL		
(3) TRAP substrate Soln. B	1×0.3 mL		
(4) Nuclear stain soln. ***	1×10 mL	20 pieces (0.5 mL/slide)	24-well : 40 wells 96-well : 192 wells
(5) ALP substrate soln. (premixed)	1×30 mL	60 pieces (0.5 mL/slide)	24-well : 5 plates (120 wells) 96-well : 6 plates (576 wells)

(Note)
* : Please thaw each reagent at room temperature (RT). Do not leave thawed reagents at RT for a long time.

[**After opening the kit**]
Reagent (1), (2) and (3) : Store at -20℃.
Reagent (4) and (5) : Store at 2~10℃ after slightly shaking.
** : Repeated freeze-thaw cycles of Reagent (2) may cause some precipitation in TRAP substrate soln. A. In that case, use the solution after filtration through approx. 0.2 μm filter.
*** : Since the amount of the Reanget (4) is corresponded to one-third of amount of other stain solutions, please use this solution only when it is necessary.

1. Staining of ALP and TRAP enzyme activities in bone tissue section

Staining example of GMA embedded thin section sample of non-decalcified bone

[**Precautions before operation**]
・In the case that double staining of TRAP stain and ALP stain is planned, first perform the TRAP stain, followed by microscopic observation and ALP stain.
・As TRAP stain and ALP stain images are possibly difficult to be observed due to nuclear staining, microscopic observation prior to nuclear staining is recommended.

[**Reagents and apparatuses to be prepared**]
・DDW (distilled deionized water)
・0.1 mol/L AMPD-HCl buffer, pH 9.4 (when ALP stain is performed after TRAP stain on a section)
AMPD : 2-Amino-2-methyl-1,3-propanediol
(Wako Cat. No. 015-06411 (100 g))
・Xylene (Wako Cat. No. 244-00086 (500 mL))
・Mounting reagent (water-insoluble)
・Optical microscope furnished with a camera
・Moist chamber
・Coplin-staining jars (for washing slides)
・Heater plate
・Micropipette
・Measuring pipette
・Beaker or Tube
・Coverslips
・Cover glasses

[**Procedure**]
[Preparation of sample]
(1) GMA embedded thin section samples of non-decalcified bone (2 μm thick) applied to silan coated slides.
(2) Wash samples with water.

[Preparation of *TRAP stain soln*.]
(3) Prepare *TRAP stain soln.* in the following ratio just before use.
(Do not store the solution after preparation.)
〈*TRAP stain soln.*〉

Tartaric acid soln. (×10)	1 mL
TRAP substrate soln. A	9 mL
TRAP substrate soln. B	0.1 mL

・If precipitation occurs in the prepared staining solution, gently centrifuge the precipitates, and use the supernatant.

[TRAP stain]
(4) Apply 0.5 mL of *TRAP stain soln*. on each section in a moist chamber at room temperature and allow to stand for 30 minutes at RT.
・Colorimetric time varies according to the amount and activity of TRAP in the samples. Stop the reaction in the appropriate state

while observation is carried out microscopically. Note that long reaction time may cause precipitation of the reactant and nonspecific reactions to various cells besides osteoclasts.

(5) Add a sufficient amount of distilled water to soak the sections in 3 Coplin-staining jars and wash the sections in these jars for 1 min. each.

(6) Add a sufficient amount of 0.1 mol/L AMPD-HCl buffer (pH 9.4) to soak the sections in each Coplin-staining jar, soak the sections and allow to stand for 10 minutes.

(7) Remove excess moisture on the slides.

[ALP stain]

(8) Apply 0.5 mL of *ALP substrate soln. (premixed)* on each section and allow to stand for 30 minutes in a moist chamber at room temperature.

* Colorimetric time varies according to the amount and activity of alkaline phosphatase in the samples. Stop the reaction in the appropriate state while observation is carried out microscopically.

(9) Add a sufficient amount of distilled water to soak the sections in 3 Coplin-staining jars and wash the sections in these jars for 1 min. each.

[Nuclear stain]

(10) Add a sufficient amount of distilled water to soak the sections in a Coplin-staining jar and apply 0.5 mL of *Nuclear stain soln*. on the sections.

After 4～5 seconds, immediately wash one section by moving them up and down in distilled water.

(When more than one section is stained, it is recommended to repeat the staining and washing steps one by one as immediate washing after the application of *Nuclear stain soln.* is necessary for the procedures for nuclear stain.)

(11) Add a sufficient amount of distilled water to soak the sections in each Coplin-staining jar and wash the sections.

[Observation]

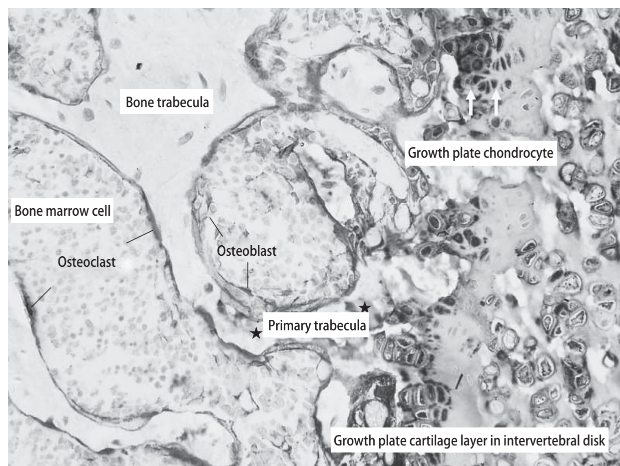
(12) Dry the sections on a heater plate at 37℃.

(13) Add a sufficient amount of xylene to soak the sections in each Coplin-staining jar and soak the sections.

(14) Mount the sections using mounting agents and perform observation.

Stained images and staining method are provided by Hajime Kawahara.

Non-decalcified mouse spinal bone GMA resin embedded 2 μm-section



2. Staining of ALP and TRAP enzyme activities in cultured cell

Staining example of cells cultured in 24-well plate

[Precautions before operation]

- In the case that double staining of TRAP stain and ALP stain is planned, first perform the TRAP stain, followed by microscopic observation and ALP stain.
- As TRAP stain and ALP stain images are possibly difficult to be observed due to nuclear stain, microscopic observation prior to nuclear stain is recommended.
- The methods for cell fixation and permeabilization are not limited to these procedures.
  If done properly, other fixation and permeabilization techniques suitable for the samples used can be employed.

[Reagents and apparatuses to be prepared]

- DDW (distilled deionized water)
- Wash buffer (Dulbecco's Phosphate buffered saline : D-PBS (-) (Wako Cat. No. 045-29795 (500 mL))
- Fixative* : Dilute 37% Formaldehyde soln. to 1/10 with cold PBS at 2～10℃ and place on ice. (＊: Prepare the fixative just before use.) Formaldehyde soln. (Wako Cat. No. 061-00416 (500 mL))
- Permeate [Ethanol / Acetone (50 : 50 v/v)]
  Ethanol (99.5) (Wako Cat. No. 057-00456 (500 mL)),
  Acetone (Wako Cat. No. 016-00346 (500 mL))
- 37℃ Incubator
- Optical microscope furnished with a camera
- Micropipette
- Microtubes

[Procedure]

Culture cells in a 24-well plate.

[Fixation of cell]

(1) Immediately after removal of the culture media, add 3 mL of PBS and rinse the cells gently.

(2) Remove the PBS added, slowly add 500 μL of pre-chilled fixative so as not to remove the cells and allow to stand on ice for 10 minutes. (Carry out the following steps at room temperature.)

(3) Dilute the fixative by adding 2 mL of PBS to the wells containing the fixative.

(4) Remove the solution in the wells and add 2 mL of PBS. Repeat this step 2 additional times.

[Permeabilization]

(5) Remove PBS, add 500 μL of permeate and incubate for 1 minute at -30～-20℃.

(6) Gently remove the solution in the wells and add 2 mL of PBS. Repeat this step 2 additional times.

[Preparation of *TRAP stain soln*.]

Just before use, mix each reagent in the following ratio according to the number of samples. (Do not store the solution after preparation.)

〈*TRAP stain soln.*〉

Tartaric acid soln. (×10)	100 μL
TRAP substrate soln. A	900 μL
TRAP substrate soln. B	10 μL

24-well multiplate : 250 μL/well
96-well multiplate : 50 μL/well
Slide : 500 μL/piece

［TRAP stain］

(7) Add 250 μL of prepared *TRAP stain soln*. in each well, cover the plate to prevent them from drying and allow them to react at 37℃ in an incubator for 15～45 minutes.
- Colorimetric time varies according to the amount and activity of tartrate-resistant acid phosphatase in the samples. Stop the reaction in the appropriate state while observation is carried out microscopically. Note that long reaction time may cause precipitation of the reactant and nonspecific reactions to various cells besides osteoclasts.

(8) Dilute the reaction solution by adding 2 mL of DDW in the wells.

(9) Remove the solution in the wells and add 2 mL of DDW. Repeat this step 2 additional times.

(10) When necessary, remove excess moisture in the wells and perform ALP stain or nuclear stain.

［ALP stain］

(11) Add 250 μL of *ALP substrate soln. (premixed)* in each well, cover the plate to prevent them from drying and allow them to react at 37℃ in an incubator for 15～45 minutes.
- Colorimetric time varies according to the amount and activity of alkaline phosphatase in the samples. Stop the reaction in the appropriate state while observation is carried out microscopically.

(12) Dilute the reaction solution by adding 2 mL of DDW in the wells.

(13) Remove the solution in the wells and add again 2 mL of DDW. Repeat this step 3 additional times.

(14) When necessary, remove excess moisture in the wells and perform nuclear stain.

［Nuclear stain］

(15) Add 250 μL of *Nuclear stain soln*. in the wells and stain for 5～15 minutes at room temperature. (The time for staining is provided only as a guide. Employ the time suitable for the sample used.)

(16) Add 2 mL of DDW to dilute *Nuclear stain soln*. in the wells.

(17) Remove the solution in the wells and add again 2 mL of DDW. Repeat this step 2 additional times.
Repeat this step until the DDW added to the wells becomes clear.

［Observation］

(18) If the samples get dry, drop some DDW on them and perform observation.

［Example of staining results］

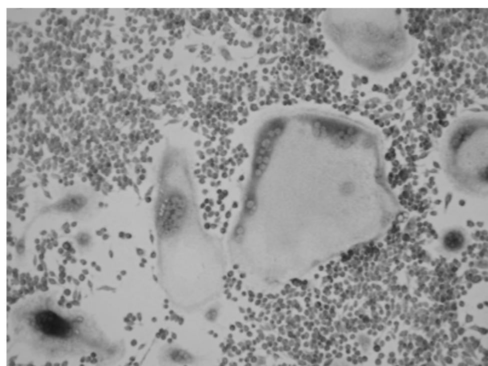


Figure 1. Enzyme activity staining of tartrate-resistant acid phosphatase (TRAP) in RAW264 cells

RAW264 cells (cell line derived from Mouse leukemic monocyte, differentiate into osteoclast-like cells) were cultured in the presence of sRANKL. On day 6 of culture, the cells were fixed in neutral formalin and treated with Ethanol/Acetone (50 : 50 v/v) for permeabilization, and then TRAP stain was performed.
TRAP-positive, multinuclear osteoclast-like cells were observed.

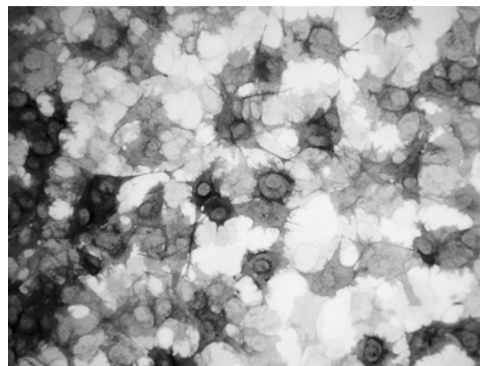


Figure 2. Enzyme activity staining of alkaline phosphatase (ALP) in MC3T3-E1 cells

MC3T3-E1 cells (cell line derived from Mouse calvaria, differentiate into osteoblasts) were cultured in the presence of BMP-2. On day 7 of culture, the cells were treated with Ethanol/Acetone (50 : 50 v/v) for permeabilization and then ALP stain was performed.

【Expiration Date】

24 months after the manufacturing. (Indicated on each label.)

【Storage】

［Before opening the kit］ Store at -20℃.

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FUJIFILM

Wako

<研究用试剂>

产品编号：294-67001（60 tests）

病理研究用

（用于抗酒石酸酸性磷酸酶和碱性磷酸酶的酶活性染色）

TRAP/ALP双染试剂盒

【前言】

正常的骨代谢是通过成骨细胞诱导骨生长与破骨细胞诱导骨吸收来维持平衡。当这个平衡被打破，破骨细胞的骨吸收异常增加时，会导致骨量下降，继而引起骨质疏松。因此，目前正在开展各项研究，以了解破骨细胞和成骨细胞的代谢机制、从而推动相关疾病的治疗及特效药的开发。

目前，成骨细胞标志酶——碱性磷酸酶（ALP）和破骨细胞标志酶——抗酒石酸酸性磷酸酶（TRAP）在组织切片或培养细胞中被用作成骨细胞和破骨细胞存在的标记。

本试剂盒利用组织切片和培养细胞的ALP/TRAP酶活性，可通过观察成骨细胞和破骨细胞在组织及培养细胞中的染色成像，从而确认细胞的分化状态和骨组织内细胞分布情况。

【特点】

(1) 使用时仅需将3种溶液混合，即可制备TRAP的酶活性染色所需的显色底物溶液（对酒石酸盐敏感的酸性磷酸酶等酸性磷酸酶进行酶活性染色时，仅需混合除酒石酸钠溶液以外的2种溶液）。
(2) 使用预混底物溶液即可轻松进行碱性磷酸酶活性染色。
(3) 可实现双重染色，即TRAP的活性部位呈红紫色，碱性磷酸酶的活性部位呈偏蓝色的茶褐色。
(4) 可用于培养细胞及骨组织切片（GMA树脂包埋切片）。

【试剂盒组成】

(1)	酒石酸钠溶液（×10）Tartaric acid soln.（×10）	3 mL	1支
(2)	抗酒石酸酸性磷酸酶底物溶液A TRAP substrate soln. A	30 mL	1支
(3)	抗酒石酸酸性磷酸酶底物溶液B TRAP substrate soln. B	0.3 mL	1支
(4)	核染色试剂Nuclear stain soln.	10 mL	1支
(5)	碱性磷酸酶预混底物溶液ALP substrate soln.（premixed）	30 mL	1支

（备注）

1. 每种试剂需在室温（RT）下解冻，解冻的试剂切勿长时间置于室温（RT）下。
2. 本产品在培养细胞中可供24孔板使用5次，96孔板使用6次，是骨组织切片约60张的用量。（每张切片使用500 μL）。
3. 产品开封前后的注意事项
   开封前存储在-20°C，开封后请参考以下注意事项。
   ① 酒石酸钠溶液（×10）、TRAP底物溶液A、TRAP底物溶液B在开封后也必须在-20°C下存储。TRAP底物溶液A在反复冻融时会产生少许沉淀，可使用约0.2 μm的滤膜过滤后再使用。
   ② 核染色试剂和ALP预混底物溶液解冻后，轻轻搅拌并在2~10°C下存储。

【操作方法】

1. 骨组织切片ALP和TRAP酶活性的染色
使用未脱钙骨GMA包埋切片样品的染色示例

【操作前的注意事项】

※ 进行TRAP和ALP双重染色时，需先进行TRAP染色，在显微镜下观察后，再进行ALP染色。

※ 某些情况下核染色可能会使TRAP染色和ALP的染色成像难以观察，因此，推荐使用显微镜观察后再进行核染色。

【除试剂盒外所需的试剂和器材】

- 蒸馏水
- 0.1 mol/L AMPD-HCl缓冲液（pH 9.4）（对切片进行TRAP染色后，进行ALP染色时）
  AMPD：2-氨基-2-甲基-1,3-丙二醇（产品编号：015-06411）
- 二甲苯（产品编号：244-00086）
- 封片剂（非水溶性）
- 光学显微镜
- 湿润箱等容器
- 染色缸（清洗切片）
- 37°C展片机
- 微量移液器
- 刻度移液管
- 烧杯或冻存管等容器
- 盖玻片（Coverslips）
- 盖玻片（Cover glasses）

【操作步骤】

【样品准备】

⑴ 在硅烷涂层载玻片放上未脱钙骨GMA包埋切片样品（2 μm）

⑵ 用水清洗样品

【制备TRAP染色液】

⑶ 使用前请按照以下比例制备TRAP染色液。
（现配现用，切勿在制备后储存溶液）

酒石酸钠溶液（×10）	1 mL
TRAP底物溶液A	9 mL
TRAP底物溶液B	0.1 mL

※ 已制备的染色液中出现沉淀时，可轻轻离心沉淀，使用上清液。

【TRAP染色】

⑷ 在湿室（室温）内将0.5 mL TRAP染色液滴在每个切片上，室温静置30 min。
※ 显色时间因样品中抗酒石酸钠磷酸酶的量和活性而异。使用显微镜观察，并在适当状态下终止反应。但需要注意，反应时间过长时，可能会出现反应物沉淀或与破骨细胞外的细胞发生非特异性反应。

⑸ 在3个染色缸中加入足以浸没切片的蒸馏水，并清洗1 min。

⑹ 在染色缸中加入足以浸没切片的0.1 mol/L AMPD-HCl缓冲液（pH 9.4），浸泡切片并静置10 min。

⑺ 去除载玻片上多余的水分。

【ALP染色】

⑻ 向每个切片上滴加0.5 mL ALP预混底物溶液，在湿室内（室温）静置30 min。
※ 显色时间因样品中ALP的量和活性而异。使用显微镜观察，并在适当的状态下终止反应。

⑼ 在3个染色缸中加入足以浸没切片的蒸馏水，并清洗1 min。

【核染色】

（10）在染色缸中加入足以浸没切片的蒸馏水，向每个切片上滴加0.5mL的核染色试剂。4~5秒后迅速将切片放入蒸馏水中清洗。

（在核染色步骤中，滴加染色液后需要立即洗净，因此对多张切片染色时，建议对每张切片重复染色和漂洗步骤。）

（11）在染色缸中准备足以浸没切片的蒸馏水，清洗切片。

【观察】

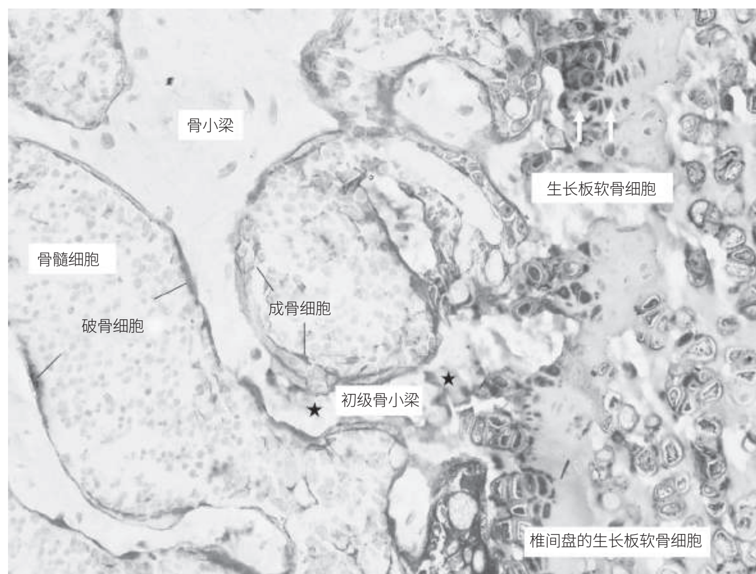
（12）使用37°C的展片机烘干切片。

（13）在染色缸中加入足以浸没切片的二甲苯，放入切片。

（14）使用封片剂进行封片，并进行观察。

染色图像及染色方法由河原 元老师提供。

小鼠脊柱 未脱钙GMA树脂包埋2 μm切片



2. 培养细胞的ALP和TRAP酶活性染色

使用24孔板培养的培养细胞染色示例

【操作前的注意事项】

※ 进行TRAP/ALP的双重染色时，建议先进行TRAP染色并在使用显微镜观察后，再进行ALP染色。

※ 核染色可能导致TRAP和ALP染色图像难以观察，建议在核染色前先使用显微镜观察一次。

※ 细胞固定和渗透的方法不局限于以下步骤。无特殊情况，也可以根据所用样品使用相应的固定和渗透方法。

【除试剂盒外所需的试剂和器材】

- 蒸馏水
- 清洗缓冲液：D-PBS (-) （产品编号：045-29795）
- 固定液：使用在2~10°C下冷却的PBS溶液将37%甲醛溶液稀释10倍，并放置在冰块上。（※ 固定液需现配现用）（产品编号：061-00416）
- 渗透剂：乙醇（产品编号：057-00456） / 丙酮 （50 : 50 v/v）（产品编号：016-00346）
- 37°C孵育箱
- 光学显微镜
- 微量移液器
- 微量离心管

【操作】

在24孔板中进行细胞培养。

【细胞固定】

(1) 去除培养液后，迅速添加3 mL PBS并轻轻冲洗细胞。
(2) 去除PBS后，为避免细胞分离，应缓缓加入500 µL提前冷却好的固定液，并在冰块上静置10 min。(之后的操作需在室温下进行)
(3) 向含有固定液的孔中加入2 mL PBS，稀释固定液。
(4) 去除孔中的液体，加入2 mL PBS。重复此操作2次。

【渗透处理】

(5) 去除PBS，添加500 µL渗透液，在-30°C~-20°C下孵育1 min。
(6) 去除孔中的溶液，加入2 mL PBS。重复此操作2次。

【制备TRAP染色液】

使用前，根据样品数量，按照以下试剂比例制备TRAP染色液。

※ 使用时请按照以下比例制备TRAP染色液，请勿存储已制备的溶液。

酒石酸钠溶液(×10)	100 µL
TRAP底物溶液A	900 µL
TRAP底物溶液B	10 µL

24孔板：250 µL/well，96孔板：50 µL/well，每切片：500 µL

【TRAP 染色】

(7) 将已制备的250 µL染色液添加至各孔中，盖上盖子以防止挥发，并在37°C孵育箱中反应15~45 min。

※ 显色时间因样品中含有的抗酒石酸盐酸性磷酸酶量和活性而异，使用显微镜观察，并在适当状态下终止反应。但需要注意，反应时间过长时，可能会出现反应物沉淀或与破骨细胞外的细胞发生非特异性反应。

(8) 向孔中添加2 mL蒸馏水，稀释反应液。
(9) 去除孔中的液体，并添加2 mL蒸馏水。重复此操作2次。
(10) 必要时需去除孔中多余的水分后再进行ALP染色或核染色。

【ALP染色】

(11) 将250 µL ALP预混底物溶液添加至孔中，盖上盖子以防止挥发，在37°C孵育箱中反应15~45 min。

※ 显色时间因样品中含有的抗酒石酸盐酸性磷酸酶量和活性而异，使用显微镜观察，并在适当状态下终止反应。

(12) 向孔中添加2 mL蒸馏水，稀释反应液。
(13) 去除孔中的液体，再次添加2 mL蒸馏水。重复此操作3次。
(14) 必要时需去除孔中多余的水分后再进行核染色。

【核染色】

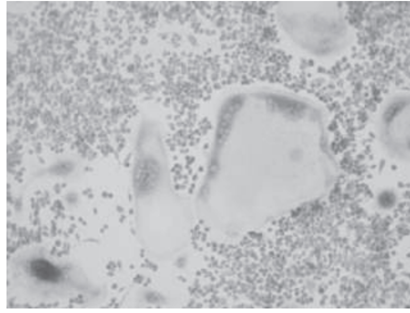
(15) 将250 µL核染色试剂添加至孔中，室温下进行5~15 min染色。(染色时间仅供参考，请根据样品的具体情况选择合适的染色时间)
(16) 向孔中添加2 mL蒸馏水，稀释核染色试剂液。
(17) 去除溶液，再次向孔中添加2 mL蒸馏水。重复此操作2次。
向孔中添加蒸馏水直至变得透明。

【观察】

(18) 样品干燥时，需在样品中滴加蒸馏水后进行观察。

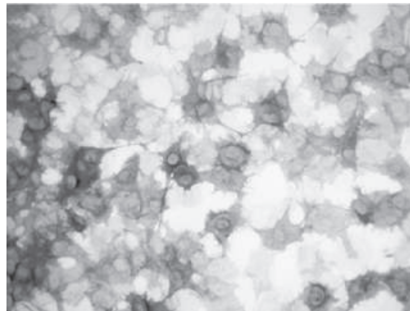
【染色结果示例】

图1. RAW264细胞的抗酒石酸酸性磷酸酶（TRAP）活性染色



RAW264 细胞（小鼠白血病单核细胞来源的细胞系，可分化为破骨细胞样细胞）在sRANKL存在的条件下进行培养，培养至第6天时，使用中性福尔马林固定，乙醇/丙酮（50 : 50v/v）进行渗透处理后，进行TRAP染色。
可以观察到TRAP阳性和多核破骨细胞样细胞。

图2. MC3T3-E1细胞的碱性磷酸酶（ALP）活性染色



MC3T3-E1细胞（小鼠颅骨来源的细胞系，可分化为成骨细胞）在BMP-2存在的条件下进行培养，培养至第7天时，使用乙醇/丙酮（50 : 50v/v）进行渗透处理后，进行ALP染色。

本产品可供培养细胞24孔板使用5次，96孔板使用6次，是骨组织切片约60张的用量（每张切片使用500 µL）。

【保质期】

24个月（详见包装标签）。

【储存条件】

开封前-20°C。

【开封后的注意事项】

① 酒石酸钠溶液（×10）、TRAP底物溶液A和TRAP底物溶液B开封后需在-20°C下存储。
　※ TRAP底物溶液A在反复冻融时会产生少许沉淀，请使用约0.2 µm的滤膜过滤后再使用。
② 核染色试剂和ALP预混底物溶液解冻后，需轻轻搅拌并在2~10°C下存储。

【参考文献】

1. 河原 元：「硬組織標本作製法」，検査と技術，29，1169（2001）.

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