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Your Molecular & Cell Technology Partner

SafeForever™

Ensuring Eternity

Protocol booklet

SafeForever™ Preserved Sample Processing Protocol

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Instructions for use

The conventional method of biospecimen collection, transport and storage is complicated, expensive and laborious. SafeForever™, is an innovative all in one solution whichis designed to overcome such barriers.

SafeForever™ is a gel-based formulation wherein the biospecimens can be storedat room temperature and used in medical diagnostics, forensics and research. The biospecimens include blood, saliva, tissue biopsies, and cell lines from humans and animals. Plant specimens such as leaves, stems, roots, flowers, fruit, and seeds can also be preserved. Bacterial, fungal and viral specimens also can be stored in SafeForever™.

The biospecimens stored in SafeForever™ can be used for downstream analysis of DNA, RNA and protein isolation. Please refer below for instructions for collection, storageand downstream analysis of various biological samples.

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Sample Collection and Storage

Sample	Collection Procedure	Storage
Blood	500ul Blood + 5ml SafeForever™	Room Temperature
Saliva	500ul Saliva + 5ml SafeForever™	
Tissue	1g Tissue + 25ml SafeForever™	
Plant and Plant Parts	$3cm^2$ Leaf+ 5ml SafeForever™	
	250mg Stem + 5ml SafeForever™	
	250mg Root + 5ml SafeForever™	
Soil	1g Soil + 5ml SafeForever™	

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DNA ISOLATION FROM BLOOD

I. Sample Collection;

Sample Type	Sample to SafeForever™ Ratio
Blood	2.5ml Blood + 25ml SafeForever™

II. Reagents;

Lysis Buffer: 0.3M Sucrose
10 mM Tris Hcl
5mM MgCl2
Triton X 100 pH:8.0

TRIzol

TE Buffer 10 mM Tris
0.1 mM EDTA pH 7.5

III. Procedure;

1) Take 1000ul SafeForever™ stored blood
2) Add 500ul of Lysis Buffer. Vortex for 20 secs
3) Incubate for 30 mins
4) Add 200ul of 100% Ethanol. Incubate for 10 mins
5) Add 500ul of Trizol. Incubate for 30 mins
6) Centrifuge at 2000 rpm for 1 min
7) Transfer the supernatant to a new tube and add 350ul of 100% chloroform
8) Incubate for 2-3 min
9) Centrifuge at 15000rpm for 10 min at 40°C
10) Discard aqueous phase and add 400ul of 100% ethanol
11) Incubate for 3 - 5 min
12) Centrifuge at 15000rpm for 10min at 40°C
13) Discard supernatant
14) Wash the pellet with 70% ethanol after 5 min incubation
15) Centrifuge at 15000rpm for 5min at 4°C
16) Discard the supernatant
17) Air dry the pellet at 37°C for 15 -20 mins
18) Dissolve the pellet with 30ul of 1X TE buffer at 60°C for 10 mins

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DNA ISOLATION FROM SALIVA

I. Sample Collection;

Sample Type	Sample to SafeForever™ Ratio
Saliva	2.5ml Saliva + 25ml SafeForever™

II. Reagents;

Lysis Buffer: 155mM Ammonium chloride
10 mM KHCO3
1mM EDTA pH 7.4

Nuclei lysis buffer: 10mM Tris
10mM NaCl
2mM EDTA pH 8.2

Proteinase K 2mg/ml in 1% SDS
2mM EDTA

TE Buffer 10mM Tris
0.1mM EDTA pH 7.5

III. Procedure;

1) Take 1000ul of SafeForever™ preserved Saliva.
2) Add 1000ul of Ice-cold Lysis Buffer. Vortex for 20 secs
3) Keep it on Ice for 20 min with occasional mixing
4) Centrifuge at 15000 rpm for 10 min at 40°C
5) Decant the Supernatant
6) Resuspend the pellet in 400ul of Nuclei lysis buffer and 40 ul of 10% SDS and
100ul of Proteinase K. Vortex for 20 Secs
7) Incubate at 37°C for 3 hours – overnight
8) Add 500ul of Phenol: Chloroform: Isoamyl alcohol (25:24:1) and shake for 2 mins
9) Centrifuge at 15,000 rpm for 10 mins at 40°C
10) Carefully transfer the aqueous phase to a new 1.5ml Eppendorf tubes
11) To the aqueous phase add equal volume of 100% Ethanol and mix by inverting the tube slowly
12) Incubate for 10 mins
13) Centrifuge at 13,000 rpm for 5 mins at 40°C
14) Decant the Supernatant
15) Wash the pellet add 500ul of 75% Ethanol
16) Incubate for 5 mins
17) Centrifuge at 13,000 rpm for 5 mins at 40°C
18) Decant the Supernatant and air dry the pellet for 5 mins
19) To the pellet add 30ul of 1X TE buffer.

DNA ISOLATION FROM TISSUE

I. Sample Collection;

Sample Type	Sample to SafeForever™ Ratio
Tissue	~200mg Tissue + 5ml SafeForever™

II. Reagents;

Lysis Solution: 1M NaCl (Sterilized by autoclave)
100 mM Tris pH 8.0
25 mM EDTA pH 8.0
0.5% SDS

Proteinase K: 20mg/ml

Protein Precipitation Solution: 4M Guanidine isothiocyanate
(Sterilized by filtration)
0.1M Tris pH 7.5

RNase A Solution: 20 mg/mL

Note: Use 0.22 µm cellulose nitrate or mixed cellulose esters membranes for filtration

III. Procedure:-

1) Remove the tissue from SafeForever™ and wash with Phosphate Buffer Saline.
2) Cut the tissue into small pieces5–30 mg (wet weight) and put them inside a 1.5 mL Eppendorf vial
3) Add 300 µL of the Lysis Solution
4) Homogenize or grind the pieces inside the tube with a small mortar pestle or homogenizer
5) Add 3 µL of Proteinase K Solution and mix by vortexing
6) Incubate the sample in a thermo shaker at 55°C at 900 rpm, 15–18 h (overnight)
7) Heat the vial at 95°C for 10 min to inactive any residual Proteinase K
8) Allow the samples to cool at room temperature for 5–10 min
9) Add 4 µL of RNase A solution to the lysate and mix by tipping the bottom of the tube firmly and repeatedly
10) Incubate the samples at 37°C for 30 min
11) Leave the tubes to cool at room temperature for 5 min
12) Add 100 µL of Protein Precipitation Solution and vortex vigorously for 20 seconds
13) Centrifuge the tubes at ~16,000 g for 10 min
14) Carefully transfer the supernatant to a new 1.5 mL tube without disturbing the residual pellet

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15) Precipitate the DNA by adding 300 µL of chilled 100% isopropanol (kept inside the –20°Cfreezer)
16) Mix well by flipping the tubes several times, until the DNA pellet is visible
17) Centrifuge at ~16,000 g for 5 min
18) Discard the supernatant
19) Add 300 µL of 70% ethanol
20) Wash the DNA pellet by flipping the tubes 5–10 times
21) Centrifuge at ~16,000 g for 5 min
22) Discard the supernatant by absorbing with a micropipette
23) Air dry the pellet by leaving the tubes open until no drops of liquid are visible
24) Resuspend the DNA pellet in TE buffer
25) Incubate at 65°C for 1 h. Occasional tapping the bottom of the vial is recommended
26) Leave the tubes at 4°C overnight for complete resuspension
27) DNA can be preserved at –200C for long-time storage or kept at 4°C for subsequent PCR.

DNA ISOLATION FROM PLANT LEAF

I. Sample Collection;

Sample Type	Sample to SafeForever™ Ratio
Leaf	3cm^2 (Leaf)+ 5ml SafeForever™

II. Sample Collection;

CTAB DNA Extraction buffer: 1 M Tris. HCl (PH 8) - 10 ml (stock for 100 ml)
0.5 M EDTA - 8 ml
5 M NaCl - 28 ml
CTAB 2 g
PVPP 1g

RNase: 10 mg/ml

TE Buffer 10mM Tris
0.1mM EDTA pH 7.5

III. Procedure;

1) 250 mg leaf tissue + 500uL CTAB buffer.
2) Homogenize and incubate at 65°C for 20 min.
3) Keep on ice for 2-5 min.
4) Add 600uL Chloroform: Isoamyl (24:1) and shake vigorously.
5) Centrifuge at 13000 rpm for 5 min.
6) Transfer aqueous layer to fresh tube.
7) Add 250uL Chloroform, shake vigorously
8) Centrifuge at 10,000 rpm, for 5 min.
9) Transfer aqueous layer to fresh tube.
10) Add 500uL ice cold 100% Ethanol.
11) Mix slowly and incubate for 10 mins.
12) Centrifuge at 13,000rpm for 5 min.
13) Wash the pellet with 500uL 70% Ethanol.
14) Incubate at 5 mins and centrifuge: 13,000rpm for 5 mins
15) Air dry the pellet
16) Dissolve the pellet in 30-40uL 1xTE buffer and incubate for 30-60 mins @ 65°C

DNA ISOLATION FROM PLANT STEM & ROOT

I. Sample Collection;

Sample Type	Sample to SafeForever™ Ratio
Stem	2g Stem + 5ml SafeForever™
Root	2g Root + 5ml SafeForever™

II. Reagents;

CTAB DNA Extraction buffer: (stock for 100 ml)
1 M Tris. HCl (PH 8) - 10 ml
0.5 M EDTA - 8 ml
5 M NaCl - 28 ml
CTAB 2 g PVPP 1g

Proteinase K: 20 mg/ml

Chloroform: Isoamyl alcohol 24:1 ratio

TE Buffer
10mM Tris
0.1mM EDTA pH 7.5

III. Procedure;

1) Grind 250 mg Stem/Root with mortar and pestle or homogenize.
2) Add 1mL CTAB buffer, 20uL of 2-mercaptoethanol and 35uL Proteinase K
3) Mix to get a homogenous mixture and incubate for 1 hour @ 60°C with occasional swirling.
4) Cool the samples at RT.
5) (a) Add 600uL Chloroform: Isoamyl alcohol (24:1).
6) (b) Mix gently and centrifuge at 15,000 rpm for 15 min.
7) Transfer the aqueous phase to a fresh tube.
8) Add 140uL of 10% CTAB and 280uL of 5M NaCl.
9) Mix gently and repeat steps (a) and (b)
10) Transfer the aqueous phase to a fresh tube.
11) Add 1mL Ice cold Isopropanol.
12) Incubate for 1 hour at -200C and centrifuge at 15,000 rpm for 10 min.
13) Wash the pellet with 70% Ethanol (2 washes).
14) Air dry the pellet at 37°C for 15-20 mins.
15) Dissolve the pellet in 30uL 1x TE buffer and incubate at 65°C for 30-60 mins.

DNA ISOLATION FROM SOIL

I. Sample Collection;

1. Take 1g of Soil sample and add it to 5mL of the SafeForever™
2. Shake the tube continuously for a few seconds till the sample is distributed completely.

II. Reagents;

DNA Extraction Buffer (pH 8.0)	0.02gPVPP 100mM Na2HPO4 5%SDS
PEG solution	30% PEG-6000 1.6 M NaCl
Chloroform: Isoamyl alcohol	24:1 Ratio
TE Buffer	0mM Tris (7.8) 0.1 mM EDTA pH (8)

III. Procedure;

1. Take 1200ul amount of sample from the above tube in a microcentrifuge tube.
2. Add half the volume (600 µL) of extraction buffer (120mM Na2HPO4 (pH 7.4), 5% SDS and PVPP 0.02g) to the tube. Incubate for 1hr at 65°C. Centrifuge at 8k rpm for 10 mins, collect supernatant.
3. Add half volume of PEG solution (30% PEG-6000, 1.6 M NaCl) and one volume of NaCl to the tube. Incubate overnight(3hrs).
4. Further add one volume of Chloroform: Isoamyl alcohol (24:1)
5. Centrifuge the tube at 12,000 x g for 10 minutes at room temperature. Transfer the supernatant to a new tube.
6. To supernatant add 1/10th volume of 3M Sodium Acetate(pH5.2)
7. Add 2 volumes of 70% ethanol to the pellet and vortex the tube for 10 seconds.
8. Centrifuge the tube at 12,000 x g for 5 minutes at room temperature and Carefully remove the supernatant and discard it.
9. Air-dry the pellet for 10-15 minutes and Add 50 µL of TE buffer. Incubate the tube at 37°C for 15 minutes to dissolve the DNA.

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RNA ISOLATION FROM BLOOD AND SALIVA

I. Sample Collection;

Sample Type	Sample to SafeForever™ Ratio
Blood	500ul Blood + 5ml SafeForever™
Saliva	500ul Saliva + 5ml SafeForever™

II. Reagents;

a) Trizol
b) Chloroform
c) Isopropanol
d) 75% Ethanol

III. Procedure;

1) Take 1 ml of SafeForever™ Blood or SafeForever™ Saliva in a 1.5ml Eppendorftube.
2) Centrifuge at 15,000 rpm at 4°C for 20 min
3) Discard the supernatant
4) Add 1ml of Trizolto the pellet and pipetteseveral times followed by vortexing for 20 seconds to homogenize
5) Incubate the samples at RT for 5 mins
6) After Incubation add 200ulof Chloroform and vortex for 20 seconds.
7) Incubate at RT for 3 - 5 mins
8) Centrifuge at 15,000 rpm at 4°C for 20 min
9) Remove approximately about 700ul from upperaqueous layer and transfer it to a new tube
10) Repeat step 6 – 8 two more times
11) For the first time remove 600ul and second time remove 500ul of upper aqueous layer
12) To the 500ul of upper aqueous layer add 500ul of Ice-cold Isopropanol and vortex for few seconds
13) Incubate at -20°C for at least 30 mins
14) Centrifuge at 15,000rpm at 1°C for 20 min
15) Discard the supernatant
16) Wash the pellet with 1ml of cold 75% ethanol
17) Centrifuge at 15,000 rpm at 1°C for 5 min
18) Repeat step 16 – 17 for one time
19) Remove excess of ethanol
20) Airdry the pellet at RT for at least 5 mins
21) Resuspend the pellet in 20ul RNase free water
22) Incubate in 55°C heat block for 5 mins
23) Vortex the sample briefly and quantify.

PROTEIN EXTRACTION PROTOCOL

I. Sample Collection;

Refer Sample collection and storage section.

II. Reagents Required;

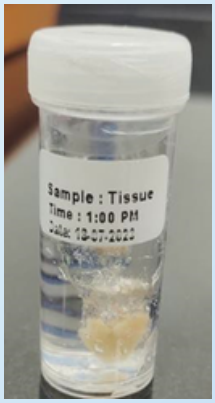
a) Trizol
b) Chloroform
c) 0.3 M Guanidine Hydrochloride in 95% ethanol
d) Isopropanol
e) 100% Ethanol

III. Procedure;

1) Take 1000 µL of SafeForever™ saliva/blood/cell line sample in a 2 mL Eppendorf tube. For Tissue Sample, take 35 – 100mg and homogenize for 40 secs with Trizol. Note: For separation of blood and plasma from SafeForever™ bloodsamples, take 1 mL of SafeForever™ blood sample and centrifuge at 3000 RPM at RT for 5 minutes which will result in separation of serum and blood cells followed by protein extraction using trizol method.
2) Add 1 mL of trizol reagent(if not already added) and mix it properly using a pipette. Incubate it for 30 minutes at RT. And briefly spin the sample and collect only trizol supernatant in a fresh Eppendorf tube.
3) Add 0.2 mL of chloroform and vortex it thoroughly. Incubateat RT for 10 minutes.
4) Centrifuge the sample at 12000 X g at 4°C for 15 minutes.
5) Collect only organic phase OR phenol-ethanol supernatant after DNA precipitation into a new 2 mL Eppendorf tube.
6) Add 1.5 mL of isopropanol to the organic phase OR phenol-ethanol supernatant.
7) Incubate for 10 minutes at RT.
8) Centrifuge at 12000X g for 10 minutes at 4°C.
9) Discard the supernatant with a micropipette.
10) Prepare a wash solution consisting of 0.3 M Guanidine Hydrochloride in 95% ethanol.
11) Resuspend the pellet in 2 mL of wash solution. Incubatefor 20 minutes.
12) Centrifuge for 5 minutes at 7500 X g at 4°C.
13) Repeat step 11 to step 12 twice.
14) Add 2 mL of 100% ethanol, then mixby vortex briefly. Incubate for 20 minutes.
15) Centrifuge for 5 minutesat 7500 X g at 4°C. Discardthe supernatant with a micropipette.
16) Air dry the protein pellet for 5-10 minutes.
17) Resuspend the pellet accordingly between 50-200 µL of 50mM Tris (pH8.0) by using micropipette.
18) Heat the sample at 50°C in a heatingblock for 10 minutes. Centrifuge the sample at 10,000 g for 10 minutes at 4°C to remove insoluble components. Transfer the supernatant with reconstituted protein into a 0.5 mL eppendorf tube.
19) Store at -80°C.

Hematoxylin and Eosin (H&E) protocol

Hematoxylin and Eosin (H&E) staining is a commonly used histological staining technique that is used to visualize the structure of cells and tissues in a sample.



Uterus tissue stored in SafeForever™

1. ~200mg of the uterus tissue was stored in SafeForever™ for 24hours
2. 1.20mg of the sample was immersed in formalin for fixation overnight.

Procedure of Hematoxylin and Eosin (H&E) Staining

1. After fixation the tissue is embedded in melted paraffin which can be subsequently solidified by cooling.
2. The embedded tissue is then subjected to sectioning, (Sectioning is the production of very thin slices from a tissue sample. The tool used for sectioning is called a microtome). The sectioned tissue is typically 5-10µm (micrometers, microns) in thickness.
3. The section is then placed on slide.
4. Deparaffinize sections in xylene three changes (5-10 mins each)
5. Hydration: Drain the xylene and hydrate the tissue slice by passing it through alcohol baths of decreasing concentration (100%, 90%, 80%, 70%) and water.
6. Nuclear Staining: Hematoxylin stain for 3 to 5 minutes.
7. Wash in running water until portions are “blue” for no more than 5 minutes.
8. Differentiation: selective dye removal from the segment). A few seconds in 1% acid alcohol (1% HCl in 70% alcohol).
9. Blueing: Rinse with running tap water. After dipping in ammonia water until the portions become blue, rinse with tap water.
10. Counterstain: Stain for 10 minutes in 1% Eosin Y.
11. Using tap water, wash for 1 to 5 minutes.
12. Dehydration: Dehydrate in rising alcohol content.
13. Clearing: To clear slides, place them in two xylene baths.
14. Mounting: Mount in DPX or another mounting format.
15. Observe under compound microscope.

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