Contents

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Introduction

E.Z.N.A.™ Blood DNA Midiprep Kits are designed for isolation of total DNA (include genomic, mitochondrial and viral DNA) from 0.2-2 mL (with standard protocol) and up to10 mL (with maximum yield protocol) of fresh, whole blood treated with any common anticoagulant such as heparin, EDTA, or acid-citrate-dextrose. This kit can also be used to purify DNA from buffy coat , lymphocytes, serum, plasma an dbone marrow. The procedure completely removes contaminants and enzyme inhibitors making total DNA isolation fast, convenient, and reliable. There is no need for phenol/chloroform extractions, and time-consuming steps such as CsCl gradient ultracentrifugation, and precipitation with isopropanol or LiCl, are eliminated. DNA purified using the E.Z.N.A.™ Blood DNA method is ready for applications such as RT-PCR*.

Principle

The E.Z.N.A.™ Blood DNA Kits combine the reversible binding properties of HiBind® matrix, a new silica-based material, with the speed of midi-column spin technology to provide fast and high quality DNA . The standard protocol Sample is first mixed with BL buffer which lyse the cell and release DNA under denaturing conditions that inactivate DNases. The cell lysate is then loaded into the HiBind® Midi- spin. DNA binds to HiBind® matrix while impurities are effectively removed after few quick wash steps. genomic DNA is purified on the HiBind® Midi spin column.

Storage

E.Z.N.A.™ Blood DNA Midiprep Kits should be stored at room temperature. During shipment crystals may form in the BL Lysis Buffer. Warm to 37°C to dissolve. All the components are guaranteed for at least 24 months from date of purchase.

*The PCR process is covered by U.S. Patents 4,683,195 and 4,683,202 (and international equivalents) owned by Hoffmann-LaRoche, Inc.

Kit Contents

E.Z.N.A.™ Blood DNA Kits	D3494-00	D3494-01	D3494-03	D3494-04
Purification	2	10	50	100
HiBind™ DNA Midi Columns	2	10	50	100
15 mL Collection Tubes	2	10	50	100
Buffer TL	5 mL	25 mL	120 mL	240 mL
Buffer BL	5 mL	25 mL	120 mL	240 mL
Buffer HB	8 mL	40 mL	160 mL	320 mL
Equilibration Buffer	3 mL	12 mL	60 mL	125 mL
DNA Wash buffer	6 mL	40 mL	160 mL	400 mL
OB Protease	6 mg	30 mg	150 mg	300 mg
RNase A	25 µL	110 µL	550 µL	1.2 mL
DNA Elution Buffer	5 mL	20 mL	100 mL	200 mL
Instruction Manual	1	1	1	1

Before Starting

Important

Reconstitute OB Protease with elution buffer before use

D3494-00 Reconstitute OB Protease with 250 µL of elution buffer
D3494-01 Reconstitute OB Protease with 1.5 mL of elution buffer
D3494-02 Reconstitute OB Protease with 7.5 mL of elution buffer
D3494-03 Reconstitute OB Protease with 15 mL of elution buffer

*We recommend that you aliquot and store vials of reconstituted protease at -20°C. Vortex briefly before use

DNA Wash buffer must be diluted with absolute ethanol before use

D3494-00 Add 9 ml of 100% ethanol to each bottle
D3494-01 Add 60 mL of 100% ethanol to each bottle
D3494-03 Add 240 mL of 100% ethanol to each bottle
D3494-04 Add 600 mL of 100% ethanol to each bottle

Harvesting and Storage of Blood

E.Z.N.A.™ DNA Midiprep Kits are designed for purification of genomic DNA from up to 10 mL whole blood. The system is not limited by DNA binding capacity of HiBind® Midi columns (which can bind up to 500 ug of DNA), but by lysis volume. Due to the abundance of erythrocytes and proteins, greater than 20 mL whole blood will significantly lower DNA quality. The relatively low DNA content of leukocytes means that the maximum binding capacity of HiBind® DNA columns can not be reached.

Samples should be collected in the presence of an anticoagulant (preferably acid-citrate-dextrose) and processed within a few hours. Minimize storage prior DNA isolation as leukocyte transcripts generally have variable stabilities.

Standard Protocol (for up to 2 mL of whole blood sample)

Equipments to be supplied by user

- All the subsequent steps must be performed using a centrifuge capable of at least 5000 x g. Ensure that an appropriate rotor adaptor is in place to prevent damage to the collecting tube.
- Water bath or heating block - preset to 70°C.
- Have a shaking water bath set to 55°C.
- Absolute ethanol (96-100%), do not use denatured alcohol as it contains other substances such as methanol and methylethyketone.
- Isopropanol
- Sterile 50 mL centrifuge tubes and 15 mL centrifuge tubes.
- Laboratory centrifuge equipped with swinging-bucket rotor
- Vacuum manifold (for Vacuum protocol)
- Vacuum source (-200 to 600 mBa) (for vacuum protocol)

1. Add up to 2 mL sample into a 15 mL centrifuge tube (not provided). If the sample less than 2 mL, bring the volume up to 2 mL with 10 mM Tris-HCl, PBS, or Elution Buffer provided.
2. Add 140 µL OB Protease and mix the sample throughly by vortexing.
3. Add 2.1 mL of Buffer BL. Vortex 5 minutes to mix thoroughly.
4. Add 10 µL RNase A solution to each sample to remove RNA.
5. Incubate sample at 70°C for 10 min.
6. Briefly vortex the tube once during incubation.
7. Add 2.2 mL of isopropanol to lysate and mix.

   Note: We recommend that you aliquot and store vials of reconstituted protease at -20°C. Vortex briefly before use
8. Take a HiBind® DNA Midi Column pre-inserted in a 15 ml collection tube (supplied). **Add 1 ml Equilibration Buffer into the column. Let the column sit for 4 minutes at room temperature to equilibrate the membrane.** Centrifuge at 3000 x g for 3 minutes
9. Transfer the half volume of sample into the column and centrifuge at 3,000 x g for 3 min to bind DNA. Discard the

flow-through and reuse the collection tube for nest step.

10. Insert the column back into the 15 mL collection tube from step 8 and add reminder of lysate from step 7. Centrifuge at 3000 x g for 3 minutes. Discard the collection tube and flow-through liquid.

11. Place the column into a second 15 mL tube and wash by pipetting 3 mL of HB Buffer into the column. Centrifuge at 4,000 x g for 5 minutes. Discard the flow-through liquid and re-use the collection tube.

12. Place the column into a **same 15 mL tube** from step 10 and add 3 mL of DNA Wash Buffer. Centrifuge at 3,000 x g for 3 minutes. Discard the flow-through liquid and re-use the collection tube.

13. Insert the column into **same collection tube from step 11**. Add 3 mL of DNA Wash Buffer into the column and centrifuge at 4500 x g for 15 minutes. ***This step is critical for removal of trace ethanol that might otherwise interfere with downstream applications***. If the centrifugal force is below 4000 x g, incubate the column in 70°C vacuum oven to evaporate the residue ethanol.

14. Place the column into a nuclease-free 15 mL centrifuge tube (not provided) and add 200-500 µL of preheated (70°C) Elution Buffer (10mM Tris-HCl, pH 8.5) or water. Allow tubes to sit for 2 min at room temperature.

15. To elute DNA from the column, centrifuge at 4,000 x g for 5 min. Retain flow-through containing the DNA. For maximum yield, place column into a second 1.5 mL tube and repeat elution step with another 200-500 µL of preheated Elution Buffer or water. Discard column.

Note: First elution typically yields 60%-70% of the DNA bound to the column. Second elution can increase the yield by 20%. However, increasing elution volume reduces the concentration of the final product. Volumes lower than 200 µL greatly reduce yields**.** reduces the concentration of the final product. To obtain DNA at higher concentrations, elution can

be carried out by load the eluted DNA back into the column and perform the second elution.

Maximum Yield Protocol (for up to 10 mL whole blood)

Prepare the Red Blood Lysis Buffer (RBL) as following	
NH_4Cl	155mM
$KHCO_3$	10mM
Na_2EDTA	0.1mM
Adjust to pH 7.4 with 1M Hcl or NaOH	

1. To 1 volume of whole fresh blood (maximum of 10 mL) add 5 volumes of RBL Buffer. For example add 5 mL Buffer RBL to 1 mL blood. Mix by vortexing.

2. Incubate for 15 min on ice, mixing by brief vortexing twice. Lysis of red blood cells is indicated when the solution becomes translucent. Blood samples from individuals with an elevated hematocrit or ECR, extend incubation time to 20 min.

3. Pellet leukocytes by centrifuging at 450 x g for 10 min at 4°C. Completely remove and discard the supernatant containing lysed red blood cells.

4. Wash the white blood cell pellet with 2 volumes of Buffer RBL per volume of whole blood used in step 1. Thoroughly vortex to resuspend cells.

   Tip: If you used 10 mL of whole blood, wash with 20 mL of Buffer RBL.

5. Centrifuge at 450 x g for 10 min at 4°C. Again, completely remove and discard the supernatant.

6. Add 2 mL TL buffer to the pelleted white blood cells and vortex thoroughly to mix.

7. **Add 140ul of OB preotease (D3494) solution, vertex to mix well, and incubate at 55°C in a shaking water bath to effect complete lysis**. If no shaking water bath is available, vortex every 20-30 minutes. Lysis time depend on amount and type of tissue, but usually under 2 hours.

8. Add 10 uL RNase A and mix by briefly vortexing.

9. **Add 2.1 mL Buffer BL and vortex to mix. Incubated** at **70°C** for 10 minutes. A wispy precipitate may form on addition of Buffer BL, but does not interfere with DNA recovery.

10. **Add 2.2 mL absolute ethanol and mix thoroughly by vortexing.** If precipitation can be seen at this point, break the precipitation by passing through a needle using a syringe.

IMPORTANT: THIS AND ALL SUBSEQUENT STEP MUST BE PERFORMED USING A CENTRIFUGE EQUIPPED WHICH IS CAPABLE OF 6,000 X g.

11. Take a HiBind® DNA Midi Column pre-inserted in a 15 ml collection tube (supplied). **Add 1 ml Equilibration Buffer into the column. Let the column sit for 4 minutes at room temperature to equilibrate the membrane.** Centrifuge at 3000 x g for 3 minutes

12. Transfer the 3 mL sample from step 10 into the column including any precipitate that may have formed. Centrifuge at 3,000 x g for 5 min to bind DNA. Discard both flow-through liquid and reuse the collection tube. **Note: Since the column can only contains around 4 mL sample volume, it is necessary to load the column twice.**

13. Place the column back into the 15 mL collection tube and load the rest of the sample from step 10 into the column. Centrifuge at 3000 x g for 5 minutes. Discard the flow-through and collection tube.

14. Place the column into a **new 15 mL collection tube** and wash by pipetting 3 mL of Buffer HB Buffer. Centrifuge at 3,000 x g for 3 mins. **Discard the flow-through liquid and reuse the collection tube**

15. Place the column into same collection tube from step13 and wash the column by pipetting 3.5 mL of DNA Wash Buffer into the column. Centrifuge at 3,000 x g for 3 min. **Discard the flow-through liquid and reuse the collection tube.**

16. Place the column back into same collection **tube** from step 14 and wash by pipetting 3 mL of DNA Wash Buffer into the column. Centrifuge at 4500 x g for 15 min. Discard flow-through liquid and collection tube. **Note: it is critical for removing traces of ethanol that may otherwise interfere with downstream applications. Remove any traces of ethanol from the column using a pipette.**

Option: If the centrifugal force is less than 4000 x g It might be necessary to dry the column further by placing the column in a

vacuum oven at 70°C for 10 minutes.

17. Place column into a clean 15 mL centrifuge tube. Add 200-500 µL Elution Buffer (10 mM Tris-HCl, pH 8.5) or sterile deionized water onto the center of column matrix and centrifuge 5 min at 4500 x g to elute DNA. This represents approximately 70% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration. Alternatively, a second elution may be performed using the first eluate to maintain a high DNA concentration. Preheating the water to 70°C and allowing the column to soak 2 min at room temperature before elution may significantly increase yields.

Vacuum Protocol (for up to 2 mL of whole blood sample)

1. Add up to 2 mL whole blood to a 15 mL centrifuge tube. If the sample less than 2 mL, bring the volume up to 2 mL with 10 mM Tris-HCl, PBS, or Elution Buffer provided.

2. Add 140 µL OB Protease and mix the sample throughly by vortexing.

3. Add 2.1 mL of Buffer BL. Vortex 5 minutes to mix thoroughly.

4. Add 10 µL RNase A solution to each sample to remove RNA.

5. Incubate sample at 70°C for 10 min.

6. Briefly vortex the tube once during incubation.

7. Add 2.2 mL of isopropanol to lysate and mix. ***For buffy coat, isolated leukocytes, and cultured cells, yields will improve if 260 µL absolute ethanol is used in place of isopropanol.***

8. Take a HiBind® DNA Midi Column pre-inserted in a 15 ml collection tube (supplied). **Add 1 ml Equilibration Buffer into the column. Let the column sit for 4 minutes at room temperature to equilibrate the membrane.** Centrifuge at 3000 x g for 3 minutes

9. Insert the HiBind® DNA Midi-spin column on a outlet of vacuum manifold. Transfer the half volume of sample into the column. Apply the vacuum until all the sample pass through the membrane. Turn off the vacuum.

10. Load the remainder of the sample into the column. Apply the vacuum until all the sample pass through the membrane. Turn off the vacuum.

11. Add 3 mL HB Buffer into the column. Apply the vacuum until all the liquid pass through the column. Turn off the vacuum.

12. Add 3 mL DNA wash Buffer into the column. Apply the vacuum until

all the liquid pass through the column. Turn off the vacuum.

13. Add another 3 mL DNA wash Buffer into the column. Apply the vacuum until all the liquid pass through the column. After all the liquid pass through the membrane, apply maximum vacuum for additional 15 minutes to dry the column.

    Note: it is critical for removing traces of ethanol that may otherwise interfere with downstream applications. Remove any traces of ethanol from the column using a pipettor. It might be necessary to dry the column further by placing the column in a vacuum oven at 70°C for 10 minutes.

14. Turn off the vacuum and remove the column from manifold. Insert a HiBind® DNA Midi-spin column in a 15 mL collection tube (provided).

15. Add 200-500 µL Elution Buffer (10 mM Tris-HCl, pH 8.5) or sterile deionized water onto the center of column matrix.

16. To elute DNA from the column, centrifuge at 4,000 x g for 5 min. Retain flow-through containing the DNA. For maximum yield, place column into a second 15 mL tube and repeat elution step with another 200-500 µL of preheated Elution Buffer or water. Discard column.

    Note: Each elution typically yields 60%-70% of the DNA bound to the column. Thus two elutions generally give >80%. However, increasing elution volume reduces the concentration of the final product. To obtain DNA at higher concentrations, elution can be carried out by load the eluted DNA back into the column and perform the second.

Yield and quality of DNA: determine the absorbance of an appropriate dilution (20- to 50-fold) of the sample at 260 nm and then at 280 nm. The DNA concentration is calculated as follows:

DNA concentration = Absorbance$_{260}$ × 50 × (Dilution Factor) µg/mL

High copy number plasmids generally yield up to 1 mg of DNA from 500 mL culture. The ratio of (Abs_{260})/(Abs_{280}) gives an indication of nucleic acid purity. A value greater than 1.8 indicates greater than 90% nucleic acid. Alternatively, quantity (as well as quality) can sometimes best be determined by agarose gel/ethidium bromide electrophoresis by comparison to DNA samples of known concentrations. Typically, the majority of the DNA eluted is in monomeric supercoil form, though concatamers may also be present.

Troubleshooting Guide

Problem	Possible Cause	Suggestions
Clogged Column	Incomplete lysis	Extend incubation time of lysis with Buffer BL and protease. Incubate for specified time at 70°C. It may be necessary to extend incubation time beyond 10 min.
	Sample too large	Do not use more than maximum starting sample volume specified in the protocol.
	Sample too viscous	Divide sample into multiple tubes, adjust volume with 10 mM Tris-HCl proportionately. Replace ethanol instead of isopropanol in step 7.
Low DNA Yield	Clogged column	See above
	Poor elution	Repeat elution or increase elution volume (see note on page 4). Incubation of column at 70°C for 5 min with Elution Buffer may increase yields.
	Improper washing	Wash Buffer Concentrate must be diluted with absolute ethanol (96%-100%) as specified on page 3 before use.
Low A_{260}/A_{280} ratio	Extended centrifugation during elution step.	Resin from the column may be present in eluate. Avoid centrifugation at speeds higher than specified. The material can be removed from the eluate by centrifugation — it will not interfere with PCR or restriction digests.
	Poor cell lysis due to incomplete mixing with Buffer BL	Repeat the procedure, this time making sere to vortex the sample with Buffer BL immediately and completely.
	Incomplete cell lysis or protein degradation due to insufficient incubation.	Increase incubation time with Buffer BL and protease.
	Samples are rich in protein.	After applying to column, wash with 3 mL of a 1:1 mixture of Buffer BL and ethanol and then with DNA Wash Buffer.
No DNA Eluted	Poor cell lysis due to improper mixing with Buffer BL.	Mix thoroughly with Buffer BL prior to loading HiBind® column.
	Absolute ethanol not added to Buffer BL.	Before applying sample to column, an aliquot of Buffer BL/ethanol must be added. See protocol above.
	No ethanol added to Wash Buffer Concentrate.	Dilute Wash Buffer with the indicated volume of absolute ethanol before use.