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Introduction

DNA isolation from fish or avian blood sample can be difficult because it contains nucleated red blood cells. E.Z.N.A.® NRBC Blood DNA Kit is designed for isolating genomic DNA from fresh, or ethanol preserved blood samples containing nucleated red blood cells. The kit allows single or multiple, simultaneous processing of samples in under 60 minutes. There is no need for phenol/chloroform extractions, and time-consuming steps such as CsCl gradient ultracentrifugation are eliminated. Purified DNA obtained with the E.Z.N.A.® Blood DNA Kit will be ready for applications such as PCR, Southern Blotting, and Restriction Digestion.

E.Z.N.A.® NRBC Blood DNA Kit uses the reversible nucleic acid-binding properties of HiBind® matrix, combined with the speed of mini-column spin technology. A specifically formulated buffer system allows genomic DNA 30-60 kb to bind to the matrix. Samples are first lysed under denaturing conditions and then applied to the HiBind® DNA spin columns to which DNA binds, while cellular debris, hemoglobin, and other proteins are effectively washed away. High quality DNA is finally eluted in sterile deionized water or low salt buffer.

Storage and Stability

All components of the E.Z.N.A.® NRBC Blood DNA Kit, except the Proteinase K should be stored at 22°C-25°C. Once reconstituted, Proteinase K must be stored at -20°C. Under these conditions, DNA has successfully been purified and used for PCR after 24 months of storage. Under cool ambient conditions, a precipitate may form in the Buffer BL. In case of such an event, heat the bottle at 37°C to dissolve. Store Buffer BL at room temperature.

Expiration Date: All E.Z.N.A.® NRBC Blood DNA Kit components are guaranteed for at least 24 months from the date of purchase when stored at 22-25°C.

Binding Capacity

Each HiBind® DNA column can bind approximately 100 µg DNA. Using greater than 10 ul ml whole blood is not recommended.

Product Number	D0715-00	D0715-01	D0715-02
Purification times	5 Preps	50 Preps	200 Preps
HiBind® DNA columns	5	50	200
2 ml Collection Tubes	15	150	600
RL1 Buffer	5 ml	20 ml	60 ml
Buffer BL	5 ml	20 ml	60 ml
Buffer HB	5 ml	30 ml	110 ml
DNA Wash Buffer	1.5 ml	15 ml	3 x 25 ml
Equilibration Buffer	1.5	7 ml	25 ml
Elution Buffer	5 ml	40 ml	160 ml
Proteinase K	155 µl	3 mL	8 mL
Proteinase Storage Buffer	5 ml	25 ml	25 ml
User Manual	1	1	1

Buffer BL contains a chaotropic salt. Equilibration Buffer contains Sodium Hydroxide. Use gloves and protective eyeware when handling this solution.

Before Starting

IMPORTANT	1 Reconstitute Proteinase K in Proteinase Storage Buffer as follows: D0715-00 Add 150 µl of Proteinase Storage Buffer D0715-01 Add 1.5ml of Proteinase Storage Buffer D071502 Add 6.0 ml of Proteinase Storage Buffer Vortex vial briefly prior to use. We recommend that you aliquot and store vials of reconstituted protease at -20°C.
	2 DNA Wash Buffer Concentrate must be diluted with absolute ethanol (96-100%) as follows: **D0715-00** Add 6 ml absolute ethanol **D0715-01** Add 60 ml absolute ethanol **D0715-02** Add 100 ml absolute ethanol per bottle ***Store diluted DNA Wash Buffer at room temperature***

All centrifugation steps must be carried out at room temperature.

A. Protocol for Isolating DNA from Blood with Nucleated Red blood Cells

Materials and equipments Supplied by User

- **Tabletop microcentrifuge and sterile 1.5 ml tubes.**
- **Water bath -** set to 65°C.
- **Ethanol -** approximately 0.3 ml per sample.
- **RNase A -** Prepare a stock solution of RNase A at 25 mg/ml.

NOTE: The procedure below has been optimized for use with FRESH or ethanol fixed blood samples of 1 to 15 µl in volume.

Bring samples and Proteinase K solution to room temperature and have a water bath equilibrated to 65°C. Preheat an aliquot of Elution Buffer (approximately 0.4 ml per sample) at 65°C. **Carry out all centrifugation steps at room temperature**.

1. **Add 1 - 4 µl of blood sample to a sterile microcentrifuge tube. Add 250 µl of RL1 Buffer**. Mix throughly by vortexing for 10 seconds.

2. **Add 25 µl Proteinase K (20mg/ml) and 275 µl of Buffer BL.** Vortex at maxi speed for 15s to mix thoroughly.

3. **Incubate the sample at 65°C for 10 min.**

4. **Briefly vortex the tube once during incubation**. Optional: If RNA-free genomic DNA is required, add 5µl RNase A (25mg/ml) to each sample and incubate at room temperature for 5 minutes .

5. **Add 275 µl of absolute ethanol (room temperature, 96-100%) to lysate and vortex at maxi speed for 20s to mix thoroughly.** Briefly centrifuge the tube to collect any drops from the inside of the lid.

6. **Assemble an HiBind® DNA column in a 2 ml collection tube (provided).** Add 100µl of Equitation Buffer to each column. Wait 3-4 minutes at room temperature. Centrifuge at maximum speed for 30 seconds.

7. Transfer the lysate from step 5 into the column and centrifuge at ≥10,000 x g for 1 min to bind DNA. Discard the collection tube and flow-through liquid.

8. **Place the column into a second 2 ml tube (provided) and add 500 µl of Buffer HB.** Centrifuge at ≥10,000 x g for 1 min. Discard flow-through liquid and reuse the collection tube for next step.

9. **Place the column into a same 2 ml tube from step 7 and wash the column by pipetting 700 µl of DNA Wash Buffer diluted with ethanol.** Centrifuge at ≥10,000 x g for 1 min. Again, dispose of collection tube and flow-through liquid.

   ***Note**: DNA Wash Buffer is provided as a concentrate and must be diluted with absolute ethanol as indicated on the bottle and page 3. If refrigerated, the*

diluted DNA wash buffer must be brought to room temperature before use.

10. **Using a new collection tube, wash the column with a second 700 µl of DNA Wash Buffer and centrifuge as above.** Discard flow-through and re-use the collection tube for next step.

11. **Place the empty column into the same 2 ml collection tube form step 10, centrifuge at maximum speed for 2 min to dry the column.** This step is crucial for ensuring optimal elution in the following step.

12. **Place the column into a sterile 1.5 ml microfuge tube and add 100-200 µl of preheated (65°C) Elution Buffer (10mM-Tris-HCl, pH 8.5).** Allow tubes to sit for 5 min at room temperature.

13. **To elute DNA from the column, centrifuge at maximum speed (≥14,000 x g) for 1 min. Retain flow-through containing the DNA**. Place column into a second 1.5 ml tube. Elute DNA again as step 11-12. Discard column and store the eluted DNA at -20°C

Note: *First elution typically yields 60%-70% of the DNA bound to the column. Thus two elution generally give >90%. However, increasing elution volume reduces the concentration of the final product. To obtain DNA at higher concentrations, elution can be carried out using 50 µl to 100 µl Elution Buffer. Volumes lower than 50 µl greatly reduce yields. Alternatively use the first eluate to perform the second elution.*

If necessary the DNA can be concentrated. Add sodium chloride to a final concentration of 0.1 M followed by 2 x volume of absolute (100%) ethanol. Mix well and incubate at -20°C for 10 min. Centrifuge at 14,000 x g for 10 min and discard supernatant. Add 700 µl of 80% ethanol and centrifuge at 10,000 x g for 2 min. Discard supernatant, air dry the pellet (2 min) and resuspend DNA in 20 µl sterile deionized water or 10 mM Tris-HCl, pH 8. .

B. Vacuum/Spin Protocol

1. **Prepare Blood lysate by following step 1-6 of Spin Protocol on page 4.**

2. **Insert the HiBind® DNA column into the vacuum manifold.** Carefully apply the 700µl lysate to an HiBind® DNA column. Turn on the vacuum source to draw all liquid through the column. Turn off the vacuum.

   Note: *If the lysate has difficulty to pass through the column at this stage. Place the column into a collection tube (supplied). Close the lid and centrifuge at 10,000 x g for 5 minutes or until all liquid pass through the column. Place the column into another collection tube (supplied) and continue step 7 of the spin protocol.*

3. Apply remaining lysate into the column. Turn on the vacuum source to draw all liquid through the column. Turn off the vacuum.

4. **Add 500 µl of Buffer HB into the column**. Turn on the vacuum source to draw all liquid through the column. Turn off the vacuum.

5. **Wash the column by pipetting 700 µl of DNA Wash Buffer diluted with ethanol into the column.** Turn on the vacuum source to draw all liquid through the column. Turn off the vacuum.

6. **Close the lid of HiBind® DNA column, remove it from the vacuum manifold.** Insert the column into a collection tube (supplied) and centrifuge at maximum speed for 2 minute to completely dry the column.

7. **Place the column into a sterile 1.5 ml microfuge tube and add 100-200 µl of preheated (65°C) Elution Buffer (10mM Tris-HCl, pH 8.5).** Allow tubes to sit for 5 min at room temperature.

8. **To elute DNA from the column, centrifuge at maximum speed for 1 min.** Retain flow-through containing the DNA. Place column into a second 1.5 ml tube. Elute DNA again as step 7-8. Discard column and store the eluted DNA at -20°C

C. Isolate DNA from NRBC Blood from Buccal Swab or dried blood spots collected with filter paper

Material and Equipments required

- **Tabletop microcentrifuge and sterile 1.5 ml tubes**
- **Water bath -** set to 65°C
- **Absolute Ethanol -** approximately 0.3 ml per sample
- **RNase A(Optional)** - Prepare a stock of RNase A at 50mg/ml

1. **Place the buccal swab or paper contains dried blood spot in a 2 ml centrifuge tube and add 500 µl RS1 Buffer in the tube**. If RNA-free DNA is required for downstream application, add 4 µl RNase A (25mg/ml) into the sample.

2. **Add 25µl OB Protease and 500µl Buffer BL into sample.** Mix by vortexing at maxi speed for 30s.

3. **Incubate at 65°C for 10 minutes.** Collect any liquid drop from lid by brief centrifugation.

4. Centrifuge at 14,000 x g for 10 minutes. Carefully transfer the supernatant to a new 1.5 ml tube.

5. **Add equal volume of absolute ethanol (room temperature, 96-100%) to the sample and mix by vortexing at maxi speed for 15 seconds**. Collect any liquid drop from lid by brief centrifugation.

6. **Assemble an HiBind® DNA column in a 2 ml collection tube (provided).** Add 100µl of Equitation Buffer to each column. Wait 3-4 minutes at room temperature. Centrifuge at maximum speed for 30 seconds.

7. **Carefully apply entire sample into HiBind® DNA.** Centrifuge at 10,000 x g for 1 minute. Discard the flow-through and collection tube.

8. Place the column into a new 2 ml collection tube (provided). Centrifuge at 10,000 x g for 1 minute. Discard the flow-through and collection tube.

9. Place the column into a new 2 ml collection tube (provided). **Add 500µl of HB Buffer into the column.** Centrifuge at 10,000 x g for 1 minute. Discard the flow-through and reuse the collection tube in next step.

10. Place the column into a same 2 ml collection tube from step 7. **Add 700µl of DNA Wash Buffer diluted with absolute ethanol into the column.** Centrifuge at 8000 x g for 1 minute. Discard the flow-through and reuse the collection tube in next step.

    Note: DNA Wash Buffer is provided as a concentrate and must be diluted with absolute ethanol as indicated on the bottle and page 3. If refrigerated, the diluted wash buffer must be brought to room temperature before use.

11. Place the column into a same 2 ml collection tube from step 8. **Centrifuge at maximum for 2 minutes to completely dry the HiBind® DNA column**. Discard the flow-through and the collection tube.

12. Place the column into a sterile 1.5 ml microcentrifuge tube and add 50-100 µl of preheated 65^{0}C) Elution Buffer (10mM Tris-HCl, pH 8.5). Allow tubes to sit for 5 min at room temperature.

13. To elute DNA from the column, centrifuge at maximum for 1 min. Retain flow-through containing the DNA. Place column into a second 1.5 ml tube. Elute DNA again as step 10-11. Discard column and store the eluted DNA at -20^{0}C.

Troubleshooting Guide

Problem	Possible Cause	Suggestions
Clogged Column/ low yield	Incomplete lysis	Add the correct volume of Buffer RL1 and BL and incubate for specified time at 70°C. It may be necessary to extend incubation time by 10 min.
	Sample too large	If using more than 10 µl of blood, increase volumes of OB Protease/Proteinase K, Buffer RL1, Buffer BL, and isopropanol. Pass aliquots of lysate through one column successively.
	Sample too viscous	Divide sample into multiple tubes, diluted sample with PBS.
	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	DNA Wash Buffer Concentrate must be diluted with absolute (100%) ethanol as specified on page 5 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.
	Hemoglobin remains on column	After application of sample to column, wash once with 300 µl Buffer BL.
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.
Washing leaves colored residue in column	Incomplete lysis due to improper mixing with Buffer BL.	Buffer BL is viscous and the sample must be mixed thoroughly.
	No ethanol added to DNA Wash Buffer Concentrate.	Dilute Wash Buffer with the indicated volume of absolute ethanol before use.
Eluted material has red/brown color	Sample volume too large.	Reduce sample volume and follow directions