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invitrogen bead separations

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Dynabeads® M-270 Carboxylic Acid

For research use only

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1. PRODUCT DESCRIPTION

Dynabeads® M-270 Carboxylic Acid are uniform, monosized superparamagnetic beads composed of highly cross-linked polystyrene with magnetic material precipitated in pores evenly distributed throughout the particles. The particles are further coated with a hydrophilic layer of glycidyl ether, concealing the iron oxide inside the Dynabeads. Carboxylic acid groups are then introduced on the surface.

Their hydrophilic surface ensures low non-specific binding, excellent dispersion abilities and easy handling in a wide variety of buffers. Dynabeads M-270 Carboxylic Acid are supplied in an aqueous suspension.

2. PRINCIPLE

The Dynabeads M-270 Carboxylic Acid is designed to act as a solid support for a wide variety of biomagnetic separations and manipulations. Their size makes them particularly suitable for isolation of proteins.

They can be applied to protein applications such as sample preparation, bioassays or the selection of affinity binders. The very rapid and gentle coupling chemistry of the ligand-immobilisation reaction make them very useful in coupling labile proteins, peptides and functional enzymes for the isolation of a wide variety of targets (e.g. hormones, receptors, disease markers, bacteriophages etc).

Due to the gentle pull to the magnet, the 2.8 µm Dynabeads can also be used for the isolation of fragile cells. For cell separation in general, Invitrogen Dynal recommends the use of the larger 4.5 µm Dynabeads.

Activation of the Dynabeads M-270 Carboxylic Acid can be performed with a carbodiimide followed by coupling of an amine containing ligand, resulting in a stable amide bond between the bead and the ligand. The mechanism and optimisation of carbodiimide mediated amide bond formation is extensively discussed in the literature (1,2,3,4). Alternatively, a bifunctional cross-linker may be used to introduce other functional groups like thiol, amine, maleimide etc.

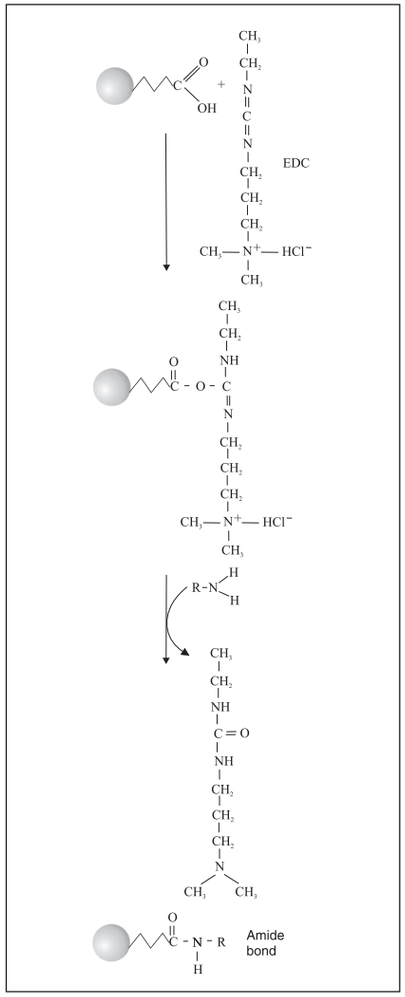
If the ligand to be bound is an oligonucleotide, it does not contain a primary amino function. This can be introduced by e.g. using 5'-amino modified oligonucleotides. Please note that other amino-groups in the oligonucleotide might to some degree react with the carboxylic acid groups on the beads, resulting in coupling via the internal bases.

Once coupled with your ligand, the Dynabeads can be added to a cell lysate or other suspensions containing your target molecule. After a short incubation allowing affinity capture of the target, the Dynabeads are pulled to the side of the test-tube by the use of a magnet (Dynal® MPC™) allowing aspiration of unbound material. Furthermore, the magnetic separation facilitates washing and concentration of the isolated target bound to the beads. Dynabeads with bound target molecule can be used directly in downstream bioassays, or can be boiled in application buffer and analysed on SDS-PAGE. Alternatively, the target molecule can be eluted off the Dynabeads with conventional elution methods such as high salt, low pH etc.

3. INSTRUCTIONS FOR USE

For coating of a ligand to Dynabeads M-270 Carboxylic Acid, Invitrogen Dynal recommends to use 20 µg pure protein or ~700 pmol oligonucleotides/peptides per mg Dynabeads and a final concentration of 10-30 mg beads per ml during incubation.

The suggested protocols described in sections 3.1 and 3.2, illustrate an example using 3 mg of Dynabeads, but should be scaled up or down to suit specific needs. It is recommended that the protocols are optimised to meet your requirements (e.g. sample volume, concentration of ligand/beads/EDC, MES buffer volume and pH).



Calculations:

The concentration of the supplied beads is 30mg/ml. 3mg beads = 100 µl.
Protein concentration is in this example set to 1mg/ml.
Given that 20 µg protein is required per mg Dynabeads, the required amount of protein in this case is 60 µg. This corresponds to a volume of 60 µl ligand.
The required amount of EDC varies depending on the performed coating procedure in below.
The final sample volume should be 100 µl to meet the recommendation of final Dynabeads concentration of 30 mg/ml.
In brief, according to the calculation above you require:
100 µl of washed Dynabeads (see the washing step under each protocol)
60 µl ligand.
MES Buffer to adjust the final volume to 100 µl.

3.1 Activation and Coupling of Ligand

The traditional procedure for ligand coupling is the formation of an amide bond between a primary amino group of the ligand and the carboxylic acid groups on the surface of the Dynabeads, mediated by carbodiimide activation. The intermediate product of the reaction between the carboxylic acid and the carbodiimide is very labile and will hydrolyse quickly. To get the desired immobilisation of the ligand it is therefore important to have the ligand immediately available. Alternatively, the activated Dynabeads can be captured as a less labile intermediate, like an N-hydroxyl succinimide ester (use NHS, MW 115.1 or sulfo-NHS, MW 217.1), and then react with the ligand over a longer period.

There are several alternative protocols for carbodiimide-mediated immobilization of ligand by amide bond formation:

One-step protocol (see protocol 3.1a below):

1. Mix ligand and Dynabeads.
2. Add carbodiimide and incubate the reaction mixture.
3. Wash the Dynabeads and resuspend in buffer.

Two-step protocol (see protocol 3.1b below):

1. Add carbodiimide to the Dynabeads.
2. After 30 minutes incubation, quickly wash the Dynabeads in cold water followed by cold buffer.
3. Add the ligand to the Dynabeads and incubate further.
4. Wash the Dynabeads and resuspend in buffer.

Two-step protocol with NHS (see protocol 3.1c below):

1. Add a mixture of carbodiimide and N-hydroxy succinimide to the Dynabeads.
2. After 30 minutes incubation, wash the Dynabeads to remove excess carbodiimide.
3. Add the ligand to the Dynabeads and incubate further.
4. Wash the Dynabeads and resuspend in buffer.

The one-step protocol is recommended when using ligands that do not contain carboxylic acid groups (e.g. oligonucleotides). If the ligand does contain carboxylic acid groups, these may react with the carbodiimide and cause polymerisation of the ligand. Since this method is less laborious and generally gives higher yields, it may however still be advantageous to use this method if it is possible to add ligand in excess to compensate for the loss due to polymerisation.

The two-step protocol is preferred when the ligand contains carboxylic acid groups and you have limited amounts of the ligand available. The two-step protocol without NHS requires a very fast wash of the beads in cold buffer after the activation. The two-step protocol with NHS should be used if the ligand is in an alkaline buffer or a buffer with high phosphate concentration.

1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, MW 191.7) is the carbodiimide most commonly used. It is very soluble in water and relatively stable as an aqueous solution. For some applications, other carbodiimides may slightly improve the results. As an example: When coupling IgG or IgM, without using NHS, the more hydrophobic carbodiimide N-cyclohexyl-N'-(2-morpholinoethyl) carbodiimide methyl-p-toluensulfonate (CMC, MW 423.6) generally improves yield and orientation of the immobilised antibody.

3.1a One-Step Coating Procedure

1. Wash Dynabeads M-270 Carboxylic Acid twice with 25 mM MES, pH 5, using the equal volume of Dynabeads (100 µl) pipetted out of the vial, for ten minutes with good mixing (en-over-end or similar).
2. Add the required amount of ligand (60 µg) in 25 mM MES, pH 5 (60 µl) to the washed Dynabeads. Mix well and incubate with slow tilt rotation at room temperature for 30 minutes.
3. Immediately before use dissolve EDC in cold 100 mM MES, pH 5 to a concentration of 100 mg/ml.
4. Add 30 µl EDC solution (3 mg) to the Dynabeads/ligand suspension. Mix well.
5. Add 10 µl of 25 mM MES, pH 5 to final volume of 100 µl.
6. Incubate for 2 hours or longer (over night) at 4°C with slow tilt rotation.
7. Wash the coated Dynabeads as described below (see protocol 3.2).

3.1b Two-Step Coating Procedure (without NHS)

I) Activation with EDC

1. Wash Dynabeads M-270 Carboxylic Acid twice with 0.01 M NaOH, using the equal volume of Dynabeads (100 µl) pipetted out of the vial, for ten minutes with good mixing (en-over-end or similar).
2. Wash three times with 100 µl de-ionised water in the same manner. Remove excess liquid.
3. Dissolve the EDC in cold, de-ionised water to 19-76 mg/ml (0.1-0.4M). Add 100-200 µl of EDC-solution to the Dynabeads. Vortex to mix properly.
4. Incubate for 30 minutes at room temperature with slow tilt rotation.
5. After incubation, place the tube on the magnet for 4 minutes and remove the supernatant. Wash once with cold, de-ionised water and once with 50 mM MES, pH 5, as quickly as possible to avoid hydrolysis of the activated carboxylic acid groups.

The Dynabeads are now activated and ready for coating with a ligand containing primary amine groups. Activated beads cannot be stored and you should proceed directly to the next step:

II) Immobilisation of ligand after activation

1. Remove the wash solution used in step 5 above. Add the required amount of ligand (60 µg) in 50 mM MES, pH 5 (60 µl) to activated Dynabeads.
2. Add 40 µl of 50 mM MES, pH 5 to final volume of 100 µl. Vortex to ensure good mixing.
3. Incubate for at least 30 minutes at room temperature, or 2 hours at 4°C, with slow tilt rotation.
4. After incubation, place the tube on the magnet for 4 minutes and remove the supernatant.
5. Wash the coated Dynabeads as described below (see protocol 3.2).

3.1c Two-Step Coating Procedure using NHS

I) Activation with EDC and NHS:

1. Wash Dynabeads M-270 Carboxylic Acid twice with 25 mM MES, pH 5, using the equal volume of Dynabeads (100 µl) pipetted out of the vial, for ten minutes with good mixing (en-over-end or similar).
2. Immediately before use dissolve EDC in cold 25 mM MES, pH 5 to a concentration of 50 mg/ml.
3. Similarly, prepare a 50 mg/ml solution of NHS in 25 mM MES, pH 5.
4. Add 50 µl of EDC solution and 50 µl of NHS solution to the washed Dynabeads. Mix well and incubate with slow tilt rotation at room temperature for 30 minutes.
5. After incubation, place the tube on the magnet for 4 minutes and remove the supernatant. Wash twice with 100 µl of 25 mM MES, pH 5.

The Dynabeads are now activated and ready for coating with a ligand containing primary amine groups.

II) Immobilisation of ligand after activation

1. Add the required amount of ligand (60 µg) in 25 mM MES, pH 5 (60 µl) to activated Dynabeads.

2. Add 40 µl 25 mM MES, pH 5 to final volume of 100 µl. Vortex to ensure good mixing.
3. Incubate for at least 30 minutes at room temperature, or 2 hours at 4°C, with slow tilt rotation.
4. After incubation, place the tube on the magnet for 4 minutes and remove the supernatant.
5. Wash the coated Dynabeads as described below (see protocol 3.2).

3.2 Washing of Coated Beads

All immobilisation procedures require washing of the coated Dynabeads to remove excess ligand and to block un-reacted surface.

NOTE: In order to quench the non reacted activated carboxylic acid groups, incubate the Dynabeads coated with ligand with either 50 mM Tris pH 7.4 for 15 minutes or 50 mM ethanolamine in PBS pH 8.0 for 60 minutes, both at room temperature with slow tilt rotation.

1. Wash the coated Dynabeads a total of four times with 100 µl PBS or 50 mM Tris. Blocking protein like BSA or skimmed milk powder may be added to a concentration of 0.1 - 0.5 % when it does not interfere with downstream applications of the Dynabeads. Also 0.1% Tween-20 or Triton X-100 can be added during washes to reduce nonspecific binding.
2. Resuspend the coated Dynabeads to the desired concentration in PBS or a Tris storage buffer. The Dynabeads are now ready for use.

Store the coated Dynabeads at 2-8°C. Addition of 0.1 - 0.5 % protein (BSA) and/or 0.01 - 0.1 % Tween-20 or Triton X-100 is recommended to stabilize the immobilised ligand and increase the ease of handling. Coated Dynabeads can usually be stored for several months at this temperature, depending on the stability of the immobilised ligand. A final concentration of 0.02% (w/v) sodium azide (NaN_3) may be added as a bacteriostatic agent. If the coated Dynabeads are stored for more than two weeks, they should be washed twice for five minutes with a buffer suitable for the application prior to use.

3.3 Isolation of Target Molecule

Efficient isolation of target molecules using Dynabeads is dependent on the bead-concentration, target molecule concentration, the ligand's affinity for the target molecule and time. Binding is performed from 10 minutes to 1 hour, at a recommended concentration of 1-10 x 10^9 beads/ml. Target-ligand equilibrium is reached after approximately 1 hour.

1. Add sample containing target molecule to the coated Dynabeads (3 mg beads). For a 100 kD protein, use a volume containing approximately 25 µg target molecule to assure an excess of this molecule.
2. Incubate the mixture with tilting and rotation for one hour to capture the target (incubation times as low as 10 minutes can be used with concentrated protein samples in volumes close to what was originally pipetted from the vial).
3. Place the tube on the magnet for 4 minutes to collect the Dynabeads at the tube wall. For viscous samples, increase the time on the magnet. Pipette off the supernatant.
4. Wash the Dynabeads 3 times using 1 ml PBS each time and exchanging buffers by the use of the magnet, according to protocol 3.2.

Efficient isolation of target molecules using Dynabeads is dependent on the bead-concentration, target molecule concentration, the ligand's affinity for the target molecule and the specific binding kinetics involved.

The concentration of required Dynabeads will depend on the size of your specific molecule. Also the salt-concentration and pH of the chosen binding, washing and elution buffers can be varied depending on the type of molecule to be immobilised. Similarly, the selected buffer used in the downstream application should be optimised for the specific application.

The size of the Dynabeads M-270 Carboxylic Acid presents a high surface area per mg beads and a corresponding high capacity for the target molecule. The effective binding capacity will depend on the size of the specific molecules to be immobilized.

As the Dynabeads M-270 Carboxylic Acid will not inhibit enzymatic activity, bead-bound material can be used directly in downstream analysis. Alternatively, the target molecule can be eluted off the Dynabeads following conventional elution methods.

3.4 Target Protein Elution Procedure

Conventional elution methods can be applied for the elution of target protein from the Dynabeads. Low pH (2.8-3.5), change in ionic strength, affinity elution, electrophoresis, polarity reducing agents, deforming eluants can be applied, or even boiling the bead-target complex in SDS-PAGE application buffer for direct characterization of protein on SDS-PAGE. The method of choice depends on the affinity of the specific target molecule to the ligand coated onto the Dynabeads, the stability of the target molecule and the downstream application and detection method. Most proteins will be eluted off at pH 3.1 following the procedure described below, but some protein functionality might be lost under such harsh conditions. If maintaining functionality of the target molecule is important, try milder elution conditions first such as high salt (e.g. 2M NaI) or stepwise elution reducing pH from 6 down to 3. This is also recommended if the bead-bound ligand must remain functional to allow reuse of the Dynabeads.

1. Add 30 µl 0.1 M citrate (pH 3.1) to the Dynabeads with immobilized target.
2. Mix well by tilting and rotation for 2 minutes.
3. Place the test tube on the magnet and transfer the supernatant, containing purified target, to a clean tube.
4. Add additional 30 µl 0.1 M citrate (pH 3.1) to the Dynabeads to elute any remaining target.
5. Mix well by tilting and rotation for 2 minutes.
6. Place the test tube on the magnet, pipette off the eluate and pool the supernatants containing pure target molecule.

Total collected volume = 60 µl

To ensure reuse of the Dynabeads and functionality of the isolated target molecule, bring both the Dynabeads and the target molecules back to physiological pH (7.4) immediately after elution.

4. GENERAL INFORMATION

Invitrogen Dynal AS complies with the Quality System Standards ISO 9001:2000 and ISO 13485:2003.

4.1. Product Characteristics

Typical characteristics for any given batch of this product:

Diameter:	2.8 µm
Density:	1.6 g/cm^3
Specific surface area:	2-5 m^2/g beads
Active chemical functionality:	150 µmol/g beads
Concentration:	2 x 10^9 beads/ml (approx. 30 mg/ml)

Certificate of Analysis (CoA) is available upon request. Material Safety Data Sheet (MSDS) is available at 'www.invitrogen.com'.

4.2. References

1. Nakajima N and Ikade Y, "Mechanism of Amide Formation by Carbodiimide for Bioconjugation in Aqueous Media", Bioconjugate Chem. 1995, 6(1),123-130.
2. Gilles MA, Hudson AQ and Borders CL Jr, "Stability of water-soluble carbodiimides in aqueous solution", Anal Biochem. 1990 Feb 1;184(2):244-248.
3. Sehgal D and Vijay IK, "A method for the high efficiency of water-soluble carbodiimide-mediated amidation", Anal Biochem. 1994 Apr;218(1): 87-91.
4. Szajani B et al, "Effects of carbodiimide structure on the immobilization of enzymes", Appl Biochem Biotechnol. 1991 Aug;30(2):225-231.

4.3. Additional Material Needed

- Magnetic device (Dynal MPC, Magnetic Particle Concentrator)
- Mixing/rotation device
- Test tubes, glassware and pipettes
- Ligands
- Buffers/solutions (see below)

4.4. Recommended Buffers/Solutions

Listed below are some recommended buffers for use with Dynabeads M-270 Carboxylic Acid.

0.01 M NaOH: 0.4 g NaOH (MW 40.0) dissolved in 1,000 ml distilled water.

100 mM MES pH 5: 2.13 g MES (2-[N-morpholino]ethane sulfonic acid, MW 213.25). Dissolve in 90 ml distilled water, adjust to pH 5 and adjust to 100 ml.

0.05 M Tris pH 7.4: 0. 79 g Tris HCl (MW 157.6) Dissolve in 90 ml distilled water, adjust to pH 7.4 and adjust to 100 ml.

0.1 M Citrate pH 3.1: 2.10 g citric acid ($C_6H_8O_7$ x H_2O, MW 210.14). Dissolve in 90 ml distilled water, adjust to pH 3.1 and adjust to 100 ml.

2 M NaI: 3 g NaI (MW 149.9) to 10 ml distilled water.

PBS pH 7.4 (phosphate buffered saline): 0.26 g NaH_2PO_4 x H_2O (MW 137.99) 1.44 g Na_2HPO_4 x $2H_2O$ (MW 177. 99) 8.78 g NaCl (MW 58.5). Dissolve in 900 ml distilled water, adjust pH if necessary and adjust to 1,000 ml. PBS with 0.1% (w/v) BSA/HSA/skimmed milk: Include 0.1% (w/v) BSA/HSA/skimmed milk (0.1 g) in 100 ml PBS (above). PBS/Tween 20/Triton X: Include 0.5-1.0 % (w/v) Tween 20/Triton X (50-100 mg) in 100 ml PBS (above).

If a preservative is needed for storage of coated Dynabeads, a final concentration of 0.02% (w/v) sodium azide (NaN_3) may be added to the storage buffer. This preservative is cytotoxic and must be carefully removed before use by washing. Required safety precautions must be followed when handling this material.

4.5. Storage & Stability

When stored in unopened vials at 2-8°C, Dynabeads M-270 Carboxylic Acid are stable until the expiration date printed on the label.

Dynabeads M-270 Carboxylic Acid coated with antibody may be stored at 4°C for several months without loss of antigen binding capacity. Coated Dynabeads M-270 should be washed once before use.

Dynabeads M-270 Carboxylic Acid should not be autoclaved, but can be incubated with ethanol (70%, 1 hour) or gamma irradiated after freeze drying.

Dynabeads M-270 Carboxylic Acid should be washed once before use (see washing procedure above).

Precautions should be taken to prevent bacterial contamination of the antibody-coated Dynabeads. If cytotoxic preservatives are added, these must be carefully removed before use by washing.

Primary antibody coated Dynabeads stored for more than two weeks should be washed once for 5 min in PBS/BSA before use.

4.6. Warnings & Limitations

This product is for research use only. Not intended for any animal or human therapeutic or diagnostic use unless otherwise stated.

Sodium azide is toxic if ingested. Avoid pipetting by mouth.

Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. When disposing through plumbing drains, flush with large volumes of water to prevent azide buildup.

Intellectual Property Disclaimer

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4.9 Warranty

The products are warranted to the original purchaser only to conform to the quantity and contents stated on the vial and outer labels for the duration of the stated shelf life. Invitrogen Dynal's obligation and the purchaser's exclusive remedy under this warranty is limited either to replacement, at Invitrogen Dynal's expense, of any products which shall be defective in manufacture, and which shall be returned to Invitrogen Dynal, transportation prepaid, or at Invitrogen Dynal's option, refund of the purchase price.

Claims for merchandise damaged in transit must be submitted to the carrier.

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