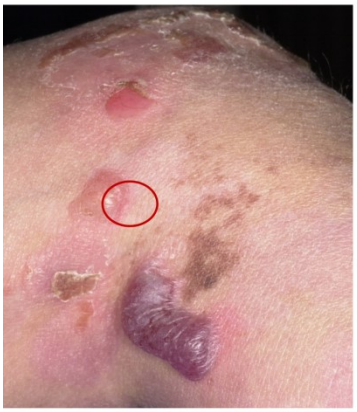
# Supporting information

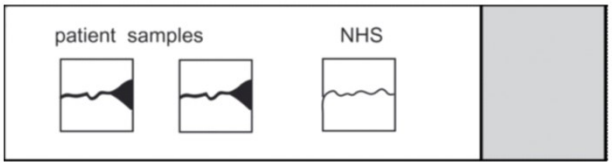
## Appendix 2. Supplementary Figures

**Supplementary Figure 1. Biopsy and IFM for EB laboratory diagnosis.** a) Biopsy for IFM including part of a fresh blister. b) Slide preparation for IFM: frozen sections of patient´s and normal human skin (NHS) placed on one slide. c) Immunofluorescence images on NHS and EB skin using specific monoclonal antibodies to collagen IV, keratin 14, laminin-332 and collagen VII. Upper panels: epidermolysis bullosa simplex (EBS). Note EBS (AR) skin: no immunoreactivity for keratin 14, as compared to normal staining in NHS. Middle panels: junctional epidermolysis bullosa (JEB). In lesional skin, note the absent staining of laminin-332 as compared to normal staining pattern in NHS; normal staining of collagen IV and VII to the floor of the blister (*). Lower panels: dystrophic epidermolysis bullosa (DEB). In lesional skin, note the absent staining of collagen VII as compared to normal staining pattern in NHS; normal staining of collagen IV, laminin-332 and keratin 14 to the roof of the blister (*).

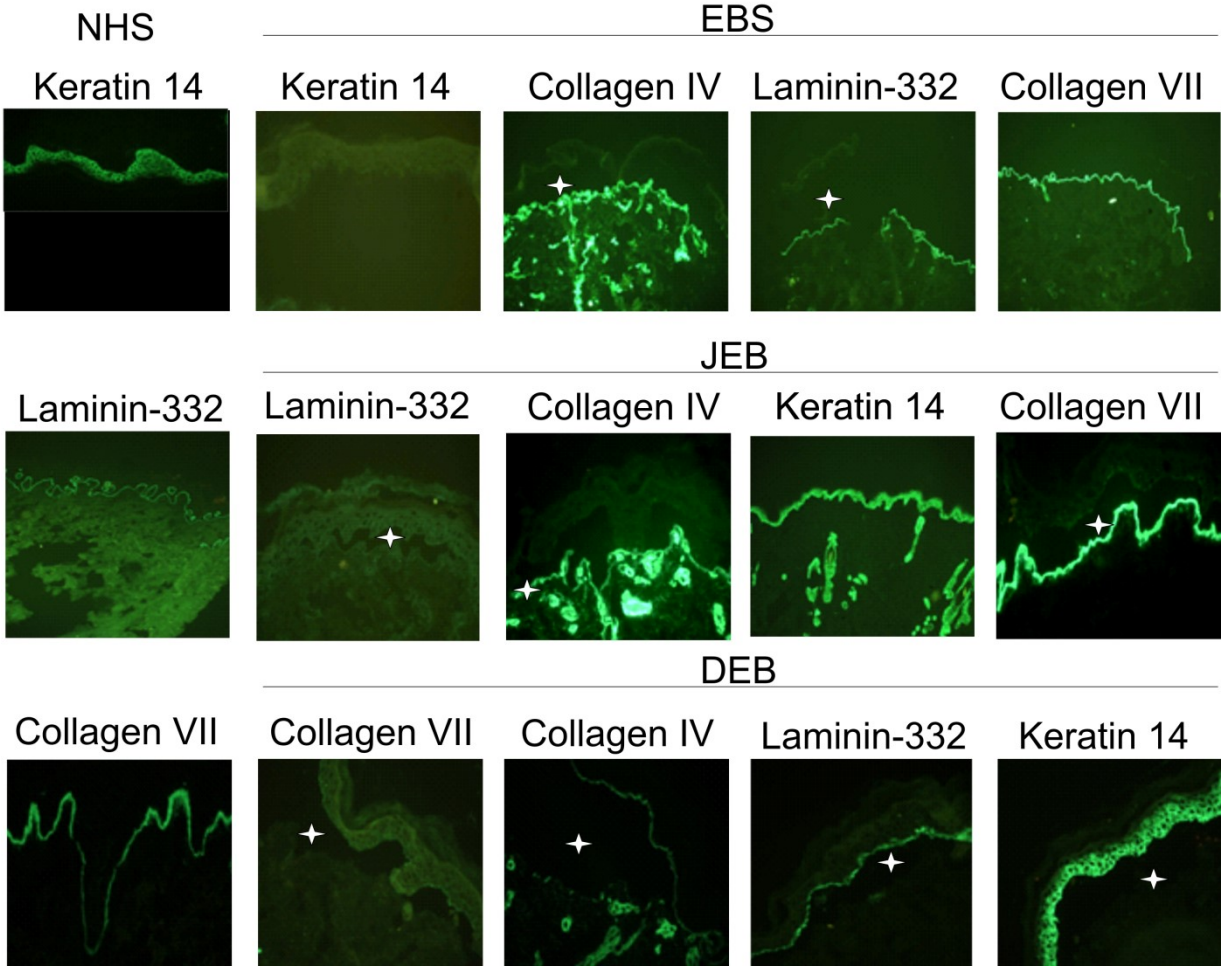
a



b


patient samples
NHS

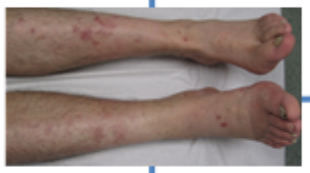
c


NHS
EBS
Keratin 14
Keratin 14
Collagen IV
Laminin-332
Collagen VII
JEB
Laminin-332
Laminin-332
Collagen IV
Keratin 14
Collagen VII
DEB
Collagen VII
Collagen VII
Collagen IV
Laminin-332
Keratin 14

**Supplementary Figure 2. Example of how the guideline works in clinical practice.**

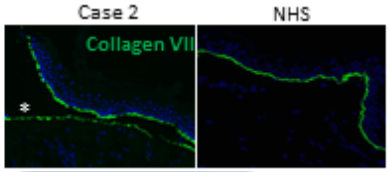
Case 2 is an adult female with clinical features and family history suggestive of EB; with signed informed consent, she was referred to an EB diagnostic centre as a pregnancy was envisaged. IFM confirmed DEB, and with *COL7A1* as the unique candidate gene, direct bidirectional SS of *COL7A1* was performed. Identification of two pathogenic sequence variants (one highly prevalent and one novel) discloses recessive inheritance which was confirmed in the patient's parents and affected sibling. Pathogenicity of the novel mutation assessed according to ACMG. Analysis of the patient's partner to reduce risk of RDEB offspring and proper genetic counselling were provided.

Case 2
Clinical phenotype of EB: **YES**
**Began at the age of 1 year**



Skin fragility/blistering: **pretibial, hands and feet, milia, dystrophic toenails and loss of some.**
Mucosal fragility: NO
Family history: **YES (unaffected, unrelated parents; affected sibling)**

Informed consent: **YES**




IFM: **YES**
Skin cleavage at dermal level

TEM: NO

Skin biopsy: **YES**

Genetic testing: **YES**

Choose diagnostic approach

One candidate gene:

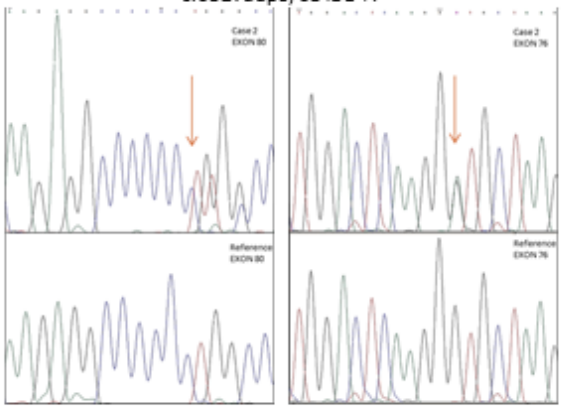
**YES, *COL7A1***

SS

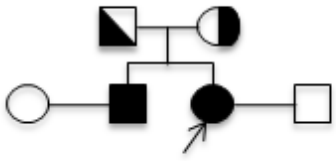
PATHOGENIC VARIANT?

**Yes**
Novel

*COL7A1*
c.6527dupC; 6341G>A



| Case 2 | *COL7A1*: c.6527dupC<br>Recurrent pathogenic variant | *COL7A1*: c.6341G>A<br>Novel<br>Co-segregates in 4 families<br>MAF < 0.01<br>In silico pathogenic | Other genetic tests |
|---|---|---|---|
| **Father** | Healthy carrier | Non carrier | |
| **Mother** | Non carrier | Healthy carrier | |
| **Affected sibling** | Heterozygous | Heterozygous | |
| **Case 2 partner** | Non carrier | Non carrier | Non carrier of recurrent *COL7A1* pathogenic variants |



EB diagnostic: *COL7A1* disease-causing variants support IFM results and clinical manifestation of RDEB. Genetic counselling was provided.

# Appendix 3. Supplementary Tables

## Supplementary Table 1. Proteins involved in EB

| Proteins | Characteristics | Localisation, tissue expression | Functions |
|---|---|---|---|
| **BPAG1** | Part of the plakin protein family | Inner plaque of hemidesmosomes in basal epidermal keratinocytes, skin and brain-specific isoforms exist | Cytoskeleton, signalling and tissue integrity |
| **CD151, syn. Tetraspanin 24** | Tetraspanin with four transmembrane domains | In hemidesmosomes at the basal pole of basal keratinocytes, podocytes | Stabilizes $\alpha6\beta4$ and $\alpha3\beta1$ integrins |
| **Desmoplakin** | Part of the plakin protein family, specifically a cytoplasmic component of desmosomes | Epithelial and cardiac tissues | Cell-to-cell adhesion, structure and cell integrity |
| **Exophilin 5, syn. Slac2-b** | Intracellular protein that interacts with keratin network | Keratinocytes and other hormone-related tissues | Plays a role in intracellular vesicle trafficking and secretion |
| **Integrin $\alpha3$ subunit** | Transmembrane integrin receptor | At the basal pole of basal keratinocytes in the skin, kidney and lung | Structure and cell integrity, signalling, development and extracellular matrix (ECM) organisation |
| **Integrin $\alpha6\beta4$** | Cellular transmembrane adhesion proteins that bind to laminin-332 in the ECM and keratins in the cell cytoplasm | Component of hemidesmosomes at the basal pole of basal keratinocytes, normal epithelial and endothelial cells | Signalling, ECM organisation |
| **Kelch-like protein 24** | Intracellular protein contributing to keratin network stability | Keratinocytes and many other tissues, including brain and heart | Cytoskeleton and tissue integrity |
| **Keratin 5 and 14** | Keratin polypeptides contain of rod domains and build heterodimers which assemble into intermediate filaments | Basal keratinocytes in epidermis and oral mucosa | Cytoskeleton and tissue integrity, signalling, intracellular transport |
| **Kindlin-1** | Focal adhesion protein required for integrin binding and activation | Epithelial expression | Signalling, cell adhesion, migration, proliferation, differentiation and ECM deposition assembly |
| **Laminin-332, syn. laminin 5** | Heterotrimeric protein located at the basement membrane zone binding integrin $\alpha6\beta4$ from the basal keratinocyte and type VII collagen from the ECM | At the basal pole of basal cells in epithelial tissues | Epidermal adhesion, cell migration |
| **Lysyl-hydroxylase 3** | Member of the 2-oxoglutarate-dependent dioxygenase family | Secreted and present in the extracellular space; expressed in a variety of tissues | Enzyme able to hydroxylate lysyl residues and glycosylate hydroxylysyl residues in collagens |
| **Plakoglobin, syn. gamma-catenin** | Part of the plakin protein family, specifically a cytoplasmic component of desmosomes | Most tissues, including keratinocytes and cardiac muscle | Cell-to-cell adhesion, structure and cell integrity |
| **Plakophilin-1** | Part of the plakin protein family, specifically a cytoplasmic component of desmosomes | At the nucleus and desmosomes in many epithelial tissues, including the skin | Cell-to-cell adhesion, structure and cell integrity |
| **Plectin** | Large protein part of the plakin protein family | Inner plaque of hemidesmosomes in | Cytoskeleton, signalling and tissue integrity |

| | | | |
|---|---|---|---|
| | (adhesion junction plaque proteins), which act as cytoskeleton linkers | basal epidermal keratinocytes, muscle and many other tissues | |
| **Transglutaminase 5** | Enzyme which catalyses the formation of protein cross-links between glutamine and lysine residues | Suprabasal epidermis in the skin and oesophagus | Protein modification and stabilization, keratinocyte differentiation |
| **Type XVII collagen** | Transmembrane collagen component of hemidesmosomes | Epithelial hemidesmosomes of skin, mucous membrane and eye | Epidermal adhesion, ECM organisation |
| **Type VII collagen** | Polypeptide with central collagenous domain and C- and N-terminal noncolleagues domains, triple helix and assembles extracellularly into collagen fibrils | Anchoring fibrils beneath the lamina densa of the basement membrane Skin, mucous membranes, component of conduits in the spleen | Dermal-epidermal adhesion, ECM organisation |

Legend: ECM, extracellular matrix; N-terminal, amino-terminal, C-terminal, carboxy-terminal

**Supplementary Table 2. Comparison of genetic testing techniques which can be used for EB laboratory diagnosis**

| **Biomaterial** | **DNA** | | | | | **RNA** |
|---|---|---|---|---|---|---|
| **Method** | **Sanger Sequencing** | **NGS – targeted EB gene panel** | **Whole exome sequencing (WES)** | **Multiplex Ligation-dependent Probe Amplification (MLPA)** | **Quantitative Fluorescent-PCR (QF-PCR) for microsatellite analysis** | **Transcriptome sequencing (RNA-Seq); reverse transcription (RT-PCR) and Sanger sequencing** |
| **Aim** | Detection of known and novel variants (nucleotide substitutions and small deletions, insertions, inversions) | Detection of known and novel variants (nucleotide substitutions and small deletions, insertions, inversions) | Detection of known and novel variants (nucleotide substitutions and small deletions, insertions, inversions)<br><br>Haplotype analysis<br><br>Detection of loss of heterozygosity (LOH) | Detection of known and novel large rearrangements (deletion/insertions of whole exons or genes) | Haplotype segregation for: indirect carrier detection; indirect evidence of large rearrangement(s); confirmation of *de novo* sequence variant event(s) or uniparental isodisomy (UPD); preimplantation genetic haplotyping (PGH); maternal contamination testing in prenatal diagnostics | Detection of altered transcripts |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Advantages:** | Golden standard for genetic human disease diagnosis<br><br>Straightforward approach if candidate gene is obvious or the familial sequence variant(s) is (are) known<br><br>Simple equipment<br><br>Easy to set up (compared to NGS)<br><br>Enables detection of mosaic variants (if the variant is present in>20% of the DNA)<br><br>The cheapest option for analysis of a few selected exons | Rapid and effective approach in absence of a candidate gene<br><br>Multigenic analysis per sample in a single tube improving cost/efficiency<br><br>Enables detection of mosaic variants<br><br>Relatively low-cost method for analysis of several / large genes | Effective approach in absence of a candidate gene<br><br>Multigenic analysis per sample in a single tube improving cost/efficiency<br><br>Discovery of new genes<br><br>Enables detection of mosaic variants | Analysis of large rearrangements (deletions/duplication)<br><br>Detection of loss of heterozygosity (LOH)<br><br>Easy to set up | Rapid and low-cost<br><br>Easy to set up | Confirmation of effect of new variants on splicing<br><br>Identification of novel sequence variants located in exons and introns as a result of new splicing variants<br><br>Enable detection of mosaic variants |
| **Limitations and uncertainty** | Candidate gene is mandatory (laborious and expensive for several genes per patient)<br><br>No detection of larger ins/del<br><br>No detection of variations in other EB genes<br><br>Allele “dropout” in the PCR due to lack of primer hybridization (e.g., an SNP in primer region, deletion of one allele)<br><br>15-50% of variants are undiagnosed, depending on EB type | Uncovered regions (uncaptured/ low coverage)<br><br>Computational capabilities are needed<br><br>Not (yet) for detection of larger insertions/deletions<br><br>Bioethical challenges- enable detection of unsolicited findings[1] | Uncovered or poorly covered regions (uncaptured/ low coverage/deep introns) can vary in different sequencing platforms<br><br>Not (yet) for detection of larger ins/del<br><br>Less coverage for target genes than EB-panels, including non-coding sequences<br><br>Computational capabilities are needed[2]<br><br>Bioethical challenges: detection of unsolicited findings (non-EB genes) | Available for a limited number of genes<br><br>Limited available positive controls<br><br>Reliable results depend on high DNA quality | Not 100% reliable as a paternity test.<br><br>Microsatellites not always informative for all families. | Skin biopsy is not always possible.<br><br>Mutant mRNA allele maybe degraded by nonsense-mediated mRNA decay (NMD). Not always easy to amplify both allele products. |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Other considerations** | Recommended primer design, to avoid reported SNP-containing regions (Association for Clinical Genetic Science (ACSG))<br><br>Software based analysis is mandatory<br>Additional visual evaluation highly recommended to detect mosaic cases (ACSG) | Recommended, to include all EB genes and introns<br><br>Mandatory to confirm by SS.<br><br>Unable to detect sequence variants in introns/regulatory regions (if introns are not included) | Mandatory to confirm results by SS<br><br>Unable to detect sequence variants in introns/regulatory regions | Recommended PCR–based confirmation for each deletion to rule out that the probe complimentary DNA regions contains SNP(s). Preferential amplification of a smaller allele (in case of deletion) may occur | Recommended to use a genetic analyzer capable of 2 bp allele resolution and peak area/peak height quantification<br><br>Stutter products are common, therefore, experience in microsatellite analysis is required | Recommended to test for possible aberrant splicing in unreported sequence variants |
| **Internal and external quality control (search for references)** | Blind sample in each run; negative (no sequence variant) and positive (with sequence variant) controls<br><br>Verification of sequence variant in parents<br><br>External quality assessment (EQA) schemes | NGS quality metrics (www.eurogentest.org/) | NGS quality metrics (www.eurogentest.org/)<br><br>EQA schemes | Blind sample and negative (no sequence variant) and positive internal controls are needed in each run | Blind sample in each run | |

**Supplementary Table 3. Classification of sequence variants**

| Class | Variant name | Clinical significance | Main criteria* | Clinical application | Recommendation |
|---|---|---|---|---|---|
| **1** | Clearly benign | Variant is not considered to cause EB in the patient | Variant does not segregate with the disease | Genetic test is considered negative | Keep searching for pathogenic variant/s |
| **2** | Likely benign | Variant is not likely the cause of EB in the patient | Minor allele frequency (MAF) in control populations < 0.001 (1000G and ExAC) | Genetic test is considered negative | Keep searching for pathogenic variant/s |
| **3** | Uncertain significance (VUS) | Insufficient or inconsistent evidence to ensure that variant is not causing EB in the patient | Variant is novel or very rare<br>Predicted to be deleterious<br>In an EB gene associated to the patient's clinical presentation | Genetic test is considered uncertain | Not for clinical decision-making<br>Not for risk calculation<br>Family member testing may be useful to gain information to reclassify the variant but not for genetic counselling<br>Keep searching for pathogenic variant/s |
| **4** | Likely pathogenic | Variant is considered the probable cause of EB in the patient | Loss of gene function established as a pathogenic mechanism in EB: premature truncation (frameshift, nonsense, or consensus splice site (+/- 1, 2))<br><br>Variant is novel or very rare in control ethnically matched populations | Genetic test is considered positive<br>Cautious clinical decision-making | Family member testing<br>Genetic counselling<br>Not for risk calculation<br>Re-evaluate the status of the variant periodically |
| **5** | Clearly pathogenic | Variant is considered causative of EB in the patient | Family co-segregation well established<br><br>Publications support pathogenicity | Genetic test is considered positive<br>Clinical decision-making<br>Risk disease calculation | Family member testing<br>Genetic counselling |

* For further criteria: http://www.mgz-muenchen.com/files/Public/Downloads/2018/ACMG%20Classification%20of%20Sequence%20Variants.pdf (Richard , 2015)
MAF, minor allele frequency

**Supplementary Table 4. The most useful websites and online bioinformatics tools** ✓

(Modified after Richard et al., 2015)

| |
|---|
| **Databases:** |
| GnomAD http://gnomad.broadinstitute.org/ |
| ClinVar https://www.ncbi.nlm.nih.gov/clinvar/ |
| HGMD http://www.hgmd.cf.ac.uk/ac/index.php (registration is mandatory, free version contains data published up to 3 years ago) |
| SNPdb *https://www.ncbi.nlm.nih.gov/snp* |
| International registry of dystrophic epidermolysis bullosa (DEB) patients and associated *COL7A1* pathogenic variants: www.deb-central.org |
| Intermediate filament database: www.interfil.org |
| LOVD http://www.lovd.nl/3.0/home |
| **Reference Sequences:** |
| LRG http://www.lrg-sequence.org |
| NCBI https://www.ncbi.nlm.nih.gov/refseq/rsg/ |
| **Primer design:** |
| Primer3 |
| BLAST |
| SNPcheck |
| PCR in SILICO |
| **Bioinformatic services:** |
| Varsome (contain ACMG scoring system and integrates several other computational tools) |
| Missense prediction: |
| Poly-Phen-2 |
| SIFT |
| Mutation Taster |
| Splice site predictions: |
| GeneSplicer http://www.cbcb.umd.edu/software/GeneSplicer/ gene_spl.shtml |
| Human Splicing Finder http://www.umd.be/HSF/ |
| MaxEntScan http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html |
| NetGene2 http://www.cbs.dtu.dk/services/NetGene2 |
| NNSplice http://www.fruitfly.org/seq_tools/splice.html |
| FSPLICE http://www.softberry.com/berry.phtml?topic=fsplice& group=programs&subgroup=gfind |
| **Pathogenic variant designation:** |
| HGVS http://varnomen.hgvs.org/ |
| Mutalyzer http://www.humgen.nl/mutalyzer |

**Supplementary Table 5. The formula for the Michel's medium**

| |
|---|
| 1 м citrate buffer pH 7.4, 2.5 ml |
| 0.1 м magnesium sulphate, 5 ml |
| 0.1 м N-ethyl maleimide, 5 ml |
| ammonium sulphate 55 g |
| distilled water, 87.5 ml |
| **total volume 100 ml** |
| adjusted to pH 7.4 with 1 м sodium hydroxide |

## Supplementary Table 6. Standard protocol for IFM for EB diagnosis ✓

| Step | Procedure | Time |
|---|---|---|
| **1** | An appropriate number of slides are air dried | 10 minutes |
| **2** | Incubate with the primary antibodies against specific structural proteins (dilutions depend on primary antibodies; they are included in the information provided by the provider and may vary depending on the charge) | 30 minutes to 2 hours or over night |
| **3** | Wash the slides twice in PBS | 15 minutes each |
| **4** | Incubate with different specific secondary antibodies depending on the source of the primary antibody<br>Secondary antibodies are covalently bound to a fluorescent dye, most commonly fluorescein-iso-thio-cyanate, FITC) and derive from mouse, rabbit or rat (dilution 1:50, but must be tested before use) | 30 minutes to 2 hours |
| **5** | Wash of the slides twice in PBS | 15 minutes each |
| **6** | Embed in glycerol or fluorescence mounting medium | |
| **7** | Analyse with a fluorescence ultraviolet microscope at 450–490 nm (e.g. Axioscop, Carl Zeiss GmbH) | |

Legend: PBS, phosphate-buffered saline; FITC, fluorescein-iso-thio-cyanate

## Supplementary Table 7. Antibodies recommended for IFM in EB[a] ✓

| Antigen | Name / clone | Host |
|---|---|---|
| **Minimal panel[b,c]** | | |
| **Type IV collagen** | CIV-22<br>IV-4H12 | Mouse<br>Mouse |
| **Type VII collagen** | LH 7.2 | Mouse |
| **Type XVII collagen** | Ab28440 | Rabbit |
| **Keratin 14** | LL 002<br>RCK107 | Mouse<br>Mouse |
| **Laminin β3 chain** | 6F12 | Mouse |
| **Extended panel** | | |
| **BPAG1** | 279 | Mouse |
| **CD151** | 11G5a | Mouse |
| **Exophilin 5** | Polyclonal | Rabbit |
| **Desmoplakin** | 2Q400<br>DP2.15 | Mouse<br>Mouse |
| **Integrin β4 subunit** | 3E1 | Mouse |
| **Integrin α6 subunit** | GOH3<br>NKI-GoH3 | Rat |
| **Integrin α3 subunit** | P1B5 | Mouse |
| **Keratin 5/6**<br>**Keratin 5** | D5/16 B4<br>SP27<br>MAB3224 | Mouse<br>Mouse<br>Mouse |
| **Laminin α3 chain** | # 546215<br>P3H9-2 | Mouse |
| **Laminin γ2 chain** | D4B5 | Mouse |
| **Plectin** | 10F6<br>31 | Mouse<br>Mouse |
| **Plakophilin 1** | PP1-5C2<br>3G250 | Mouse<br>Mouse |
| **Plakoglobin** | PG5.1 | Mouse |
| **Transglutaminase 5** | H-15 | Rabbit |

[a],only commercially available antibodies are included in this recommendation; for several antibodies multiple providers are available, which change over time; [b], if clinical features indicate a specific EB subtype (i.e. pyloric atresia), the corresponding antigen should be included in the minimal panel;
[c], nuclear staining may enable distinction between cleavage within the basal keratinocytes or within the lamina lucida.

## Appendix 4. Example of the Report for EB Laboratory diagnosis in Case 1

Laboratory data Date:

Referring clinician:

# Molecular genetic analysis for Epidermolysis Bullosa

Last Name: Smith

First name: Jane

Date of birth: 15/01/2017

Gender: Female

Place of birth: Warsaw

Ethnic origin: Polish

Reason for referral: Female new born with congenital skin defects on upper and lower limbs, mechanically induced skin blisters and milia. Family history was negative, parents were not related.
Immunofluorescence mapping: Skin biopsy was performed in the second day of life with an extended panel of 18 antibodies to proteins of the dermal-epidermal junction zone (according to Has and He, 2016). No skin cleavage detected, all markers stained comparable to the normal skin. The result is not conclusive but excludes severe types of JEB and DEB, and autosomal recessive EBS.

**RESULT:**

***KRT5:*** **c.548T>A, p.Ile183Asn, in a heterozygous state.**
**Genotype according to HGVS: NM_000424.3: c.[548T>A];[=] or NP_000415.2: p.[Ile183Asn];[=]**

**No mutations in other analyzed regions of remaining genes were detected**

**(**Key: [=] denotes normal allele present)

**INTERPRETATION:**

- **This result shows that Jane Smith is heterozygote and has a c.548T>A (p.Ile183Asn) pathogenic variant in a single allele of *KRT5*.**
- **Genetic testing by Sanger sequencing excluded this pathogenic variant in the parents' DNA, indicating that mutation arose *de novo* in the patient or results from germline mosaicism of one of her parents.**
- **The c.548T>A (p.Ile183Asn) ~~is~~ variant is previously reported in individuals with Epidermolysis bullosa simplex (EBS) (Kim et al., 2017) and not detected in general population (GnomAD) (ACMG equals class 5).**
- **The result indicates that Jane Smith has autosomal dominant EBS due to a *de novo KRT5* pathogenic variant. The clinical subtype is severe generalized EBS.**
- **The future risk of having a child affected by EBS due to *KRT5* mutation calculated for Jane is high and equals 50% for every pregnancy.**
- **Consultation with Genetic Counselor is highly recommended.**

**Analysis performed by** **Approved by**

*Molecular biologist Y* *Laboratory director Z*

Additional information:
Analysis was based on next generation sequencing (NGS) and included coding regions and exon-intron junctions of the following genes: *CD151*, *COL17A1, COL7A1, DSP, DST, EXPH5, FERMT1, ITGA6, ITGB4, ITGA3, JUP, KLHL24, KRT5, KRT14, LAMA3, LAMB3, LAMC2, PKP1, PLEC, TGM5.* Important: gross rearrangements, introns (beside exon-intron junctions) and regulatory regions were not analyzed!
Library was prepared using (Reagents name, lot and producer) and sequenced on (sequencer name)
The results were analyzed using following bioinformatics tools: Real Time Analysis Software (RTA), MiSeq Reporter (MSR), VariantStudio, Annovar.(or other, according to individual laboratory pipelines)
UCSC hg19 was used as a reference human genome.
Following databases were used for variant annotations SNPdb (NCBI), ExAC, Ensembl, OMIM, GnomAD, ClinVar, HGMD Professional (or other).
Quality parameters: Mean Region Coverage Depth , coverage > 20x: .; Q30

Pathogenic variants identified by NGS were confirmed using Sanger Sequencing. The sequences were analyzed using Mutation Surveyor v.3.10 Network (SoftGenetics®) (or other). .
Mutations have been classified according to GenBank Accession Number: NM_000424.3 and named according to HGVS recommendations (HGVS 15.11)

Please note that DNA has been stored from this patient's sample at this center,
and will be kept indefinitely unless a written request for its disposal is received from the patient or his parent/guardian
Individual elements of this report should not be copied or transferred to other systems;
the report should only be copied in its entirety.

## References

1 Matthijs G, Souche E, Alders M, *et al.* Guidelines for diagnostic next-generation sequencing. *European Journal of Human Genetics* 2016; **24**:2–5.

2 Roy S, Coldren C, Karunamurthy A, *et al*. Standards and Guidelines for Validating Next-Generation Sequencing Bioinformatics Pipelines: A Joint Recommendation of the Association for Molecular Pathology and the College of American Pathologists. *The Journal of Molecular Diagnostics* 2018; **20**:4–27.